Investigation of Two Australian Medicinal Plants *Viola odorata* and *Euphorbia peplus* for Anticancer Agents

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INVESTIGATION OF TWO AUSTRALIAN MEDICINAL PLANTS *Viola odorata* AND *Euphorbia peplus* FOR ANTICANCER AGENTS

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by

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DECLARATION

I, Susil Francis Fernando, hereby declare that this submission entitled, " Investigation of Australian Medicinal Plants for Anticancer Agents"" is my original work and that, to the best of my knowledge and belief, contains no material previously published elsewhere or extracted in whole or in part by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at any other university or other institute of higher learning, except where due acknowledgement is made in the text.

Susil Francis Fernando

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THESIS ABSTRACT

In the absence of a clear understanding of the aetiology of diseases and measurable parameters for the attributes of drugs described in the traditional systems of medicine, an approach encompassing a broad spectrum of biological activities on the whole body that may be ascribable to the attributes of the drugs and aetiology of a disease described in the traditional medical systems such as Ayurveda, taken to investigate putative anticancer plants, is presented.

Extracts of the putative anticancer plants, *Euphorbia peplus* L. (petty spurge) and *Viola odorata* L. (sweet violet), which grow in Australia, were investigated for *in vitro* anticancer activity using DPPH free radical scavenging, Fe-(III)-TPTZ reducing (FRAP) and linoleic peroxidation inhibitory (TBARS and FTC) antioxidant activities; human complement inhibitory (CP and AP), anti-platelet aggregating (impedance method), COX-2 inhibitory immunomodulatory activities as well as the conventional *in vitro* anticancer activities using CEM [^{3}H] thymidine uptake, MTS and US-NCI's sulforhodamine B assay using 60 cell line humour tumour screen. Results were compared with relevant standards. Isolation of active extracts and chemical studies were carried out using HPLC-PDA, UV-VIS, TLC, GC-MS, ESI-MS, and AAS.

Although the two plants have failed in the NCI's conventional *in vitro* anticancer screen, our results showed that the extracts of both plants indicated a combination of biological activities. These results raise the possibility that broad-spectrum approach comprising a number of different biological systems may help to explain the purported *in vivo* anticancer activity of *Euphorbia peplus* L. and *Viola odorata* L. Preliminary chemical studies showed the presence of the flavonoid, quercetin among other compounds, in the antioxidant extracts of both plants. The ESI-MS and GC-MS profiles may be used to characterise the active extracts. Presented results may be used to design further *in vivo* studies towards utilising natural resources as anticancer agents.

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ABBREVIATIONS

ABS	Australian Bureau of Statistics
$ABTS^{\cdot+}$	2,2'-azino-bis (3-ethyl benzthiazoline-6-sulphonic acid)
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
$CHCl_3$	chloroform
COMPARE	computer analysed growth mean graph patterns
COX-1	cyclooxygenase-1 enzyme (prostaglandin-endoperoxide synthase, (EC 1.14.99.1)
COX-2	cyclooxygenase-2 enzyme (prostaglandin-endoperoxide synthase, (EC 1.14.99.1)
CTR or CTRL	control sample
DBA/2	mice
DPPH	1,1-diphenyl-2-picryl hydrazyl
ED_{50} value	concentration causing 50% growth inhibition (ED_{50}) value
ESI-MS	electro spray ionisation-MS
EtOH	ethanol
FL	flowers
FRAP	ferric reducing antioxidant potential
FRSA	free radical scavenging activity
FTC	ferric thiocyanate method
G_1, S & G_2/M	phases of the cell cycle
GC-MS	gas chromatography & mass spectrometry
GI50	50% growth inhibitory potency
GSH	glutathione
HMDS	hexamethyldisilazane
HTS	high throughput screening
KB cells	human epidermoid carcinoma of the nasopharynx
L1210	leukaemia cell line
LC50	"net cell killing" or cytotoxic effects
LDL	low density lipoproteins
LF	leaves
MAC	membrane attack complex
MDA	malondialdehyde
MED	the minimum dose producing a significant effect
MeOH	methanol
MTD	the maximum tolerated dose
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium, inner salt
N/A	(data) not available
NCI	National Cancer Institute of the United States of America
OP	*Oenothera paradoxa*
P388	mouse leukaemia cell line
PAF	platelet activation factor
PD-ECG	platelet-derived (antiogenetic) endothelial cell growth factor
PG	poly gallate
PGE_2	prostaglandin E_2

PGI_2	prostacyclin
PL	whole plant
PMS	phenazine methosulphate
PPP	platelet-poor plasma
PRP	platelet-rich plasma
RFRAOA	ferric reducing antioxidant activity
ROM	reactive oxygen metabolites
ROS	reactive oxygen species
RT	roots
SOD	superoxide dismutase
ST	stems
T/C value	the ratio of test (T) to control (C) tumour weight, or survival time of test animal
TBARS	thiobarbituric acid reacting substances
TGI	total growth inhibition or cytostatic effects
TMS	trimethylchlorosilane
TPTZ	2,4,6-tripyridyl-*S*-triazine
TXA2	thromboxane A2
WA	Walker carcinosarcoma 256 (subcutaneous)
WHO	The World Health Organisation

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CHAPTER 1 GENERAL INTRODUCTION AND LITERATURE REVIEW

ABSTRACT

Despite numerous approaches taken towards the development of anticancer drugs, the disease cancer still causes large number of deaths worldwide. Allopathic medicine has been partly successful in curing cancer but severe adverse side effects are often a major problem in treating cancer. On the other hand, traditional systems of medicine have existed for thousands years, curing patients over a wide range of diseases including certain cancers. The crude plant extracts prescribed in the traditional medical systems have been known to cure diseases with minimal or no adverse side effects. Although several plants used in traditional systems of medicine have reportedly alleviated various cancers in human, such plants when screened in cytotoxicity based assay systems have failed to indicate any promising anticancer activity. This resulted in the rejection of the putative anticancer plants as being of no use for treating cancers.

More recent approaches have targeted a number of specific activities, which include antioxidants, immunomodulators, inhibitors of platelet aggregation and cyclooxygenase-2, *inter alia* as potential uses for treating cancers. In a crude plant extract, a number of different constituents may exert synergistic effects on the whole body during the cancer healing process. In the absence of complete information on the mode of action and the chemical structures of their anticancer constituents, it is difficult to design assays to reveal the anticancer activity of the putative anticancer plants. However, if a whole body approach is taken and if a broad spectrum of anticancer related assays is used, the success rate of discovering a successful treatment from plant extracts may increase markedly. In this study a pragmatic approach was used which entailed screening plant extracts for immunomodulatory, antioxidant as well as cytotoxic activities from two putative anticancer plants *Euphorbia peplus* L. and *Viola odorata* L. In this chapter, a number of cancer-related drug screening approaches are described including the traditional Ayurvedic method of prescribing medicaments as well as the broad-spectrum screen, which includes a summary of activities to monitor plants used in traditional systems of medicine.

1 Cancer and anticancer drugs

1.1 The disease

Cancers have been termed as neoplastic (Gr. *neos* **new**, *plasma* **formation**) diseases and have been defined as a group of diseases having similar characteristics, which lead to uncontrolled proliferation of cells. These cells are often made up of premature, undifferentiated cell types, which usually survive at the risk of the host (Furst, 1963).

It is estimated that cancer claims the life of 7 million people per annum worldwide (WHO, 1996). Cancer is the leading cause of death in most of the industrialised countries including Australia (ABS, 1997a). In Australia about 70,000 new cases of cancer are diagnosed each year and the annual deaths due to cancer were estimated to be 34,316 (26.5% of all deaths) in 1997 (ABS, 1997b).

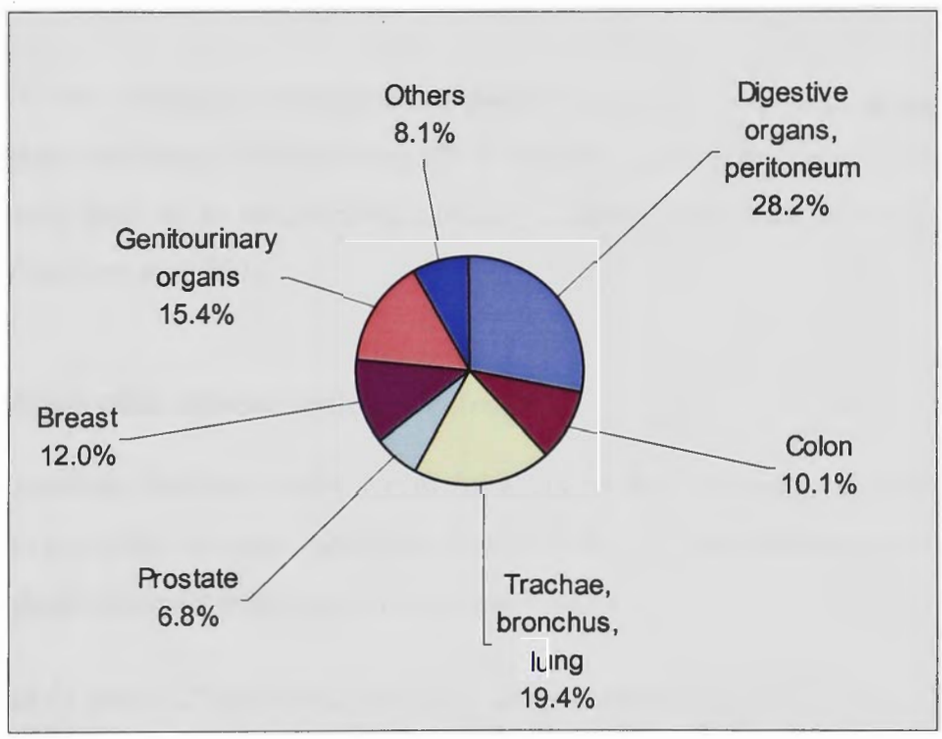


Figure 1 **Percent Cancer deaths according to the type of cancer in Australia during 1997-1998 (ABS, 1997c).**

Due to the intrinsic differences of the affected tissues of the body, cancers are categorised based on the location and the nature of the diseased tissue. Percent cancer deaths in

Australia during 1997-1998 (ABS, 1977c) according to the type of cancer are given in Figure 1. As illustrated in Figure 1, cancers of digestive organs and the peritoneum caused the highest number of cancer deaths (28.2%) in Australia during 1997.

1.1.2 Suggested causes of cancer

Although there is no conclusive evidence, free radical formation resulting from oxidative stress, UV light from the sun and other environmental and genetic factors are suggested as positive causes of cancers possible through mutations of DNA. Free radicals are reportedly involved in cell damage and tumour promotion (Cerutti, 1985). Disequilibrium between kinetics of catabolic enzymes that retard the rate of cell destruction and the normally acting anabolic enzymes that maintain the rate of cell formation leading to the accumulation of immature undifferentiated cells is cited as the preceding phenomenon to a cancerous cell growth. Although there is no conclusive evidence for specific cancer enzymes to date, reduced activity of catalase and xanthine oxidase in tumour tissues has been observed (Furst, 1963).

According to the findings of recent human genome research, it has been suggested that gene mutations that trigger the switching off of tumour suppressor genes or switching on of oncogene(s) leads to an uncontrolled division of tissue cells which, in turn, results in tumours (Van Gent *et al*, 2001).

1.2 Problems with current anticancer drugs

Allopathic medicine has been partly successful in controlling or curing cancer but severe side effects are often a major problem. Some of the known adverse effects of the anticancer drugs currently being used are given in Table 1.

From a total of about 62 approved anticancer drugs marketed in the US only a few have showed useful clinical antitumour activity against most common forms of primary and disseminated cancer. Most of the available drugs have a narrow spectrum of antitumour activity and a narrow therapeutic index. Often tumours that are initially responsive to a single drug rapidly become resistant to it (Boyd *et al*, 1992). Thus, multiple drug resistance is also a problem in the treatment of invasive terminal disease such as cancer. Despite extensive searches for effective anticancer drugs, the National Cancer Institute (NCI) of

Table 1 Adverse side effects of some clinically used plant derived single anticancer drugs

Drug in Clinical use	Reported contraindications	Reference
Vincristine	Neurotoxicity, genotoxicity	Wang *et al*, (2000), Nefic & Ibrulj (2000)
Vinblastine	Intestinal paralysis	Noble (1990)
Etoposide (VP-16-213)	Myelosuppression, leucopenia, cardiac toxicities	O'Dwyer *et al* (1985)
Paclitaxel	Severe hypersensitive reactions, myelosuppression, mucisitis, peripheral neuropathy, arthralgia, alopecia, cardiotoxicity, neurotoxicity, neutropenia	Suffness (1995)

the United States was partially successful in discovering only three anti-cancer drugs namely paclitaxel (Taxol®), camptothecin (topotecan and irinotecan) and homoharringtonine. All of these three compounds are isolated or derived from plants and discovered out of 35,000 samples derived from an estimated 12,000 species of plants tested using the NCI's Natural Product Screening Program during 1960 and 1982 (Cragg *et al*, 1998). In spite of the major effort used to identify anticancer agents from plants, the success rate of discovering a marketable anticancer drugs per species has been as low as 1:8000 (Farnsworth, 1994).

Although cytotoxic therapy has resulted in controlling some disseminated malignancies including acute childhood leukaemias, Hodgkin's disease and germ cell tumours, it has been unsatisfactory in curing most frequent solid tumours in the advanced metastatic stage (Schwartsmann and Workman, 1993) and have often resulted in remissions. Chemotherapy has not been successful in treating advanced adult solid tumours

(Tattersall, 1982). Hence cytotoxic drugs are often not effective as single drugs. Even the effective cytotoxic drugs have shown severe adverse side effect.

Paclitaxel (Taxol®) is an example of a single cytotoxic drug whose use has been masked by a plethora of adverse side effects (Chang *et al*, 1992). It is interesting to note the use of combination of other drugs to alleviate the undesirable side effects. For instance, pre-medication including dexamethasone has been used to prevent the severity of paclitaxel's hypersensitivity reactions (Suffness and Wall, 1995). It has been suggested that immunostimulants be used to alleviate the immunosuppression caused by most of the cytotoxic drugs (Suffness and Wall, 1995). This follows the concepts outlined by the allopathic system, which promotes the use of a number of single compounds to alleviate a disease. A similar effect has been observed in the use of natural crude extracts containing variety of compounds, which exert a balanced therapeutic effect through a number of different biological activities.

The latest allopathic treatment scheme for cancers entails alternate chemotherapy using a cocktail of drugs. For an example, primitive neuroectodermal tumours and Ewing sarcomas in children have been treated with combination of vincristine, actinomycin-D, doxorubicin, and cyclophosphamide and ifosfamide in order to achieve high therapeutic efficacy and a moderate toxicity (Rosito, 1999). Taxol® (paclitaxel) is given with cisplatin, 5-fluorouracil, and folic acid to achieve a balance between its efficacy and toxicity (Kollmannsberger *et al*, 2000).

The most successful anticancer agents isolated from plants on the basis of cytotoxicity to date have shown side effects (Table 1). However the use of cytotoxicity may have resulted in many other compounds being missed with different biological activities that may have been useful in treating cancer. Thus the possibility that compounds that activate the immune system may have been missed when cytotoxic activity is solely used to identify the active ingredients from plants.

As discussed in previous sections, cytotoxic compounds have been isolated from several plants used in the traditional medicine and have been extensively investigated for cancer therapy. During the past decade, immunomodulatory (Labadie, 1993; Bohlin, 1995), antioxidant, and anti-inflammatory (Bohlin, 1995) activity of plants used in traditional medicine and anticancer activity have also been reported, prompting further investigation

of non-cytotoxic agents, which demonstrate other biological activities, for treating cancer patients.

In order to utilise the resources from the traditional systems of medicine for treating cancer, it is important to understand the basic principles involved in them.

1.3 Traditional systems of medicine

Farnsworth (1994) defined traditional medicine as the sum total of all non-mainstream medical practices usually excluding so-called 'Western' or allopathic medicine. Because of the different degree of authentication of the medical practice involved, it is appropriate to include only the systems of medicine based on a theory (whether scientifically provable or not), formal education and a written documented history such as Ayurvedic, Traditional Chinese, Siddha, and Unani, under the title of Traditional Systems of Medicine. Farnsworth (1994) noted that the information on plants used in these systems is probably more reliable than that from other systems such as traditional healers, curanderos, shamans, 'witch doctors' and herbalists. According to the statistics of the World Health Organization (WHO), traditional systems of medicine still serve the primary health care needs of about 80% of the world population (Farnsworth *et al*, 1985). At present there is a growing demand worldwide for natural products as preventive, promotive and curative agents as evident by the inclusion of natural products section in most of the modern pharmacies that traditionally were used to sell allopathic medicines.

Although the traditional systems of medicine such as Ayurveda (Kapoor, 1990) and Chinese traditional medicinal system (Liu, 1989) have been in existence for thousands of years, treating various diseases including cancer, use of plant extracts and formulations have not received extensive study as anticancer agents, based on the principles of human body's functions, aetiology of diseases, attributes of medicinal plants and treatment methods. The basic Ayurvedic medical compendia, Charaka Samhita (1^{st} – 2^{nd} centuries BC) translated by Bhishagratna (1996) and Susruta Samhita (4^{th} century BC) translated by Sharma & Dash (1995) and the Chinese equivalent compiled in Shen Nong's Classic of Materia Medica (Xuemin, 1994) describe the principles of traditional system of medicine.

Over the last 15 years the NCI (Cardellina II *et al*, 1993) and other research groups have screened a large number of medicinal plants described in ancient medical treatises of traditional systems of medicine for anticancer activity, based on various selection criteria

and test systems, but not according to the principles of whole body approach. Although the plant derived anticancer drugs, paclitaxel and camptothecin were developed, based on cytotoxicity are currently in clinical use, neither of them have been discovered on the basis of ethnopharmacological focus (De Smet, 1997).

The interpretations of the aetiology of diseases, and the attributes of the medicaments described in the medical treatises of these systems are often non-specific compared to those of allopathic system. On the other hand, there seems to be a gap between the concepts of the traditional systems and allopathic medicine in reference to aetiology and attributes of drugs, which requires an intensive study into the philosophical concepts, identifying measurable parameters associated with these concepts. Although recent developments in the field of human genome may assist to a certain extent, it is difficult to design scientific experiments to determine the activity of drugs derived from plants, animals and minerals and elucidation of their mode of action(s).

Since Ayurveda is a system of medicine that involves medical education based on the principles described in the treatises of Charaka (Sharma and Dash, 1995) and Susruta (Bhishagratna, 1996), it is important to look at the relevant principles of Ayurveda with reference to the aetiology of a disease and the described attributes of drugs. In addition, it is important to rationalise and ascribe most appropriate scientifically measurable parameters to those principles, in order to design a scientific research strategy used to investigate the efficacy of those plants, based on the principles of Ayurvedic system of medicine. This by no means is an easy task. In order to facilitate the above task, the basic concepts of the Ayurvedic system are discussed in the following sections.

1.4 Ayurveda

Ayurveda (*Ayur* **life**, *veda* **science** in Sanskrit) is a traditional system of medicine, which originated in India around 400 A.D., in which materials of plant, animal and mineral origin are used as preventive, promotive (promotive), and curative agents (Basham, 1954).

According to the fundamental concept of Ayurveda, the entire material universe is composed of Mahabuta (five basic factors), viz., Prithivi, Apo or Jala, Tejo, Vayu and Akasa. The living being evolved from these five basic factors is composed of Dhatus, which is a combination of three somatic Doshas (Thridoshas) viz., Vata, Pitta and Kappha along with Malas (excretions), mind and soul. The Thridoshas and Malas are in dynamic

equilibrium to maintain the human body in a healthy condition. Any disturbance in this equilibrium causes a malfunction of the body and results in a disease. Hence treatment of a disease in Ayurveda, is on the basis of bringing back the physiological state of the body by treating the disease with aetiopathological antidote.

The aetiology of a disease described in the Ayurveda system of medicine is different from that accepted by the allopathic system of medicine. Some of the Ayurvedic terminology related to the aetiology of a disease cannot be explained in modern scientific terms of allopathic. For an example, it is indicated by Ayurveda that the cause of a disease of the body is due to the disequilibrium of the "Thridoshas" viz. Vata, Pitta and Kappha (in *Sanskrit*) or body-humours that function as bio-regulators responsible for physiological and biochemical functioning of the body. Manifestation of a disease is due to the disproportionate composition of "thridoshas". Udupa (1975) resembles Thridoshas to wind, bile and phlegm respectively and attempted to translate these attributes to serotonin, acetylcholine and adrenaline. An Ayurvedic treatment given to restore the disequilibrium of "thridoshas" is not used merely to correct the symptoms of the disease.

1.4.1 Ayurvedic drugs

According to Chraka Samhita (Sharma and Dash, 1995), since the human body is composed of the same five basic factors that the universe is composed of, the Ayurveda system prescribes materials composed of the same five basic factors viz. Prithivi, Apo or Jala, Tejo, Vayu and Akasa from plant, animal and mineral materials to act as a preventive, promotive and curative agents for human diseases. The five fundamental principles enters into the composition of all substances, predominance of any of them determines the character of a particular substance (Susruta Samhita (I), Bhishagratna, 1996).

The attributes of a drug in Ayurveda are different from that of the allopathy. There is a theoretical basis for the use of different plants. The attributes of a drug are described in Ayurveda as Rasa (tastes), Guna (inherent quality as vitalising, reducing, purifying and saponifying), Veerya (potency as "heat generating" or "heat absorbing"), Vipaka (digested for as pungent, sour and sweet).

These attributes of medicinal plants are described in authoritative treatises of Charaka and Susruta. A comprehensive description of Ayurvedic medicinal plants is given in terms of

Rasa, Guna, Virya, Vipaka in the Indian Materia Medica of Ayurveda (Dash and Kashyap, 1980) and in Sri Lankan Ayurvedic Pharmacopoeia (Jayasinghe, 1994).

Selection of a medicinal plant therefore is based on a systematic rationalisation of its attributes in terms of Rasa, Guna, Veerya, Vipaka and Prabhava in counteracting the disproportionated "Thridoshas" in a disease state of the body (de Silva, 1982; Ranasinghe, 1987).

1.4. 2 Rationale in counteracting a disease state

The disturbance to the equilibrium of "Thridoshas" caused one or a combination of Doshas to be predominant or aggravated. Hence a medicament possessing opposite qualities is administered with due regard to the location, dose and time, to alleviate the aroused Dosha state. In case of medicines that are not of opposite qualities but in which the opposite qualities predominate, reconciliation is not radical. Radical reconciliation can be achieved only with the medicines possessing the opposite qualities (Charaka Samhita, verses 62-63, 1996). Drugs having sweet, sour and saline taste alleviate Vata; those having astringent, sweet and bitter tastes alleviate Pitta and those having astringent, pungent and bitter tastes alleviate Kapha. A drug that possesses Madhur (sweet) and Tikta (bitter) tastes can be prescribed to alleviate a disease state caused by aggravation of both Vata and Pitta (Charaka Samhita, verses 66, 1996).

According to Ayurvedic system of medicine, diseases are caused by different Doshas and for the alleviation of each Dosha, specific groups of drugs are prescribed. Thus, the Doshas involved in the manifestation of a disease is considered when selecting a group of drugs

If the medicinal plants prescribed in Ayurvedic system of medicine, need to be investigated for a therapeutic cause, then the experimental strategy should be designed based on the aetiology described in the authoritative Ayurvedic treatise. Measurable parameters of the aetiology of a disease by means of bioassays and attributes of the medicament need to be identified and developed.

Such an approach is plausible in the absence of a disease category such as "cancers and leukemia" and "anticancer medicinal plants" in the ancient medical treatises. Moreover, 35% of indigenous diseases are not ascribable to any Western disease category and are

categorised as 'other' (Cox, 1994). There are also diseases, aetiologies of which are not well defined in the allopathy that most of the time are referred to as 'Syndromes".

Hence the whole body approach complies with the aetiology of a disease and attributes of a drug(s) prescribed in traditional systems of medicine such as Ayurveda.

1.4.3 Research problems with complex traditional drug preparations

In order to design an experimental investigation into the anticancer activity of traditional medicinal plants, it is important to identify the problems and develop strategies to circumvent them. Unavoidable problems encounter in such investigations are discussed in the following sections.

1.4.4 Problems in adopting Ayurvedic principles in drug development

Aetiology of a disease as well as the attributes of a drug are not well defined in the traditional systems of medicine. Consequently, a pragmatic approach and a comprehensive strategy based on basic theoretical concepts of the traditional medical system are lacking to date, to carry out a proper ethnopharmacological investigation into the materials described in these systems of medicine.

In the absence of a comprehensive set of measurable parameters indicative of (1) vitiated predominant Dosha(s) and (2) the attributes of the plant in terms of Rasa, Guna, Virya, Vipaka and Phabhava, it is difficult to design an experimental strategy to test the efficacy of a medicinal plant.

Treating a disease state by administering a single "active" (cytotoxic) compound isolated from an Ayurvedic medicinal preparation is not a rational approach to test the efficacy of a medicinal plant due to the exclusion of a number of compounds from the plant extract that may exert a synergistic action.

In addition, *in vitro* testing is not supported by the Ayurvedic principles, as the test system is not corresponding to the wholesome system of the body. However, *in vivo* experimentation is quite expensive for monitoring purposes requiring a compromise to use *in vitro* screening for preliminary selection of the active extract; followed by a sequence

of separation steps to isolate the active component(s), in order to study the mode of action of the active components.

1.4.5 Complexity of plant extracts

A crude plant extract due to its would be always undefined and complex chemical nature, and the lack of knowledge of its mode of action, poses many challenges when carrying out a scientific investigation.

Most of the Ayurvedic drug preparations contain a number of plant ingredients or herbo-mineral or herbo-animal mixtures given as a decoction or boiling water extract. Investigation of such a complex mixture is by no means an easy task. Hence selecting a single plant that is used for anticancer treatment would be an appropriate way to approach an investigation.

One of the known advantages of using a crude boiling water extract is that compounds such as saponins in the extract that affect surface tension enable dissolution of water insoluble compounds into the aqueous solution (Farnsworth, 1994). Similarly, the synergistic effect of using an orchestra of compounds in a crude extract versus an administration of a single drug may be justified on the basis that some compounds assist passage of other molecules through the lipoprotein membrane of the intestine. A balanced absorption avoiding toxic levels of an active compound into the system may regulate by the presence of other "auxiliary compounds". Such a synergism may be separated out in an activity-guided isolation of compounds followed by a single drug administering. However, activity-guided isolation of active compound(s) and elucidation of its structure would enable investigation of mode of action in isolation.

A biochemical investigation is difficult to design with dosage form prescribed in the Ayurvedic treatises due to minute concentrations of many compounds present in a particular extract. Such problems can be overcome by concentrating the compounds, by adopting a strategy involving an extraction method different from the prescribed dosage form, and subsequent concentration.

1.4.6 Factors affecting anticancer activity of plants

Charaka Samhita describes various procedures that should be followed in collecting, preparing and administering medicinal plants for various diseases, in order to ensure the quality of the plants for intended therapeutic effect. Hence the availability and abundance of an anticancer constituent(s) of a plant may depend on geographical location, season and maturity of harvesting, drying and storage method, and which part of the plant has been used.

1.4.7 Plant parts used and method of collection

Prospective use of plants described in traditional systems of medicine was evident from the discovery and development of the anticancer agents, vincristine (leurocristine) and vinblastine (vincaleukoblastine) from the alkaloid fraction of differential extracts (Svoboda *et al* 1959) of the plant, *Catharanthus roseus* (L.) G. Don. (Johnson *et al*, 1963). Vinblastine is used clinically for the treatment of Hodgkin's disease and choriocarcinoma, whereas vincristine is reported to be effective in treating acute lymphocytic leukaemia in children (Johnson *et al*, 1963).

Positive antileukaemic results were produced, when an alkaloid fraction from differential extracts of *C. roseus* leaves was screened against the acute lymphocytic leukaemia (P1534) implanted in mice (Johnson *et al*, 1959). However when an alcoholic extract of *C. roseus* whole plant was screened against sarcoma 180 and L1210 leukaemia in another laboratory, negative results were obtained (Leiter *et al*, 1962). An aqueous extract of *C. roseus* seed also led to negative results when screened against sarcoma 180, adenocarcinoma 755 and L1210 (Leiter *et al*, 1962). The negative results could have been due to the use of different parts of the plant, extraction solvent, and extraction method as well as the sensitivity of the cancer cell line used in the screen. Hence it is important to follow the procedures used in the traditional medical system to collect the plants and determining which part to use.

1.4.8 Method of extraction for plant materials

The method of extraction plays an important role in any activity testing as it results in complete, partial or none, extraction of the active compound(s) from the original plant material. Hence it is important to investigate the basis of the extraction methods and their efficiency, and validity in the screening program as it develops over the years.

Extraction procedures for plants used in the anticancer screening program of NCI have been evolved over 14 years. The early stage of the extraction procedure carried out according to Koppaka Roa method during 1957-1961, involved extraction of about 0.5 kg of ground plant material with 6-8 L of MeOH at room temperature and fractionation into water and petroleum ether extracts by solvent extraction. The activity against rat Walker 256 carcinosarcoma was found in extracts of all three fractions (water, petroleum ether and insoluble fraction) of the plant extracts tested (Statz & Coon, 1976).

The extraction procedure developed by Morris Kupchan, which involved preparing water and ethanol extracts, was adopted by the NCI during 1961-1964. The water extract was prepared by extracting 100 g of ground plant material with 200-300 ml of water at room temperature, fast filtering, and concentrating followed by freeze-drying. The ethanol extract was prepared by extracting 100 g of ground plant material in a soxhlet extractor with 95% EtOH for 3 hours and concentrating under vacuum at approximately 40^0C. Activity against Walker carcinosarcoma 256 (WA) was confirmed in 10% of >9000 plant extracts prepared by Kupchan procedure (Statz and Coon, 1976). Based on the observation that the same spectrum of chemically active substances could be extracted from plant materials using a 50% mixture of water and ethanol, NCI adopted a single-solvent (50% EtOH-water) extraction procedure during 1964-1974. Antitumour activity against WA was confirmed in 9% of the 1254 plants prepared by a single-solvent extraction procedure (Statz and Coon, 1976).

During the development of the extraction procedure, the tumour systems used in the primary screen also have been changed by the NCI. The WA tumour cell system was terminated due to its being non-selective in its response. Many of the extracts active against WA tumour cells were found to be inactive or marginally active after fractionation or isolation. Instead murine leukaemias L1210 and P388 were introduced for primary screening of plant extracts in 1969. Since the number of extracts, which showed activity against L1210 was insignificant; L1210 was eliminated from the NCI primary screen and included for secondary screen (Statz & Coon, 1976).

In order to increase the activity against the tumour systems, the NCI adopted a simple solvent fractionation procedure developed by Jonathan Hartwell in 1975, using a sequence of hot 95% EtOH, $CHCl_3$, hexane and the insolubles in 90% MeOH-aqueous (90:10) extracts of plant. The efficacy of the extraction methods was compared using 500

randomly selected plant extracts against P388 and 9KB tumour systems. According to the results (Statsz & Coon, 1976), most of the antitumour principles were concentrated in the 95%EtOH extract and its 90% MeOH fraction. Few active compounds were found in the water fraction, and almost none were concentrated in the hexane fraction. Hence 50% aqueous–EtOH extraction procedure was discontinued from NCI screen in 1974. According to Statz and Coon (1976), the fractionation procedure was modified to include soxhlet extraction for safety reasons and initial de-fatting with petroleum ether to eliminate emulsions formation in the $CHCl_3$ partition. The modified soxhlet fractionation procedure has been adopted since 1974, to achieve the highest percentage of confirmed activity for a given collection of plants, in the screening program against a particular tumour.

Although a series of selective extraction procedures can be developed, targeting the chemical classes of compounds such as alkaloids (vincristine, vinblastine, tylocrebrine, loclincrinine, cissamparine, monocrotalin), cardenolides (apocannoside, caloptropine, cymarin), lignans (podophyllotoxin), flavonoids (eupatroin), tannins, proteins, sesquiterpene lactones (galladrin), tetracyclic triterpenes (elatericin A & B, elaterin) which are known to cause tumour inhibition (Farnsworth, 1966), possible anti-tumour compounds that do not belong to these classes would be missed out in such a procedure, despite the laborious efforts involved.

According to O'Neill and Lewis (1993), the requirement of the sample preparation is to optimise the chances of detecting any chemical type of biologically active molecule present in the plant extract, with the exception of non-selective interfering agents. In a high throughput screen (HTS) a key project management decision needs to be made on the best use of available resources, to screen a variety of solvent extracts from each plant sample, or to screen one extract from an increased number of plant samples.

Although the procedures used in the traditional medical system for preparation of plant extracts for administering to patients may be suitable for clinical evaluation involving holistic mechanisms, including absorption into the system, such extracts may not effect a detectable response in an *in vitro* system. Hence, for mostly *in vitro*, investigational purposes of a research, an extraction method needs to be developed, to effect an exhaustive extraction of the plant constituents. An exhaustive sequential extraction

procedure starting with non-polar solvent to more polar solvent would enable extraction of most of the extractable compounds from the plant matrix.

Even if the method of extraction of anticancer agents from plants is valid, if the primary *in vitro* test system is not sensitive or selective enough to pick the activity of a potential anticancer agent, it would lead to rejection of the plant. Hence, it is important to study the basis, sensitivity, efficacy and the validity of the leukaemia and tumour test systems used in the screening program. A mild method of extraction does not always permit separation of most of the active compounds into the solvent, as there are not any universal solvents that extract all compounds likely to occur in a crude plant extract.

The extraction method, which parallels the method of administration of an herbal mixture, stipulated in medical treatise, would yield most of the compounds necessary for therapeutic activity of the crude extract. Extremely sensitive methods of detection are required to pick up the active compounds in minute quantities.

On the other hand, even though many compounds can be extracted into the solvent (s) in hot extraction procedures, thermolabile compounds would not be recovered intact. Destructive methods such as electron-impact ionisation and subsequent fragmentation in GC-MS might obscure structural information on the analytes.

Hence, extraction for structural investigations and the method of extraction for therapeutic usage may not necessarily be the same.

1.5 Anticancer activity

According to Suffness and Douros (1982), many compounds that are cytotoxic to tumour cells *in vitro* have been cited as *in vitro* anticancer or tumour inhibiting agents. Toxic substances that do not show any particular selectivity toward tumour cells as opposed to normal cells, according to Suffness and Douros (1982), have little or no hope of being useful as anticancer agents.

The terms, "cytotoxic activity", "antitumour activity" and "anticancer activity" are used depending on the hosts such as cells, animals and humans used in the experiment. Since the experiments discussed in this thesis involved *in vitro* testing of cancer cells, the

observed activity was taken to mean *in vitro* anticancer, cytotoxic or cancer growth inhibiting activity.

1.6 Anticancer drug development approaches

Most of the current efficacious drugs in clinical use have been isolated, derived from plants or synthesised based on the structural analogues isolated from medicinal plants (Cragg and Norman, 1999). The approaches taken in the anticancer drug development such as screening for cytotoxic agents, mechanism based and broad-spectrum approaches are discussed in the following sections.

1.6.1 Screening for cytotoxic agents

Table 2 Procedure for development of antitumour agents from plants according to Suffness and Douros (1979)

1. Identification and collection of plants
2. Preparation of extracts for screening
3. Screening of extracts for antitumour activity
4. Recollection of plants of interest for fractionation
5. Fractionation studies leading to isolation of pure compound
6. Characterisation of new active compounds
7. Tumour panel testing
8. Large scale procurement and production
9. Formulation and toxicology
10. Clinical trials

The five systematic approaches for the selection of plants as potential sources for novel biological agents are, (1) random collection, (2) selective taxonomic (i.e. based on family and genera), (3) chemotaxonomic (phytochemical), (4) ethnomedical leads and (5) the technical-information-managed approach (Cordell *et al*, 1993; Schwartsmann and Workman, 1993). The above criteria of selection of plants and serendipitous observations have led to the discovery of several cytotoxic anticancer compounds. The procedure according to Suffness and Douros (1979), adopted in most of these approaches is given in Table 2.

The basis of the anticancer drug development approaches to date has been the use of cytotoxicity to identify effective compounds. The cytotoxic approach however has resulted in the discovery of only a few effective compounds. The most recent findings of the human genome project have led to the development of a novel drug category aiming at suppressing oncogene(s) or alternatively activating the tumour-suppressor gene. (Van Gent *et al*, 2001).

The basis of the *in vitro* and *in vivo* cytotoxicity test system used in the early NCI screening programme is discussed in this section, in order to facilitate the interpretation of previous NCI test results that will be discussed in 1.7.1.2 and 1.7.2.2.

1.6.1.1 Basis of *in vitro* cytotoxic test system

The cytotoxicity in the NCI's earlier screen has been based on the inhibition of cell protein synthesis. The mouse nasopharynx (KB) solid tumour cell-line has been used as a sensitive system to evaluate anticancer drugs (Suffness & Douros, 1979). Selection of the KB cell line for the NCI screening programme, was based on its rapid and reproducible growth as a monolayer culture since all cells grown in monolayer culture behave similarly in their response to cytotoxic drugs (Schepartz *et al.*, 1967).

In the NCI's original screening programme, *in vitro* tests were done with very small quantities of a material as a prelude to the *in vivo* testing that require relatively large quantities of the same material. Materials having only *in vitro* cytotoxic activity were discontinued from entering into the clinical trial stage of the screening programme. Promising materials have been selected for *in vivo* screening based on the 2-stage testing system. In order to pass the first stage, the ED_{50} value, dose in μg/ml that inhibits growth

to 50% of control growth, of a material must be ≤ 4 μg/ml for a pure compound and 20 μg/ml for a plant extract. A material was considered to have passed the second stage, if it has the arithmetic mean of ≤ 1 μg/ml from the first two tests. Such *in vitro* activity level is expected to enhance the activity in the *in vivo* screen. A material that passes the second stage is sent for confirmation to a second laboratory (Schepartz *et al.*, 1967).

In addition, the criteria for determining the degree of tumour growth inhibition compared with the control have been set out by the NCI as "strong" (S) for 76-100%, "moderate" (M) for 51-75%, weak (W) for 26-50%, inactive (I) for 0-25% and enhancement (E) for negative %. Performance of the *in vitro* screen is judged by estimating reproducibility of the ED_{50} value based on tests of positive control material carried out at 4 laboratories in 10 to 47 replicates, at 5 dose levels at 0.3 log intervals (Schepartz *et al.*, 1967).

Most of the clinical anticancer agents available in the market are active against the P388 leukaemia system (Boyd *et al*, 1992). Although the leukaemia test systems have not proved to be very successful in isolating agents against solid tumours, P388 and L1210 leukaemias have been very effective as a prescreen in activity-directed fractionation based on fractional cell kill concept (Schwartsmann and Workman, 1993).

At present NCI's *in vitro* screening protocol (Paull *et al*, 1989, http:// ://dtp.nci.nih.gov/docs/compare/compare.html) entails testing of samples against a diverse panel of 60 human cancer cell lines derived from nine cancer types organised into subpanels representing leukaemia, lung, colon, central nervous system, melanoma, ovarian, renal prostate, and breast cancer (Cragg and Newman, 1999).

NCI's Criteria for retesting and designation as "Active" compounds/extracts according to NCI, Website, (2000) are;

1. 50% growth inhibitory potency (average GI50) $=10^{-7.5}$ M or 0.1 μg/ml);
2. Subpanel specificity (at least 3 cell lines of a sub panel are sensitive);
3. Differential sensitivity (10 or more cell lines are at least one log more sensitive than the average of the other cell lines);

4. Individual specificity (from 1 to 10 cell lines are at least one log more sensitive than the average response);

5. Significant calculated variance in average 50% growth-inhibition (GI50), total growth inhibition (TGI) or cytostatic effects and "net cell killing" or cytotoxic effects (LC50) endpoints;

6. Significant calculated variance in the percent growth at a particular dose, and

7. Meeting general quality control criteria.

Compounds are designated "active" after retesting, if they meet criteria 1 through 6 in a reproducible manner. Attention is paid to whether the same cell lines show differential sensitivity in the second test and the computer analysed growth mean graph patterns or COMPARE profiles, are reproducible (Paull *et al*, 1989). If reproducible COMPARE profiles consistent with the mode of action of a standard agent, the compound is designated as "active" but is of interest only if it represents a novel chemotype. Compounds that meet the criteria for further testing are evaluated by the *in vivo* hollow fiber assay that assesses the sample activity against 12 human-cancer cell lines implanted in mice. An important criterion is whether the same cell line shows differential sensitivity in the second test (NCI, http://epnws1.ncifcrf.gov:2345/ dis3d/itb/ commethods. Html #meangr, 2000).

Although it is the objective of the NCI's disease oriented screening programme to maintain a low concentration of plant extracts (<100 μg/ml), in order to emphasise the differential effects of test agents on various human cell lines, it would prevent detecting active agents in minute concentrations in a complex plant extract; even if it possesses direct *in vitro* growth inhibition of cancer cells. Present selection criteria of the NCI screening programme are highly selective to direct *in vitro* antiproliferative activity of the selected cell lines.

1.6.1.2 Basis for *in vivo* cytotoxic activity

The NCI's *in vivo* screening system was designed to select materials that have more than a specified minimum antitumour activity in at least one animal tumour system. In order to accomplish this, a multistage system had been established and a scheme of sequential analysis was developed during the 1960s. A material is considered to have passed the first

test, if it had produced a T/C value (the ratio of test (T) to control (C) tumour weight, or survival time of test animal) of less than 54% and it would then be retested at the same dose. At the end of the second test, the product of the T/C values, expressed as a decimal, for the first two tests was calculated and if the value > 0.20, the material is rejected. If the material passes the second stage, it is tested a third time. For a material to pass the third stage, the product of the T/C values for the three tests must be less than 0.08. Such sequential system was designed to enable the most work to be done on the most active materials (Schepartz *et al*, 1967).

A clearly inactive material is rejected after only one test. Borderline results, which have passed the lower limit of T/C=0.54, will usually fail on the second trial which requires a mean T/C value of 0.45, or 55% inhibition as opposed to only 46% inhibition. The third trial imposes a still tougher limit by requiring an average T/C value of 0.42, or 58% inhibition, for all 3 trials. Truly active materials will go through 3 trials, followed by a minimum of 3 confirmation tests (Schepartz *et al*, 1967).

Table 3 NCI's three-stage sequential schemes for selection of materials

Scheme 1	Scheme 2
Stage 1: T/C < 0.54	Stage 1: T/C < 0.64
Stage 2: T/C < 0.20	Stage 2: T/C < 0.24
Stage 3: T/C < 0.08	Stage 3: T/C < 0.09

As additional tumour testing systems were added to the screen, it was observed that different sequential schemes were necessary, depending on the reproducibility and sensitivity of the system. For simplicity, acceptance of materials tested against all tumour systems was assigned to one of the two schemes summarised in Table 3 (Schepartz *et al*, 1967).

The human Friend virus leukaemia system was assigned to more "liberal" scheme 2. All the other tumour systems used for testing compounds and plant extracts have been assigned to Scheme 1. Shepartz *et al* (1967) observed that these acceptance levels were far below those required for a significance test such as the *t*-test. A significance test has been considered a necessary but not sufficient criterion for acceptance, since it accounts only for the variation among test objects.

Based on prior observation, all materials passing the 3-stage screen in one of the solid tumour systems are given 3 additional tests, for which a mean T/C value of 42% is required for acceptance (NCI's Instruction 14 (Rev.9), 1985).

In the case of a survival time measurement of the test animal, the L1210 was assigned 2-stages of the sequential scheme 2. For the first stage, it is necessary for the T/C ratio to exceed 125% meaning 25% increase in the mean survival time of the test animal over control. To pass the test, a material must produce an effect such that the product of the T/C values of the 2 tests, expressed as a decimal, is ≥ 1.56. The confirmation procedure consists of a dose-response experiment, at doses both higher and lower than the dose at which the sequential tests were done. If at least one dose of a material produces a T/C $\geq$ 125%, it is considered confirmed. Specific weight change differences for each solid tumour system have been considered evidence for toxicity based on prior experience (NCI's Instruction 14 (Rev.9), 1985).

Final acceptance of a compound as sufficiently active in a solid tumour system, for clinical evaluation has been based on a reasonable spread between the maximum tolerated dose (MTD), determined by death, by host weight loss and the minimum dose producing a significant effect (MED) (i.e. T/C $\leq$ 42% for SA). Reproducibility in more than one laboratory is also a prerequisite. A reproducible T/C of less than 10% at a dose below the MTD is also accepted as sufficient activity. For SA and for WA a therapeutic index, ratio of MTD/MED, of 1.5 and 2.0 is required respectively (Schepartz *et al*, 1967). For an *in vivo* test system of L1210, the criterion for acceptance of a material, as a potential candidate for clinical trial is at least 50% reproducible increase in survival (Schepartz *et al*, 1967; NCI's Instruction 14 (Rev.9), 1985.).

Reproducibility and stability have been considered as important characteristics of a screening system. As a measure of reproducibility the "between-test" log standard

deviation of the T/C values of the tumour systems has ranged from 0.11 to 0.25. Experimental groups and sequential schemes were assigned based on this standard deviation. Inherent variability of the animal systems has caused variation in T/C values from test to test. Hence close T/C values of two successive tests are not a result of unusual reproducibility of the material, but merely a chance occurrence. According to Schepartz *et al* (1967), to ascribe any special qualities to a material because of closely similar T/C values is to misread the results of the animal system.

Owing to the complex nature of a crude plant extract, high reproducibility of the results is very difficult to achieve. Hence, a crude plant extract would often fail in an animal screen due to the problem of reproducibility in results. Performance of the NCI's early *in vitro* screen was judged by estimating reproducibility of the concentration causing 50% growth inhibition (ED_{50}) value based on tests of positive control material carried out at 4 laboratories in 10 to 47 replicates, at 5 dose levels at 0.3 log intervals (Schepartz *et al*, 1967 and NCI's Instruction 14 (Rev.9), 1985).

1.6.1.3 NCI test criteria and development of Taxol® (Paclitaxel)

The validity of the above *in vitro* criteria was observed by Cragg (1998) citing the development of the first clinically effective new class of anticancer drug paclitaxel (Taxol®) from the tree *Taxus brevifolia* Nutt, belongs to *Taxaceae* family.

Although the use of *Taxus brevifolia* and other *Taxus* species (e.g. *candensis*, *baccata*) by several American tribes for the treatment of some non cancerous conditions has been reported (Hartwell, 1982), the leaves of *Taxus baccata* have been used as a traditional Asian medical treatment in Ayurveda (Kapoor, 1990), with one reported use in the treatment of "cancer" (Hartwell, 1982).

Of the collected two samples of stems and fruit (PR-4959) and stems and bark (PR-4960) of *T brevifolia* Nutt, only the ethanol extract of the latter showed *in vitro* cytotoxicity against the human epidermoid carcinoma of the nasopharynx (KB) cells. The mouse *in vivo* screening against L1210 leukaemia cells proved toxic at 500 mg/kg/day and retesting at a dose of 250 mg/kg/day showed no activity. However, tests in the Dunning leukaemia model and P-1798 lymphosarcoma system found no anticancer activity. Yet the decision to begin fractionation studies of *T. brevifolia* Nutt., bark was made on KB cytotoxicity

alone, based on the observation made during the isolation studies of camptothecin which indicated that there was an excellent correlation between the *in vitro* KB cytotoxicity and *in vivo* anticancer activity. Fractions of the sample P-4960, were found to be active against mouse *in vivo* P-1534 leukaemia, Walker 256 carcinosarcoma and P388 leukaemia (Wani *et al*, 1971; Suffness and Wall, 1995).

According to Cragg (1998), Taxol® initially exhibited only a moderate *in vivo* activity against the P388 and L1210 murine leukaemia models, like many other agents it was not considered to be a promising candidate for further development when compared with a number of other available compounds. It was the strong activity observed against the B16 melanoma in the NCI panel in 1975 that stimulated interest in Taxol® as a drug candidate for preclinical development. Having demonstrated the mechanism of Taxol action in promoting tubulin polymerisation and stabilisation of microtubules against depolymerisation (Schiff and Horwitz, 1979) and after completing the formulation studies in 1980, Taxol was approved by the NCI for Phase I clinical trials in 1983.

Initial clinical trials with Taxol® resulted in serious safety problems such as toxicity, allergic reactions including anaphylaxis due to poor solubility of Taxol® in aqueous systems as well as relatively high dose requirements. Taxol® was very close to being dropped from the clinical study Cragg (1998b). Isolation of Taxol was effected by carefully following the bioactivity of the compound using in vitro KB cytotoxicity and in vivo antitumour activity mainly against Walker 256 carcinosarcoma and P1534 leukaemia systems. Considerable variation was observed in the KB assay data between runs of the fractions. Although the same fractions were consistently cytotoxic, the degree of their cytotoxicity was poor. Hence it had been very difficult to determine whether the activity was concentrated or not, as the evaluation of the fraction relied heavily upon the *in vivo* results, requiring gram quantities of extracts and fractions. Although HPLC provides a good fractionation on an analytical scale, at the time of the Taxol® isolation, preparative HPLC was not available. The isolation of Taxol® involved ethanol extraction of 12 kg bark followed by partitioning between $CHCl_3$ and water. The activity was transferred into the $CHCl_3$ phase and this material was then passed through a series of Craig counter current distributions yielding 0.5 g of pure Taxol® (Suffuses and Wall, 1995).

Taxol® was found to be quite potent at inhibiting the growth of both human and murine tumour cell lines. Taxol® has shown IC50 (50% inhibition of tumour cell growth) values ranging from 1-10 μg/ml using the *in vitro* human tumour stem cell assay whereas the majority of the NCI human panel gave <2.5 nM and less sensitive UO-31 (renal carcinoma), HCT-15 (colon carcinoma), and OVCAR-5 (ovarian carcinoma) gave 2 μM, 214 nM, and 11 μM as IC50 values, respectively. However the 50% cell kill endpoint (LC_{50}) could not be achieved at maximum Taxol® concentration tested, 25 μM, and the majority of the NCI cell lines required a Taxol® concentration of ≥25 μM for 100% growth inhibition. Hence, in excess of 10,000-fold more Taxol® was typically required for complete inhibition of cell growth, in comparison with the dose needed to inhibit cell growth by 50% (Rose, 1995).

Taxol® has not shown any subpanel or histological cell type selectivity but general cytotoxicity against several cancers (Rose, 1995) and is now widely used in the treatment of a range of different cancers. Results of four clinical trials with Taxol® indicated responses in 20-50% of the patients with ovarian cancer achieving mostly partial remission. Also three separate clinical studies have shown that Taxol® was effective in producing responses in patients with metastatic breast cancer (Cragg, 1998).

1.6.1.4 Screening for disease specific cytotoxic agents

The NCI has been conducting a large-scale anticancer drug-screening programme for over 30 years. As the screening programme of the NCI progressed, alternative screening strategies have been introduced. Most of the anticancer drugs in clinical use were found to exert limited activity against the common adult solid tumours (Masters *et al*, 1992). Hence the concept of the "disease-oriented screen" using a panel of 60 human tumour cell lines from nine different cancers (i.e. leukaemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancers) *in vitro* was introduced in 1995. Most of the anticancer drugs in clinical use were found to exert limited activity against the common adult solid tumours (Masters *et al*, 1992). The aim of the disease-oriented screen was to identify compounds with differential or tumour-selective growth inhibitory properties (Boyd, 1989).

1.6.1.5 Latest development of the NCI screen

Since about 85% of the compounds screened in the human 60-cell line panel gave no evidence of antiproliferative activity, in 1999, a prescreen consisting of highly sensitive three-cell line panel was introduced before advancing to the full 60-cell line. The reason for the introduction of three-cell line panel comprising MCF-7 (breast carcinoma), NCI-H460 (lung carcinoma), and SF-268 (glioma) is that these highly sensitive three-cell lines have detected antiproliferative activity in ≥95% of the compounds that were ultimately considered for further *in vivo* testing. This indicates that the most sensitive representative cancer cell line from the disease-oriented screen is a significant common invasive characteristic of the disease cancer. In the case of natural products extracts, a prescreen is carried out with the same three-cell lines at a concentration of 100 μg/ml. Those extracts that show total growth inhibition in any of the three cell lines are selected for further testing on the panel of 60-cell lines. In addition, the hollow fibre assay was subsequently introduced to the NCI screen. (NCI, http://epnws1.ncifcrf.gov:2345/dis3d/itb/commethods.html#meangr, 2000).

1.6.1.6 Combination cytotoxic chemotherapy

Chemotherapy for cancer involves the use of cytotoxic and antiproliferative drugs either alone or in combination with other treatments such as surgery and radiation. The cytotoxic and antiproliferative drugs can be classified into five functional groups such as alkylating agents, antimetabolites, natural products or derivatives, synthetics, and steroids.

Combination chemotherapy with more than one drug has shown better results in controlling cancers than with a single drug. Einhorn (1990) reported the cure of metastatic testicular germ cell tumours of men after treating them with combination therapy. This is an example of using more than one cytotoxic active principle to exert a therapeutic effect. However, this approach does not include combination of different biological activities

1.6.1.7 Dereplication approach

Another approach for discovering novel compounds from plant involved a simplification step called dereplication. Dereplication is the avoidance of repeated testing of crude extracts for commonly occurring or previously known active compounds in order to

minimise the effort lost in their isolation as early as possible (Suffness, 1986). In an effort to simplify the extraction procedure, which was targeted towards new chemical structures from crude extracts of natural products including plants, a dereplication or elimination procedure to exclude commonly occurring tannins and anionic polysaccharides from the extract was followed (Cardellina II *et al*, 1993).

Computerised COMPARE pattern-recognition algorithms which correlate similar patterns of differential cytotoxicity produced by extracts, fractions and pure compounds tested in NCI's 60-cell-line-panel are used for preliminary biological dereplication (Cardellina *et al*, 1993).

Constant and Beecher (1995) suggested the use of a HPLC/electrospray mass spectrometry technique that provides a direct method of determining the molecular weights of compounds in a crude extract with little or no fragmentation as a tool for preliminary dereplication. Such information when combined with the information given in a scientific databases (i.e. NAPRALERT) enable a matching of the compound with the known ones in the same genus. Although such an approach has enabled the discovery of novel active compounds for a specific target, the possibility of a known compound escaping an important bioactivity screen or the failure of a novel compound to exert an *in vivo* effect in combating cancer is still a major problem.

1.6.2 Mechanism based approach

In this section several anticancer drug development approaches targeting various mechanisms of cancer progression and inhibition, such as catabolic anabolic enzyme kinetics of cancer cells, induction of non-clonogenic cytotoxicity, induction of apoptosis, and cell cycle phase-specific inhibition are discussed. As described in section 1.1.2, the restoration of an imbalance between catabolic and anabolic enzyme kinetics leading to carcinogenesis may be modified by enzyme activators i.e. Zn, Cu, Mg, Mn or Co or enzyme inhibitors such as Hg, Ag, and Au (Furst, 1963).

A major emphasis in developing anticancer drug has been to use assays to screen drugs exerting non-clonogenic cytotoxicity based on cell damage, in the whole tumour population, rather than in the proliferative or clonogenic cell fraction (Fridborg *et al*, 1995). Such drugs are purported to act differentially, killing the whole cancer cell population whilst leaving the normal cells of the body intact. Another category of

anticancer drugs that destroys the whole cancer cell population, is based on apoptosis where the selected drug induces the expression of a specific gene that triggers the programmed death of cancer cell population.

Activity of an anticancer drug can also be classified according to its mechanism of action on a particular stage of the cell cycle of cancer cells. The phases of the cell cycle progression and the activity of some of the anticancer drugs against respective phase are illustrated in Figure 2. The progression of the eukaryotic cell cycle checkpoints is regulated by cyclin-cyclin dependent kinase (cdk) complexes, by initiating a phosphorylation cascade leading to the transcription of genes required respectively for proliferation, the phosphorylation of nuclear lamins and histones for nuclear membrane disruption and chromosome condensation (Hunter and Pines, 1994).

Although the phase of the cell cycle at which the drugs exert their activity, is important (Figure 2), other factors including the concentration, exposure time and the tissue type may alter the phase specific effect of a drug.

Vinblastine and vincristine have shown arrest of HeLa cells in mitosis by dissolution of the mitotic spindle. Cells exposed to 0.3 μg/ml of vincristine for 3 hours have shown more sensitivity in S and in late G_1 than in early part of G_1 or in G_2, whereas cells exposed to vincristine (0.1 μg/ml) have shown effectiveness during the S phase. Also vinblastine has shown an immediate interphase death by lysis of cells treated in late G_1 phase (Madoc-Jone and Mauro, 1968).

The naturally occurring spindle poisons, namely, vincristine, vinblastine, podophyllotoxins and paclitaxel (Taxol®) were found to inhibit the proliferation of the cell cycle at the G_2-M phase thereby blocking mitosis. However, the mitotic inhibition of paclitaxel is unique because it stabilises microtubules and inhibits depolymerization back to tubulin (Suffness, 1995).

The plant flavonoid, apigenin showed significant inhibition of UV-induced mouse skin tumorigenesis by reversing G_2/M arrest in cultured keratinocytes (Lepley *et al*, 1996). The mechanism of apigenin, according to Lepley *et al* (1996) is partly due to inhibition of the mitotic kinase activity of $p34^{cdc2}$, and perturbation of cyclin B1 levels.

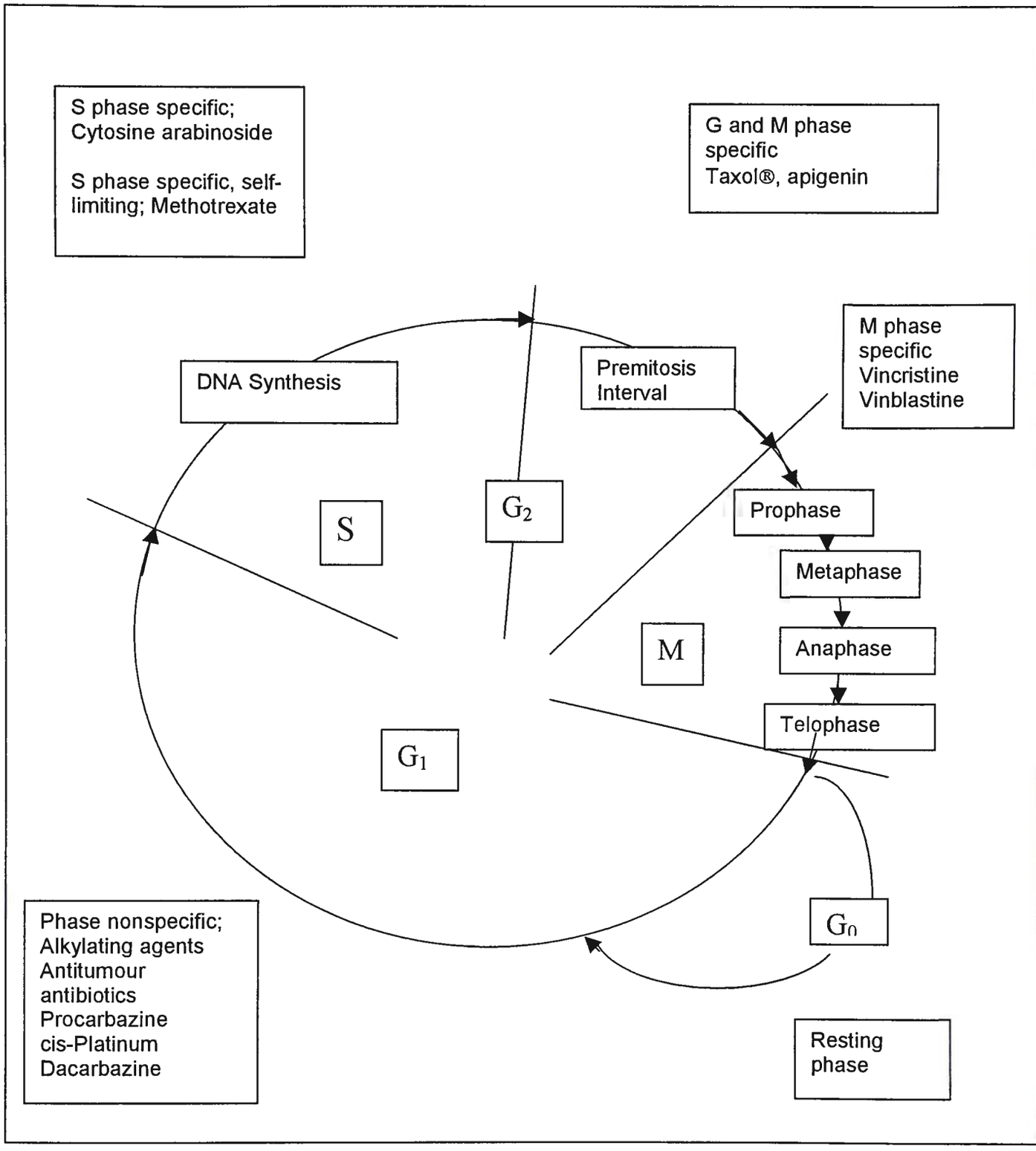


Figure 2 **Cell Cycle showing stages at which some of the useful anticancer drugs have been suggested to play their inhibitory role (modified from Furst, 1963; Lepley *et al*, 1996)**

The assay of percentage of cells retained in G_1, S and G_2/M phases of the cell cycle has been carried out by Lepley *et al* (1996) using flow cytometry followed by the data analysis with the aid of the CellFit Cell Cycle Analysis computer programme. The protein complexes affecting the progression of cell cycle were assayed by using Western blot analysis and immune complex $p34^{cdc2}$ kinase assay, whereas the uptake of the anticancer agent by the cancer cells was assayed using an aliquot of radioactive tritium incorporated drug (Lepley *et al*, 1996).

The NCI has screened over 50,000 compounds for antitumour activity *in vitro*, which generally targeted DNA, RNA, and protein synthesis or function. Although the understanding of molecular and tumour biology has advanced tremendously during the past decade, development of cytotoxic anticancer agents as single drugs in clinical use has been a failure or has achieved only a partial success.

Table 4 Approach of anticancer drug screening including variety of molecular targets

Tumour vasculators
Angiogenesis inhibitors
Disruption of matrix-vasculature interactions
Destruction of tumour vasculature
Invasion and metastasis
Modulation of adhesion molecules
Matrix metalloproteinase inhibitors
Cell cycle controlling/cell signalling
Modulation of cell cycle checkpoints and cell signalling
Modulation of gene expression
Mechanism of apoptosis
Modulation of bcl-2
Immunological recognition and response
Induction of immune response to tumour antigens
Immunotoxins and targeted reagents
Catabolic enzyme stimulation
Cox-2 enzyme inhibition

As the knowledge about enzymes and receptors increases, new biological targets can emerge which will create new bioassays more relevant to the clinical effects. Some of the assays have appeared to be more relevant to clinical anticancer effect (see Table 3). The NCI recently expanded its screening portfolio to include a wide variety of new molecular and cellular therapeutic targets and approaches (Christian *et al*, 1997), some of which are given in Table 3. Although these assays have been developed mostly as single assays by researchers working in separate research areas, their possible use and efficacy as a battery of assays aiming at the broad-spectrum activity of a plant extract in the whole body need to be investigated.

1.6.3 Broad spectrum approach aiming at whole body

As discussed in the above sections, cytotoxicity based anticancer drug development approaches have failed to yield an effective drug with minimum adverse effects. However, investigations that have been carried out separately during the past decade have indicated indirect anticancer properties such as antioxidant, immunomodulatory activity including cyclooxygenase-2 enzyme inhibitory and inhibition of platelet aggregation. Hence it is important to investigate the possibility of using these broad spectrum of activities in future anticancer drug screening and development. Some of these indirect anticancer activities that may be used in a broad-spectrum approach that complement the whole body efficacy of a complex mixture of drugs or a crude plant extract are discussed in this sections.

1.6.3.1 Antioxidant activity

In this section, disturbances to the redox balance resulting oxidant-state leading to a cancer, sources of free radicals, and body's defence are discussed in order to understand the role played by antioxidants as anticancer agents. In addition, types of different antioxidants and reported anticancer activity of some antioxidants are also discussed.

1.6.3.1.1 Redox balance and health

Oxidants and antioxidants (reductants) have well defined functions and reside in specific cellular compartments of the body. Control of oxidant-antioxidant balance or redox homeostasis is important to maintain the body in healthy state. The redox state is defined as the ratio of the reduced form to the total (reduced and oxidised) form (Hwang *et al*,

1992). Rose (1989) suggested that the relative amounts of oxidised and reduced forms of major antioxidants in the body might be either a determinant or a useful indicator of a disease state. Rose and Bode (1995) further stressed the importance of measuring the levels of the endogenous biological antioxidants as an index of tissue health because of their possible role in maintaining the health.

1.6.3.1.2 Oxidative stress and cancer

Oxidative stress, a disturbance in the prooxidant-antioxidant balance in favour of the former (Sies, 1993) and the cell damage by oxygen radicals and lipid peroxidation plays a crucial and perhaps causative role in the pathogenesis of a number of chronic and acute diseases, such as cancer (Esterbauer, 1996). An oxidative stress results in formation of free radicals i.e. superoxide anion ($O_2^{\cdot}$). A superoxide anion can transform into more reactive hydroxyl radical and other reactive oxygen species that could induce tissue damage and inflammation, initiate chain reactions forming lipid peroxides and toxic compounds. Because of the potential damage caused by the reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, hydrogen peroxide and singlet molecular oxygen on DNA, proteins, carbohydrates and unsaturated lipids, the ROS are implicated in the aetiology of many diseases (Sies, 1985 and 1991; Prasad and Laxdal, 1994).

According to Yagi (1987), lipid peroxidation is strongly associated with carcinogenesis and mutagenesis. Cerutti (1994) suggested that the inactivation of tumour suppressing gene P53 by oxy-radicals along with destruction of DNA structure may promote the development of cancer. In such a situation, antioxidants may provide protection against development of tumours depending on the target tissue and the carcinogen (Cerutti, 1994; Paya *et al*, 1995).

The hypothesis that lipid peroxidation can mediate DNA damage was supported by the findings of Hruszkewy (1988). Hruszkewy (1988) showed that the DNA isolated from peroxidised rat liver mitochondrial preparations *in vitro* had a completely different electrophoretic mobility due to cross linking, from the DNA isolated from the mitochondria protected from peroxidation by adding the antioxidant, α-tocopherol.

1.6.3.1.3 Sources of free radicals

The four endogenous sources of free radicals that presumed to be the sources of most oxidant species generated within the body are given below.

1. The mitochondria generate $O_2^{\cdot}$, H_2O_2, and ·OH as by-products of aerobic respiration.

2. The phagocyte cells generate NO·, $O_2^{\cdot}$, H_2O_2, and $OCl^{\cdot}$ during the destruction of bacteria or virus-infected cells by oxidative burst.

3. Peroxisomes produce H_2O_2 during the degradation of fatty acids which is normally degraded by the enzyme, catalase. According to Kasai *et al.* (1989), some of the H_2O_2 escape degradation resulting in an oxidative stress condition.

4. Induction of cytochrome P450 enzymes during detoxification also generate oxidant by-products that damage DNA.

In addition to the above endogenic sources of free radicals, the following exogenic sources also can contribute to the load of various oxidant species:

1. The nitrogen oxides of the cigarette smoke cause oxidation of macro molecules (Frie *et al*, 1991)

2. Fe and Cu salts in excess generate the peroxide radicals via the Fenton-pathway.

3. Chlorogenic and caffeic acids from natural sources may generate oxidants by redox cycling (Halliwell and Gutteridge, 1989).

According to Ahmad (1995), O_2 dependence imposes universal toxicity to aerobic life processes. Reduction of one electron from O_2 generates the superoxide anion, $O^{\bullet -}$, from various biological redox-active autoxidizable sources such as catecholamine, oxidoreductases and subcellular organelles such as mitochondria, endoplasmic reticulum (microsomes), nuclei and chloroplasts (Ahmad, 1995).

Sulphoxide free radicals are converted to H_2O_2 via the Fenton reaction to the hydroxyl free radical, •OH. These and other forms of activated O_2 constitute reactive oxygen species (ROS) or metabolites (ROM). The most reactive forms of ROS are •OH and $^1O_2$. Their deleterious reactions include oxidation of proteins, DNA, steroidal compounds,

peroxidation of the cell membrane's unsaturated lipids to form unstable hydroperoxides, and their highly reactive peroxidation products including malondialdehyde (MDA) and hydroxynonenals that threaten cellular integrity and function.

Oxygen metabolism, carcinogen metabolism and radiation also can cause the formation of reactive oxygen free radical species. Free radicals that continue to propagate the peroxidation chain reaction called "endogenous oxidative stress", which all aerobic organisms must cope. Involvement of free radicals during conversion of the native LDL to the more atherogenic, oxidatively modified LDL was evident by the results from investigations employing antioxidants (Steinberg *et al*, 1989). According to Esterbauer *et al* (1990), several antioxidants such as α-tocopherol, γ-tocopherol, β-carotene, ubiquinol-10, and lycopene are contained in LDL itself. When these antioxidants are exhausted the oxidative modification occurs.

1.6.3.1.4 Body's multilevel defence system against radicals

The body's defence line according to Gey (1992) consists of;

1. Enzymes; superoxide dismutase, catalase, and glutathione peroxidase

2. Non-essential endogenous radical scavengers including; glutathione (GSH), proteins, uric acid, and ubiquinol-10

3. Essential radical scavengers; antioxidant vitamins C (ascorbic acid) and E (α-tocopherol)

4. Singlet oxygen quenching carotenoids such as β-carotene and vitamin A (retinol) (Di Mascio *et al.*, 1991; Sies 1985, 1991)

The free radical species generated by oxygen metabolism, carcinogen metabolism and radiation are suggested to be scavenged by the endogenous ascorbates and urates. Such scavenging reduces the potential of the free radical to be toxic while oxidising the scavenger to a non-antioxidant form (Halliwell and Gutteridge, (1985); Becker (1993); Pryor (1984) and Ames *et al* (1981). Ascorbate oxidises to dehydro-L-ascorbic acid, urate to allantoin and glutathione (GSH) to GSSG. Some antioxidants such as GSH function by eliminating hydrogen peroxide, a free radical precursor or as a reducing cofactor in enzymatic reactions of other antioxidants (Hughes, 1964).

The level of essential antioxidants, unlike the other antioxidant defence lines, mainly depends on their dietary supply. If the free radicals are involved in pathological mechanisms, an optimum status of essential antioxidants should reduce the risk of a disease. According to Gey (1992), this optimum status of essential antioxidants is a prerequisite to the "optimum health" defined by the World Health Organisation (WHO). The basis of the above working hypothesis according to Gey (1992) is the general assumption based on many epidemiological studies world wide that vegetable-rich diets are associated with a higher life expectancy by preventing degenerative diseases such as cancer. The disease preventive and optimum health preserving effect of antioxidants is evident by the results of the research carried out by Block (1991), Ziegler (1991), Gey (1992) and Stahelin *et al* (1991).

Block (1991) and Ziegler (1991) found that the calculated dietary intake of essential antioxidants has been inversely related to the risk of major causes of death such as cancer and coronary heart disease in western societies. The measured plasma levels of essential antioxidants also revealed inverse relationship with the major causes of death.

According to the analysis of epidemiological data of the WHO/MONICA project carried out during 12 years by Gey (1992), there was a significant correlation ($n=2421$, $p<0.05$) between low plasma levels of β-carotene, vitamins A, C and E and subsequent cancer. Poor β-carotene level was predictive for most cancers. Low plasma levels of vitamin C and A significantly increased the risk of having gastrointestinal cancers. Stahelin *et al* (1991) observed that the low levels of several antioxidants e.g. β-carotene and vitamin A had an additive effect on the risk of being diagnosed with cancer.

1.6.3.1.5 Types of antioxidants

The definition of antioxidants is based on the mode of action of the antioxidant on the process of lipid peroxidation. Lipid peroxidation being the major cause of the deterioration of food quality has been studied extensively. Antioxidants are compounds that retard or prevent autoxidation processes. Autoxidation of polyunsaturated lipids of food involves a free radical chain reaction generally initiated by exposure of lipids to light, heat, ionising radiation, metal ions or metalloprotein catalysts and lipoxygenase enzymes. Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and acting as oxygen scavengers

According to Reische *et al* (1998), primary antioxidants (Type I) or chain-breaking antioxidants are defined as the free radical scavengers that delay or inhibit the initiation step and or interrupt the propagation step of autoxidation. They react with lipid and peroxy radicals converting them to more stable, non-radical products. Examples of some synthetic primary antioxidants are mono or polyhydroxy phenols, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and poly gallate (PG). The naturally occurring antioxidants, tocopherols and β-carotene are example of primary antioxidants, although their mechanism of action is different..

The Secondary Antioxidants (Type II) are compounds that chelate prooxidant metals, replenish hydrogen to primary antioxidants, and decompose hydroperoxides to nonradical species.

The above definitions of antioxidant types are basically based on the preventive mechanisms of lipid peroxidation in foods, which has been isolated from a living system. Although the main justification of using antioxidants in food is to extend their shelf life, antioxidants according to Sherwin (1978) and Coppen (1983), cannot improve the quality of already oxidised food products.

Referring to biological systems, Halliwell & Gutteridge (1995) defined an antioxidant as "any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate". Moreover, oxidative stress may lead to the formation of oxidative species other than free radicals. Because of their contribution to the redox imbalance, formation of any oxidative species may play a role in the initiation of a disease. Thus, not only the scavenging of free radicals but also the non-free radical oxidative species need to be neutralised by the antioxidant in order to act as an effective therapeutic agent.

1.6.3.1.6 Antioxidants as anticancer agents

Antioxidants may play not only a preventive but also an inhibitory role in combating cancer. The inhibitory role may include free radical scavenging or restoration of redox-state that prevents the progression of cancer cell proliferation, or induction of cancer cell death. In addition antioxidants may induce an immune response against cancer cells. Thus, the therapeutic effect of antioxidant may involve restoration of disturbed redox

balance resulted from an oxidative stress, free radical scavenging, inhibition of cancer cell proliferation, induction of cancer cell death (apoptosis), and direct or immunomodulated cell growth inhibition or cytotoxicity.

In living systems, oxidative stress and lipid prooxidation that leads to formation of free radicals is thought to be strongly associated with carcinogenesis and other degenerative diseases (Esterbauer, 1996; Yagi, 1987; Cutler (1984,1991). Previous studies have indicated that biological antioxidants including carotenoids, ascorbate (vitamin C), glutathione, α-tocopherol (vitamin E) and retinol (Vitamin A), and the endogenous antioxidant enzymes (superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione transferase and catalase) are important in counteracting the oxidative stress (Packer, 1995). Naturally occurring antioxidants may cooperate with the natural defence system and they have been proposed for use in a number of diseases including artherosclerosis and different types of carcinoma (Sies, 1991; Bast *et al*, 1991).

Purpurogallin is a phenolic antioxidant extracted from various nutgalls of oak species as an aglycone of several glycosides (Claus, 1965). Some of the reported biological functions of purpurogallin have been the protection of ventricular myocytes and aortic cells (Wu, *et al.*, 1992) and hepatocytes (Wu, *et al.*, 1991) against oxyradicals produced by xanthine oxidase and hypoxanthines.

Fujita (1988) reported the occurrence of both the antioxidant activity and the cytotoxic activity of a plant extract that was effective in reducing or inhibiting tumour growth. According to Hong (1994), tannins inhibited the lipid peroxidation in rat heart mitochondria. Their preventive therapeutic application included suppression of mutagen or carcinogen whereas Adlercreutz (1995) suggested a possible role of antioxidative phytoestrogens such as diphenolic isoflavonoids and lignans as natural cancer-protective compounds.

According to Sen and Packer (1996) the regulation of gene expression by oxidants, antioxidants and the redox state has emerged as a novel subdiscipline in molecular biology that has promising therapeutic implications and direct involvement in pathogenesis certain of diseases including cancer and AIDS. They suggest that many basic events of cell regulation including protein phosphorylation and binding of transcription factors to consensus sites on DNA, are driven by physiological oxidant-antioxidant homeostasis.

Gallic acid, a naturally occurring plant phenol with antioxidant activity was found to induce cell death using a promyeloactive leukaemia HL-60RG cell line in culture (Inoue *et al*, 1994).

Pappalardo (1996) observed a significant decrease in the antioxidant capacity of colorectal mucosa of patients affected by colorectal cancer. Efficacy of antioxidant treatment in preventing progression rate of stomach precancerous lesions is being studied by de Sanjose *et al* (1996).

Possible immunological involvement of antioxidants in cancer prevention was reported by Zhang *et al* (1995). Although the oxidative-burst may stimulate the macrophage-mediated antitumour cell destruction (Flescher, *et al* 1984), triggering a macrophage to act against tumour cells is a difficult task.

An enhanced oxidant status has been observed in cancer patients (Gey, 1992). The antioxidant activity of a medicinal preparation may, because of its neutralising effect, exert a beneficial effect in a cancer patient in an enhanced oxidant state. Thus, antioxidant activity is an important parameter in a broad spectrum of assays designed to monitor anticancer agents in a medicinal preparation. It is proposed that the inclusion of an antioxidant assay in the broad-spectrum screen would increase the probability of detecting useful anticancer agent(s) in a plant extract.

Antioxidant activity has been measured using assays including free radical scavenging, thiobarbituric acid reacting (TBARS) substances, and thiocyanate assays.

1.6.3.1.7 Assay of antioxidant activity

Although different mechanisms of action are being suggested for antioxidant activity of different antioxidants, a common function of an antioxidant is known for their function to inhibit oxidation (Kim *et al*, 1995). As discussed in the above sections, since ROSs are involved in tissue damage and DNA damage that results in degenerative diseases including cancer, development of anticancer related antioxidant assay must entail an assay system that measures the inhibition or scavenging of ROSs. In addition, given the importance of the body's total antioxidant capacity or potential, and the vital role antioxidants may play, it is important to develop an assay that measures the total antioxidant capacity or potential of a natural antioxidant. The ferric reducing antioxidant potential (FRAP) assay has been

used to measure the ferric reducing antioxidant power of plasma (Benzie and Strain, 1996).

Since the ROSs as well as the other radicals are highly unstable, it is very difficult to measure the ROS, directly. Hence the assays to determine free radicals are generally carried out by measuring its ability to inhibit the reactions catalysed by the ROSs such as superoxide dismutase (SOD) activities (Kim *et al*, 1995). Kim *et al* (1995) adopted the assay method developed by Marklund and Marklund (1976) for SOD activity, based on the involvement of SOD in the autoxidation of pyrogallol to determine the removal of SOD by natural antioxidants. However, a complex natural products extract may interfere with the absorbance measurement at 420 nm in the pyrogallol assay. Because of the radical stability, 1,1-diphenyl-2-picryl hydrazyl (DPPH) has been used as a free radical to evaluate antioxidant activity of some natural extracts (Shimada, 1992; Yen and Chen, 1995; Yen and Duh, 1995).

Banias *et al* (1992) used the oven test method developed by Economou *et al* (1991) using lard as the substrate for lipid peroxidation. Both the FTC (Ferric thiocyanate) and TBARS methods have been used to measure the lipid peroxidation inhibition. The reaction of lipid peroxides with thiobarbituric acid has been widely adopted as a sensitive assay method for the lipid peroxidation in animal tissues (Ohkawa *et al*, 1979). According to Hruszkewycz (1988), lipid peroxidation is measured as the production of thiobarbituric acid-reactive substances such as malonaldehyde. The formation of malonaldehyde has been measured at 532 nm, after the reaction with thiobarbituric acid, according to Ottolenghi (1959).

1.6.3.2 Immunomodulatory activity

Immunomodulatory activity is defined as the biological or pharmacological effects on humoral or cellular factors functioning in the immune response resulting in specific or nonspecific or combined specific and nonspecific effects (Labadie, 1993). Due to the regulatory interactions between humoural and cellular immunofactors, the *in vivo* net effect of an immunomodulator may be stimulatory (potentiating) or suppressive. Because of the possible negative-feedback mechanisms frequent in the immune system, immunosuppression may result from stimulation of inhibitory cells or humoral factors, inhibition of effector cells or activating humoral factors. In addition,

"immunostimulation" may result from stimulation of effector cells or the production of their metabolic inducers, and from inhibition of factors that limit immunogenicity (Labadie, 1993). According to Wagner (1990), immunostimulation is defined as a prophylactic or therapeutic concept aimed at the stimulation of primarily nonspecific or non-antigen dependent human immune system. This relates primarily to the non-antigen dependent stimulation of the function and efficiency of granulocytes, macrophages, complement and natural killer cells.

In the absence of scientific definitions for many diseases described in Ayurveda, the approach of screening medicinal plants for immunomodulatory activity has been taken by Labadie (1980) and De Silva (1982) in order to rationalise the therapeutic use of plants in traditional Ayurvedic systems of medicine.

Since one and the same agent is able to exert immunostimulatory as well as immunosuppressive effects depending on its dose range, the term immunomodulatory is used in this thesis, encompassing both effects.

The cell-mediated immune response is thought to be involved in the cytotoxicity of many antitumour agents. Cytotoxic and tumour reducing lectins showed not only the cell-mediated immune response but also the humoural immune response (Kuttan and Kuttan, 1992). Hence a humoural immune response may also play an important role in curing of cancer.

Hajto *et al* (1989) observed immunomodulatory activity such as infiltration of macrophages and monocytes, increase of Natural Killer cells, and Antibody Dependent Cellular Cytotoxicity (ADCC) and monocyte maturation after treatment with *V. album*. According to Kuttan and Kuttan (1992), this was accompanied by interferon or interleukin production, which augmented the cell, mediated responses. Wong *et al* (1994) reported the use of plant-derived immunomodulatory polysaccharides, to alleviate many undesirable side effects including myelosuppression.

1.6.3.2.1 Antitumour activity of natural immunomodulators

A lectin isolated from *Viscum album* extract (NSC 635 089), showing cytotoxic and *in vivo* tumour reducing effect was also reported to possess immunomodulatory activity. Hence

V. album was reported to be useful as an immunostimulant in treating tumours (Kuttan and Kuttan, 1992).

Wagner (1990) suggested that the antitumour activity of many plants with anticancer activity such as *Viscum album* (mistletoe), *Tabebuia avellanedae* (lapacho), and *Dionae muscipula* may exert their antitumour activities by a total or partial immune induced mechanism of action. A survey of a large number of immunomodulatory plant drug constituents has revealed compounds, which are neither irritant nor cytotoxic in high doses or carcinogenic (Wagner, 1990). *In vitro* and *in vivo* monitoring of *Echinacea* extracts has indicated a number of specific substances including isobutylamides, cichoric acid and polysaccharides as granulocytes, macrophages or lymphocyte stimulating immunomodulatory compounds (Wagner, 1990).

An acidic arabinorhamnogalactan, which was isolated from *Echinacea purpurea,* has been shown to stimulate granulocytes and macrophages and induced the production of monokines (IL-1, LAF) in stimulated marrow macrophages and shown high toxicity against tumour target cells as measured by the ^{31}Cr-release assay (Stimpel *et al*, 1984). Another acidic arabinogalactan isolated from *Echinacea purpurea* was reported to be effective in activating macrophages to cytotoxicity against tumour cells, and induced macrophages to produce tumour necrosis factor (TNF-α), interleukin–1 (IL-1), interferon-β_2 and oxygen radicals, but it did not activate B-cells and did not induce T-cells to produce interleukin-2, interferon-β_2 and generated only a slight increase of T-cell proliferation (Luettig *et al*, 1989).

Wong *et al* (1994) in their review of immunomodulatory and anti-tumour polysaccharides, suggested that immunomodulatory polysaccharides can be used to enhance the therapeutic effects of anticancer drugs, such as methotrexate and cyclophosphamide by restoring their suppressed immune functions subsequent to chemotherapy. This would be an example of inclusion of immunomodulators in combination anticancer chemotherapy.

1.6.3.2.2 Immunomodulators in cancer therapy

Effective utilisation of immunomodulators for cancer therapy depends on whether an antigen specifically identifiable on the cancer cell surface be made to activate the complement targeting at its lysis. Naturally occurring compounds may attach to the

specific receptors of the cancer cells resulting a cancer cell specific cytotoxic immune response. The alternative pathway of complement activation may be exploited where antigen-specific activation is not clinically effective.

An immunomodulatory extract from the plant *Viscum album* was found to be useful in cancer treatment. Some of the immunomodulatory activities observed after a cancer treatment included infiltration of macrophages and monocytes, increased NK-cell and antibody-dependent cellular cytotoxicity, monocyte maturation and production of interferon and interleukin, which augmented the cell mediated responses (Kuttan and Kuttan, 1992). Major non-antibody dependent cytotoxic mechanism against tumour cell invasion has been attributed to the Natural Killer (NK) cell activity. In addition, Herberman and Ortalde (1981) and Kuttan and Kuttan, (1992) reported an increase in the antibody dependent complement mediated cytotoxicity to Ehrlich ascites tumour cells in animals treated with the proprietary immunomodulatory extract, "Iscador" from *Viscum album* L. Furthermore, Ito (1986) suggested that a C3 component of complement activation may provide the possible mechanism of inhibition of Sarcoma 180 solid tumour growth in mice treated with natural anti-tumour agents.

Wagner (1990) suggested that when the applied quantity of drugs with claimed anti-tumour activities are so small that a direct anti-tumour effect can be excluded and an immune induced effect can be assumed. Therefore, in addition to the cytotoxicity monitors, inclusion of monitors for both cell mediated and humoural immunomodulatory activities would increase the chances of discovering active agents from plants that may be useful in cancer chemotherapy.

1.6.3.2.3 Bioassays for immunomodulatory activity

Some of the screening methods used to detect immunomodulatory activity are given in Table 5. Application of these methods in combination would generate important information about the immunomodulatory activity of a plant extract. However, selected methods have been used to monitor a more specific immune response in multi-functional experiments.

Table 5 Screening methods for immunomodulatory activity

Method	Reference
In vitro phagocytosis a) Microscopic smear test with human granulocytes b) Chemiluminescence test with human granulytes or macro-phages c) Flowcytometry	Brandt (1967) Allen (1981) Lepley *et al* (1996)
In vivo phagocytosis on mice (carbon clearance test)	Biozzi *et al* (1953)
In vitro T-lymphocyte transformation test a) with T/B-lymphocytes b) with subpopulations of T-lymphocytes (T_4/ T_8 and NK-cells)	Rulf and Gifford (1981)
Leukocyte-migration inhibition test	Cragg and Newman (1999)
Complement test ("classical" and "alternative" pathways)	Kroes *et al* (1993)
Immune-induced cytotoxicity tests (Cr or [^{3}H]-thymidine release from tumour cells or micro-organisms)	Ogle *et al* (1988)
Interferon induction test	Cragg and Newman (1999)

The *in vitro* immunomodulatory effects on part of organs (e.g., Trachea rings), whole-cell preparations, PMNLs, monocytes, macrophages (Bloksma *et al*, 1980), lymphocytes, enzyme systems and on receptor binding has been assayed. Although positive results of immunomodulatory activity in animals are not necessarily predict the same effect clinically in humans, a battery of *in vitro* and *in vivo* assays using mostly human-derived assay systems would increase the chance of finding a useful immunomodulatory agents for clinical use.

Wagner and Jurcic (1991) described selection of bioassays for immunomodulatory activity of plant extracts. According to Wagner (1990), since the *in vitro* and *in vivo* test systems that include granulocytes, macrophages, T-lymphocytes, NK-cells and the complement as target cells or molecules allow the determination of the functional state as well as the efficiency of the cellular and humoral nonspecific immune system, these test systems are appropriate for screening of plant constituents.

There is a growing trend arising from the recent findings discussed in the following sections, that the complement system possibly plays a role in selective lysing of cancer cells.

1.6.3.2.4 Complement

The human complement system is thought to play a very pronounced role in the immune defence system and in inflammation processes. Complement was described as the heat-labile activity in serum which combined with specific antibody would cause lysis of cells (Roit, 1997 Complement comprises a series of serum proteins or zymogens (pro-enzymes) that can be activated in a cascade–like sequence. The physiological consequences of complement activation are opsonization, cellular activation and lysis (Roitt, 1997).

According to Kabat and Mayer (1961), in conjunction with the appropriate antibodies, or other sensitising agents, complement kills certain susceptible bacteria, protozoa, lyses erythrocytes, promotes immune adherence reactions, phagocytosis and neutralises some viruses. Treatment of sheep erythrocytes coupled with sulfanilic acid or *p*-azo-phenyl-arsonate groups with their respective antibodies and complement has resulted in haemolysis (Kabat and Mayer, 1961).

1.6.3.2.5 Complement in cancer therapy

Polyanions like carrageenan and dextran sulphate are recognised as adjuvants, which act non-specifically to enhance immune responsiveness to a specific antigen. Such polyanions have shown *in vitro* stimulation of phagocytosis whilst strongly inhibiting the mouse mononuclear phagocytic system *in vivo*. Such contrasting differences of *in vitro* and *in vivo* activities was at least, partly attributed to the mediation of complement by which macrophage-mediated immunomodulation is achieved (Bloksma *et al*, 1980). Van Dijk *et al* (1980) made a similar observation on *in vitro* inhibition of complement activity and *in vivo*

immunostmulating activity. Thus an assay for *in vitro* inhibition of complement activity would predict the *in vivo* immunostimulatory effect via activation of complement.

Ito (1986) reported a correlation between antitumour activity and the effects on some biological properties, such as phagocyte activity of the reticuloendothelial system, the third component of complement (C3) activation, hepatic drug-metabolising activities and pentobarbital-induced narcosis. Ito (1986) found that various natural extracts including the polysaccharides from *Broncasma berna*, *Grifora umbellata*, and *Rumex acetosa* that showed *in vivo* antitumour activity against Sarcoma 180 solid tumour implanted in mice, also significantly enhanced the phagocyte activity and C3 activity; depressed aniline hydroxylase and aminopyrine demethylase activities prolonging the duration of penobarbital-induced narcosis. Ito (1986) suggested that the possible *in vivo* mechanism of Sarcoma 180 solid tumour growth inhibition was due to the complement (C3) activation, the stimulation of phagocytosis and depression of the hepatic microsomal drug-metabolising system in tumour-bearing mice. A correlation was observed by Kosasi *et al* (1990), between *in vitro* complement inhibitory activity and the *in vivo* activation of complement system by some plant immunomodulators. Ito (1986) interpreted the *in vivo* activation of C3 component of the complement system as the possible mechanism for the *in vivo* tumour inhibition.

The use of monoclonal antibodies (MAbs) that mediate complement activation and antibody-dependent cellular cytotoxicity has been tried (Rubin, 1993) in ovarian cancer chemotherapy with limited success. However, the expression of intrinsic complement regulators on ovarian tumour cells has diminished the tumour cell killing effect of the tumour specific or organ specific and complement activating MAb s (Bjorge *et al*, 1997). In such a situation the MAb in therapy must be an efficient complement activator that exceeds the activity of the intrinsic complement regulators.

Nakamura *et al* (1999) reported the induction of apoptosis in a human lung cancer cell line with antiganglio GM2 monoclonal antibody, which showed high effector functions such as complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity. The antiganglioside GM2 monoclonal antibody potentially suppressed growth and metastases of GM2-positive human cancer cells inoculated into mice.

Thus, both activators and inhibitors of complement activity can be considered as regulators or modulators of complement. With the potential involvement of complement

in diseases like cancer, complement modulators, when utilised effectively may play an important role in destruction of cancer cells. Although the effective manipulation and the exact mechanism by which these complement modulators effect anticancer activity is yet to be elucidated, complement modulatory activity may be a useful bioassay to monitor the putative anticancer plants for their efficacy.

1.6.3.2.6 Mechanism of complement activation

Activation of complement occurs via two pathways, one is an antibody-dependent "classical pathway" (CP) and the other is via an antibody-independent "alternative pathway" (AP), both leading to activation of complement component C3 by which a common lytic pathway (or terminal pathway) is initiated.

The pathways of complement activation according to Roitt (1997) and Labro (1994) is illustrated in Figure 3 and their mechanisms are described below in section 1.6.3.2.7 to 1.6.3.2.10.

1.6.3.2.7 Complement activation via the "classical pathway" (CP)

The main antibody-directed mechanism, which triggers the activation of complement, is the so-called "classical pathway". The CP is induced by binding of C1q (a subcomponent of the Ca^{2+} dependent hexamolecular complex, C1) to the Fc portion of antibody molecules (IgG or IgM) attached to their specific antigens on the membrane of the pathogenic organism or cell. Binding of C1q to the antibody–antigen complex results in activation of C1s. The activated C1s cleaves C4 into C4a and C4b. When C4b is formed, it attaches to the target surface and acts as a binding site for the zymogen C2, which is subsequently cleaved into C2a and C2b. The cleavage fragment C2b, in the presence of Mg^{2+} binds non-covalently to C4b to form C4b2b. The C4b2b is the C3 convertase enzyme of the CP that cleaves C3 into C3a and C3b (Figure 3).

1.6.3.2.8 Complement activation via the "alternative" pathway (AP)

The basis of the "alternative" pathway (AP) activity is the presence of low levels of C3 in normal serum. The activated C3 is extremely labile in normal (non-pathological) conditions. The AP is induced by binding of C3 to host or foreign surface and hydrolysis

of the intramolecular thioester bond of C3 to C3b. The C3b, in the presence of Mg^{2+} acts as a binding site for Factor B (FB). The FB-bound C3b is cleaved by factor D to Ba and Bb. The C3bBb in fluid phase is a C3 convertase enzyme that cleaves C3 to C3b, some of which covalently binds to adjacent surfaces. The surface-bound C3b binds to more FBs and initiate the amplification loop converting more and more C3s into C3bs (Figure 3).

Activation of C3 via AP allows indiscriminate binding of C3 to any adjacent surfaces. The C3 can be stabilised by binding to substrates such as polyanions (dextran sulphate, heparin), polysaccharides of the bacterial cell envelope, aggregates of IgA and IgG or formalinized yeast cells (zymosan).

1.6.3.2.9 Amplification loop

The surface of good activators of complement attaches the C3 convertase; C4b2b and C3bBb generated via CP and AP respectively. The FB binds to the C3b and become C3bB, a substrate for the serine esterase factor (D). The D cleaves C3bB into C3bBb releasing a fragment, Ba. The C3 convertase enzyme, C3bBb cleaves many more C3 molecules, some of which binds covalently to the activating surface. The C3bBb complex dissociates rapidly unless stabilised by the binding of properdin (P) forming a C3bBb (P) complex. The C3bBb (P) complex also cleaves C3 molecules (Figure 3). In the presence of this positive feed back system, C3 cleavage mechanism will cycle until all the C3 is cleaved (fixed) or regulated adequately.

1.6.3.2.10 Terminal pathway and formation of membrane attack complex (MAC)

As complement activation continues via CP or AP, the C5 convertase (C4b2b or C3bBb (P)) generated then splits C5 into a chemotactic C5a and a membrane-bound C5b. The C5b binds C6, C7 and C8 forming a C5b678 complex, which polymerize C9. Both C5b678 and poly-C9 are macromolecular complexes termed 'Membrane Attach Complex' (MAC), which disturbs the integrity of the membrane causing lysis of the cell (Figure 3).

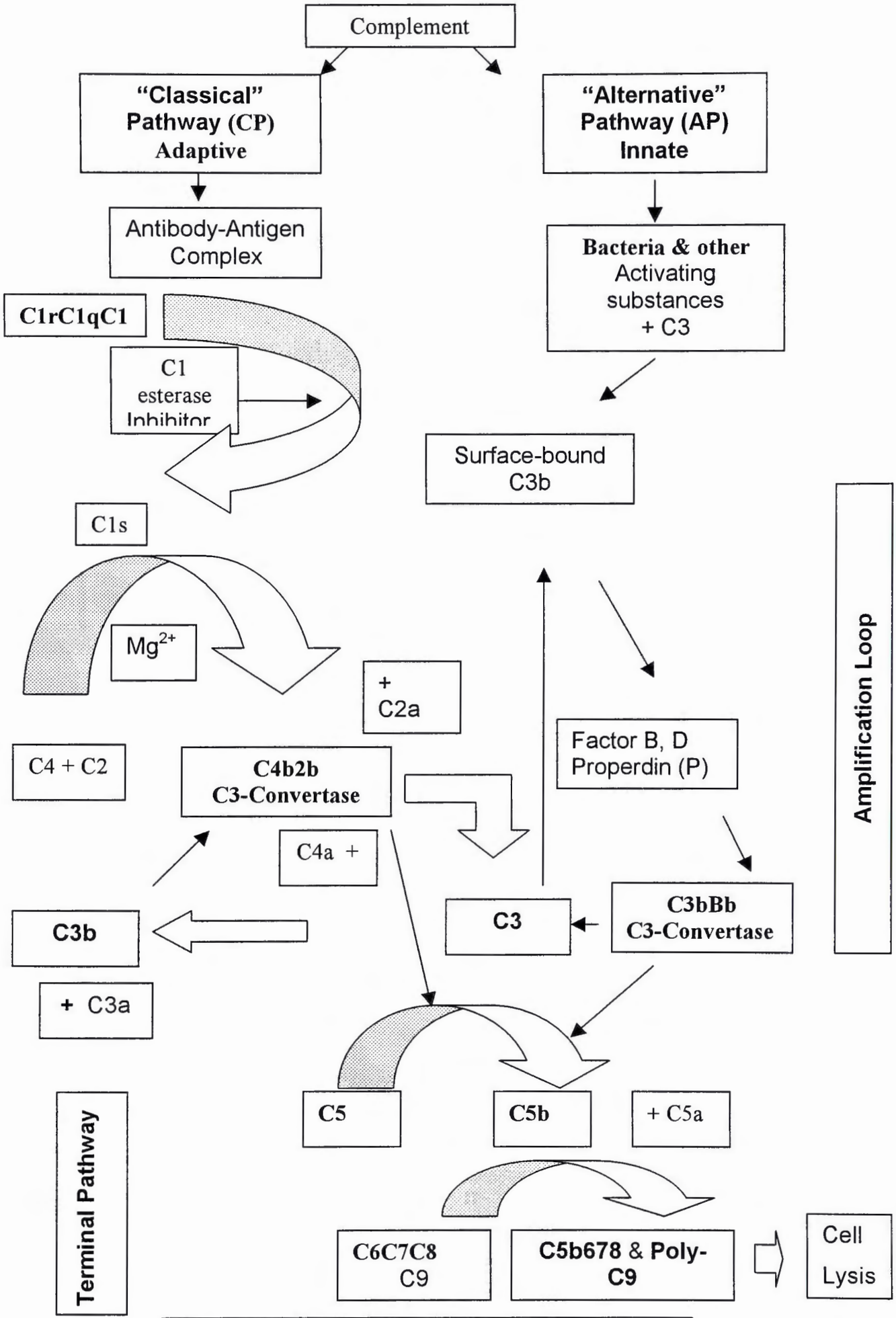


Figure 3 **Pathways of complement activation (Modified from Roitt, 1997)**

1.6.3.2.11 Assay of complement activity

Inhibition of haemolytic complement activity by an antigen-antibody complex is called "fixation of the complement". The basis of the complement fixation test is that in the presence of antigen-antibody complex, complement loses its haemolytic activity. The complement fixation test has been used for quantitative measurement of either antibody or antigen concentration (Rapp and Borsos, 1970). Specific haemolysis involves complement and antibody to erythrocyte. This antibody is referred to as haemolysin, amboceptor, haemolytic antibody or haemolytic sensitizer (Kabat and Mayer, 1961). Complement activated haemolysis or release of large haemoglobin molecules through the erythrocyte membrane was prevented by a 30% concentration of albumin or dextran (Rapp and Borsos, 1970). Screening tests that determine human complement-activation via alternative as well as the classical pathway have been used as an experimental criterion for immunomodulatory activity (Wagner, 1990; Labadie, 1993).

Although the "antitumour activity" was defined due to the involvement of macrophages, T-lymphocytes, NK-cells and their mediators for the immunomodulatory extracts or polysaccharides isolated from plants, the "complement activity" was interpreted as an antigen processing mechanism or as an anti-inflammatory activity because of the measurement of complement consumption in most of the complement tests (Wagner, 1990). An acidic arabinogalactan isolated from *Viscum album*, having similar chemical composition to that isolated from *Echinacea purpurae*, has not enhanced TNF from macrophages but has strongly activated complement (Wagner and Jordan, 1988). A correlation between *in vitro* anticomplement activity and *in vivo* antiphlogistic activity in mice has been found for a polysaccharide mixture from *Urtica dioica* (Wagner and Jordan, 1988).

Takada *et al* (1978) reported an assay method developed from those reported by Rapp and Borsos (1970), Platts-Mills and Ishizaka (1974), Mayer (1961) and Okada (1973), for inhibition of human complement via the classical and alternative pathways. Simons *et al* (1989) reported the isolation and characterisation of immunomodulatory compounds from the plant *Picrorhiza kurroa L* using assay of complement inhibition via CP and AP as a monitor for activity-guided isolation.

Citing the poor reproducibility of the haemolytic assay, Stefanski and Ruppel (1991) developed an algal assay using the algae *Euglena gracillis* for quantitative determination of the complement activity. The use of a dilution series avoids the wide variation encountered in the duel measurement algal assay.

Bamunuarachchi *et al* (1984) investigated the *in vitro* effects of medicinal plants on human complement. The potency of complement inhibition via the alternative pathway has been compared with the known inhibitor, heparin and the inhibitory potency was expressed in terms of H_{100} (100% heparin equivalent) units.

Measurement of complement includes various parameters including the determination of serum concentration giving rise to 50% haemolysis via classical pathway (CH50 unit), and the same measurement via alternative pathway (AH50), all component functions by haemolytic assay, all component levels by RID or ELISA, and split product (C3a, C4a, C5a, iC3b, Bb, C4d, SC5b-9) assays by RIA or ELISA have been developed (Giclas, 1998). In addition, INCSTAR has developed a Complement Activation ELISA (CAE) test kit to measure *in vitro* classical complement activity in human serum using a microtiter plate well. Thus *in vitro* complement activity measurement may be a useful primary tool for investigating putative anticancer plants for immunomodulatory activity.

1.6.3.3 Anti-inflammatory activity

An inflammation reaction to an infectious agent, antigen challenge or a physical damage takes place as a result of the release of soluble immune mediators, including, vasoactive amines, heparin, chemotactic factors, platelet-activating factor (PAF) and arachidonic acid metabolites, prostaglandins and leukotrienes (Bohlin, 1995).

Failure of the system to neutralise the oxy free radicals that have escaped via the oxidative burst pathway, that takes place during an immune response such as phagocytosis of a foreign organism, may induce a cancerous growth of surrounding tissues. Thus, chronic inflammation is recognised as a risk factor for human cancers including lung, bladder, bowel, breast, skin, and stomach cancers (Bennett and Birnboim, 1997).

Some of the anti-inflammatory drugs currently being used are derivatives of acetylsalicylic (aspirin), pyrazolidine and propionic acid. These drugs have been developed on the basis

Table 6 **Bioassays used to monitor *in vitro* anti-inflammatory activity (Bohlin, 1995)**

PAF-induced elastase release
Human neutrophils
PAF, test substances & SAAAVNA (peptide)
UV 405 nm
PAF biosynthesis
Human neutrophils
Acetyl-CoA (^{14}C-labelled) & test substances
Count activity of PAF
Cyclooxygenase assay
Bovine seminal vesicles
Vehicles or test solution & ^{14}C –labelled AA
Separation of unmetabolised AA
Count activity of PGs
5-Lipoxygenase assay
Human neutrophils
A23187 & test substances
analysis of LTB_4 on RP-18 HPLC

of their inhibitory activity on certain inflammatory mediators. Some of the *in vitro* bioassays utilising some of these mediators to monitor anti-inflammatory activity are given in Table 6.

As shown in Table 6, most of the anti-inflammatory drugs developed to date have been based on their specific influence on the phospholipid-derived inflammatory mediators, prostaglandins, leukotriene B4, and platelet activating factor (PAF) (Bohlin, 1995). Wagner (1993) has used inhibition of prostaglandins metabolism by cyclooxygenase-2 (COX-2) and 5-lypoxygenase enzymes as well as the inhibition of complement system via its classical and alternative pathways as *in vitro* models for developing anti-inflammatory drugs.

Some anti-inflammatory agents because of their inhibition of platelet aggregation and/or COX2 enzyme activity have also shown anticancer activity. Hence in an investigation to discover anticancer activity of a plant extract, it is important to include a screen for anti-platelet activity and the COX-2 inhibitory activity.

1.6.3.3.1 Platelet aggregation

Blood platelets have been shown to be involved in immune responses and specifically inflammation, in addition to their involvement in blood clotting. Platelets are usually activated and aggregated following a tissue injury or dysfunction of endothelium (in atheroclerosis). The term "platelet aggregation" is used to denote the adherence of one platelet to another. Since platelet aggregation is an essential event in an inflammatory process, inhibition of platelet aggregation is considered an anti-inflammatory activity.

1.6.3.3.1.2 Inhibitors of platelet aggregation activity as anticancer agents

Inhibitors of platelet aggregation have shown anticancer activities including immunomodulatory, cytotoxicity, antiproliferative and the induction of cancer cell death (apoptosis). The anticancer property of an anti-platelet or platelet aggregation inhibitor may be due to the inhibition of platelet activation factor (PAF), platelet-derived (antiogenetic) endothelial cell growth (PD-ECG) factor, or the cell adhesion molecules, which are reported to have been found in the proliferating cancer tissues.

Liao *et al* (1997) reported the importance of the antiplatelet aggregatory agents as specific PAF-receptor antagonists in the treatment of many pathophysiological situations. The platelet aggregation inhibitor, bakkenolide G, which was isolated from the plant *Petasites formosanus*, dose-dependently inhibited PAF-induced intracellular signal transductions. Therefore bakkenolide G may have the potential to interfere with the cancer cell activities.

Ginkgolides from *Ginkgo biloba* are specific and potent agonists of the inflammatory mediator, PAF that act on different types of cells (Bohlin, 1995). Ginkgolides very specifically inhibit the binding of PAF to its receptor on platelets. As shown by the studies, Ginkgolides can inhibit the suppressive effect of PAF on the proliferation of T-lymphocytes and their cytokine production. Bakkenolide G, an inhibitor of platelet aggregation, was reported as a natural inhibitor of PAF-receptor antagonist (Liao, 1997). Benett and Birnboim (1997) suggested that PAF is a mitogenic factor that contributes to the known increase in risk of malignancy associated with chronic inflammatory conditions

Jin-no *et al* (1998) reported that the level of platelet-derived endothelial cell growth factor (PD-ECGF) in plasma of HCC cancer patients could serve as a new tumour marker for the progression of the cancer. According to Schwartz *et al* (1998), the platelet derived

endothelial cell growth factor, cytokine thymidine phosphorylase has diverse functions within cells including mediation of angiogenesis, in normal and malignant (colon) cancer cells, and conversion of 5-fluorouracil (cancer chemotherapeutic agent) into its active metabolite.

Satoh *et al* (1998) reported that, thymidine phosphorylase (dThdPase) from human tumour tissues, is identical to the platelet-derived (antiogenetic) endothelial cell growth (PD-ECG) factor. Whether the inhibition of platelet aggregation is due to inhibition of the PD-ECG factor or some other factor is to be determined. Whether the PD-ECG could retard the growth of tumour tissues is also to be determined. It is possible that anti-platelet-aggregation agents may be used to inhibit the growth of tumour (cancer) cells, provided they do not affect the non-cancer cells same way.

Metastatic potentials of tumour cells are reported to be correlated with the proaggregatory and procoagulant properties or prothrombic abilities of the cells. Most of the anti-platelet activating agents are also known as potent cytotoxic agents in cancer treatment when used at high doses, and act as immunostimulators at low doses. Mammary gland tumour cell lines have shown proaggregatory and procoagulant properties that increase according to their metastatic potentials (Pasqualini *et al*, 1997). Pasqualini *et al* (1997) further suggested as a result of their findings, that the prothrombic abilities exhibited by neoplastic cells, may be acting synergistically in the dissemination of the cancer. Hence antiprothrombic agents or antiaggregatory agents could well reduce the metastatic properties and dissemination of cancer.

Silverstein and Silverstein (1998) focused on the interaction of cell adhesion molecules with intracellular components and investigated the role of cell adhesion molecules in mediating cell signal transduction. An ongoing effort to develop specific pharmacological agonists and antagonists for adhesion molecules holds great promise in clinical medicine (Silverstein and Silverstein, 1998). Abciximab (Reopro) (a monoclonal antibody inhibitor of the platelet integrin α-IIb β-3) is currently approved and available to improve vessel patency in patients undergoing angioplasty. Similar approaches to develop adhesion-based therapies to block tumour progression, and/or metastasis are under development and hold promise for patients with cancer (Silvestein and Silverstein, 1998).

Igarashi (1997) presented his findings on the functional roles of sphingolipids including sphingosine 1-phosphate, in the regulation of cell motility and platelet activation and also studied the involvement of sphingosine in cell signalling, and effects of methylsphingosine in the induction of cancer cell apoptosis. This work of Igarashi (1997) prompted the query as to whether there was any correlation between the platelet modulation and the regulation of cancer cell motility and the induction of apoptosis of cancer cells.

Chen *et al* (1997) reported the induction of apoptosis in cultured Hep 3B hepatocarcinoma, U87-MG malignant glioma, PC-3 prostate, adenocarcinoma and HeLa cervical adenocarcinoma *in vitro*, by the antiplatelet drugs pentoxifyline, dipyridamole and ticlopidine HCl. Chen *et al* (1997) suggested co-administration of antiplatelet drugs as an alternative adjunctive therapy for cancer patients since antiplatelet drugs possess *in vitro* antineoplastic activity.

Although Carr *et al* (1995) reported the activation of platelet aggregation by the antileukemic agent bryostatin 1 and developed a functional assay for antileukemic agents like bryostatin1, such *in vitro* activation of platelet aggregation may well be an immunomodulatory effect, as reported earlier by Smith *et al* (1985). Several antiplatelet aggregatory compounds, that showed anticancer activity *in vitro*, have been isolated from a medicinal plant extract (Wu, 1997). Agonists and antagonists of platelet aggregation are suggested to play an important role in cancer therapy (Borman, 1994). The inhibitors of platelet aggregation can be an important addition to the current strategies of adoptive immune therapy.

On the basis of the above facts, it is important to include the platelet aggregation assay as an activity-guided monitor to investigate and isolate anticancer compounds from medicinal plants. Since antiplatelet activity is relatively easy to measure and its potential as a monitor for anticancer agents, assay of *in vitro* antiplatelet activity is an important screen for an investigation of putative anticancer plants.

1.6.3.3.1.3 Assay of antiplatelet activity

Antiplatelet aggregation is measured using the Born aggregometer (Born, 1962). This is accomplished by allowing platelet rich plasma to aggregate and measuring the maximal possible increase in light transmission (100%), as defined by the difference in light transmission between platelet poor and the nonstimulated platelet rich plasma. Hiermann

and Bucar (1994) have used optical antiplatelet aggregation assay to study the effect of herbal extracts on prostaglandin biosynthesis.

In addition, Chang and Hsu (1991) reported the use of platelet aggregation assay in which, after incubating the test mixture with the platelet rich plasma, the platelets were spun down by centrifuge and the TXB2 in the supernatant was measured by a specific radioimmunoassay.

Although optical measurement of antiplatelet activity is the popular and conventional method, it requires a large quantity of blood to obtain platelet rich and poor plasma. On the other hand, the impedance method reported by Ingerman-Wojenski and Silver (1984) does not require a large quantity of blood as it measures impedance of the aggregate formed between two electrodes. Hence, whole blood, that represents the natural system can be used for impedance measurement. Furthermore, the impedance method is used to determine the platelet aggregation activity by Carr *et al* (1995).

Adding an aggregating agent to platelet-rich plasma (PRP) or whole blood can induce platelet aggregation. In most of the *in vitro* diagnostic assays using optical aggregometry, ADP, epinephrine, collagen and ristocetin are used. Arachidonic acid has also been used as an inducer of platelet aggregation (Saeed *et al*, 1995). Arachidonic acid metabolites are among the products biosynthesised by platelets that show intimate relevance to vascular homeostasis. Arachidonic acid is known to convert to thromboxane A2 (TXA2), which is a known potent inducer of the platelet aggregation.

1.6.3.3.2 Cyclooxygenase-2 enzyme (COX-2) inhibitory activity

Cyclooxygenase-2 (COX-2) is a key enzyme involved in the metabolism of prostaglandin and therefore plays a role in the inflammation. It follows therefore that the inhibition of COX-2 may lead to a reduction in the inflammatory reaction. Hence COX-2 has been used as a key enzyme target for anti-inflammatory activity studies (Wagner, 1993).

There are two isoforms of cyclooxygenase enzyme (prostaglandin-endoperoxide synthase, EC 1.14.99.1), constitutive cyclooxygenase-1 (COX-1) is present in cells under physiological conditions and cyclooxygenase-2 (COX-2) enzyme is induced in cells exposed to proinflammatory agents including cytokines, mitogens and endotoxin (Mitchell *et al*, 1994; Dannhardt and Kiefer, 2001). The role of COX-1 is to protect the stomach

lining and regulating the blood platelets whereas COX2 is involved in triggering pain and inflammation in response to an injury.

The cyclooxygenase enzyme pathways according to Mitchell *et al* (1994) are illustrated in Figure 4. Activation of COX-1 in platelets, endothelium, stomach mucosa, or kidney under physiological conditions lead to the release of the eicosanoids; thromboxane A_2 (TXA_2), prostacyclin (PGI_2), or prostaglandin E_2 (PGE_2). Aspirin-like drugs selectively inhibit the release of these eicosanoids effected by COX-1. Synthesis of COX-2 in cells is induced by inflammatory stimuli result in release of interleukin-1. The COX-2 enzyme catalyses the conversion of arachidonic acid to prostaglandin-H_2. The inflammation is effected by the release of PGs, together with proteases and other inflammatory mediators including reactive oxygen radicals. The inhibition of COX-2 pathway can occur at several levels by selective inhibitors of COX-2 or the inhibitors of COX-2 induction, e.g. glucocorticoids, antagonists or antibodies to cytokines and mitogens.

1.6.3.3.2.1 Inhibitors of COX-2 as anticancer agents

Malignant cells in the intestine reportedly generate the COX-2 enzyme to accelerate their growth (Gorman, 1998). In addition, an elevated level of COX-2 has been found in human prostate cancer (Uotila *et al*, 2001). Furthermore, a significant correlation of high COX-2 expression with the presence of p53 mutation in oesophageal squamous cell carcinoma (Biramijamal *et al*, 2001) and induction of apoptosis in colon cancer cells by the COX-2 inhibitor NS398 (Li *et al*, 2001) have been reported recently.

Inhibition of COX-1 can result in adverse side effects causing gastric and renal damage and failure in blood clotting whereas COX-2 inhibition may be beneficial in relieving inflammatory pain and more importantly impeding cancerous growth. Hence COX-2 inhibitors may have both anti-inflammatory as well as the anti-cancer effects.

1.6.3.3.2.2 Assay of COX-2 inhibition

Mitchell *et al* (1994) and Saeed *et al* (1995) used a method involving TLC separation and radioactivity measurement of products of radiolabelled arachidonic acid metabolism to assay the COX-2 activity.

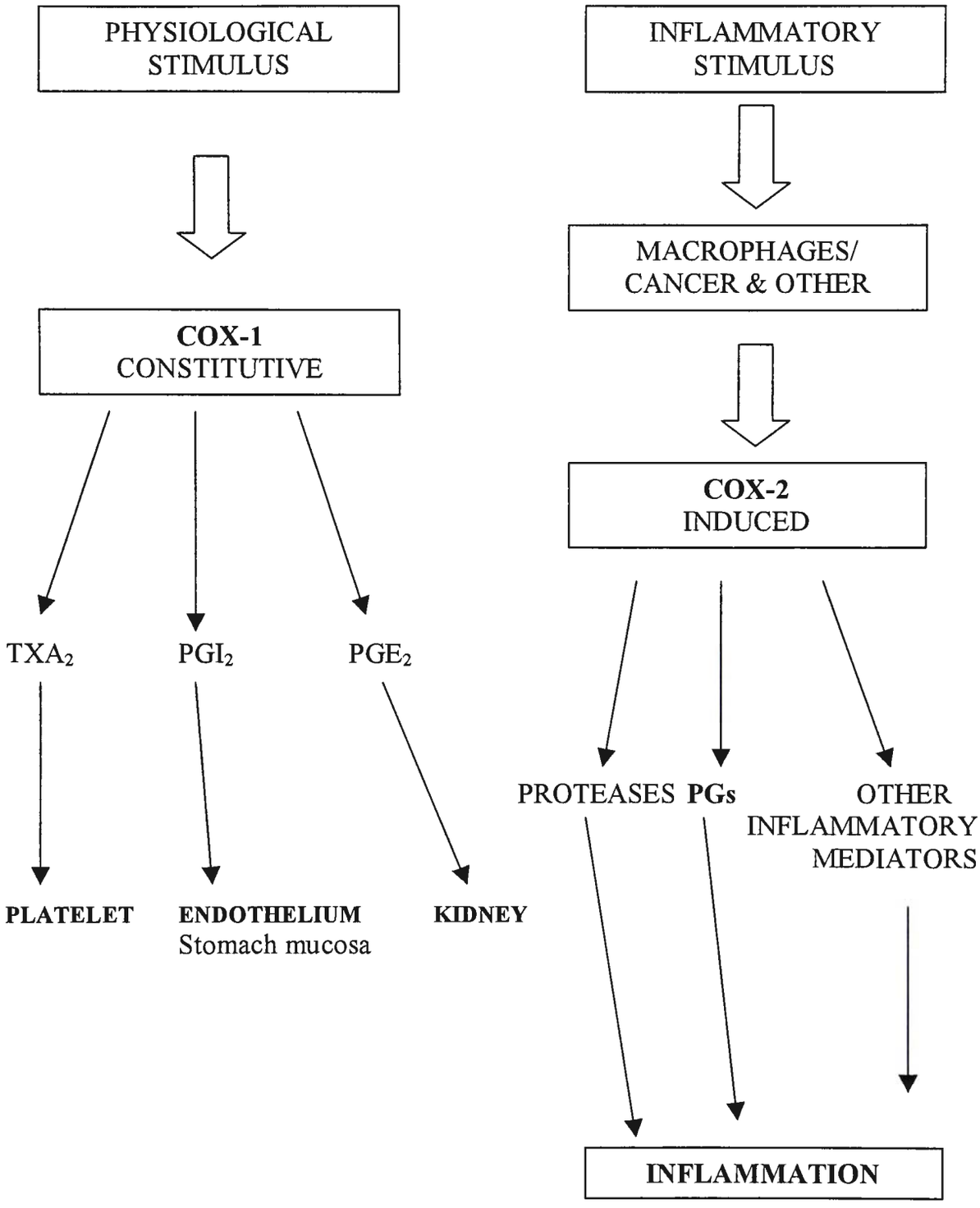


Figure 4 **Pathways of the cyclooxygenase enzymes, COX-1 and COX-2**

Mitchelle *et al* (1994) assayed the selective inhibitory activity of several drugs on COX-1 and COX-2 enzymes using purified as well as extracted enzymes. The basis of enzyme activity measurement was the conversion of [^{14}C]-arachidonic acid by the COX-1 and COX-2 enzyme into PGE_2, which after separation by thin layer chromatography was detected and quantified by performing an autoradiography. Autoradiography has been performed, by placing the TLC plate in contact with an x-ray film. Saeed *et al* (1995) have used similar method but a different TLC-solvent system to separate metabolic products of arachidonic acid on TLC. The radioactive zones of PGs and TXB2 products have been located and quantified using a Berthold Linear Analyser.

1.7 Selection of plants

Extracts of *Euphorbia peplus* L. and *Viola odorata* L. were both screened against the KB tumour cell line in the early stage of the NCI's *in vitro* screening programme in 1967. However, these leads were discontinued from further screening since they failed to indicate strong cytotoxic activity in the preliminary *in vitro* and *in vivo* screens of the NCI.

1.7.1 *Euphorbia peplus* L.

***Euphorbia peplus* L.** (Syn. petty spurge or milk weed), Family: *Euphorbiaceae. E. peplus* is a small annual herb growing in Victoria, Australia.

Figure 5, shows a *Euphorbia peplus* L. plant grown in a home garden in Victoria, Australia.

According to Everist (1974) the medicinal properties of *Euphorbia peplus* have been known since the time of Galen. Its latex has been used as a home remedy for treating corns, warts, asthma and used by physicians for rodent ulcers (Maiden, 1917). A cure of a multiple basal cell carcinoma of a person by using its milky sap was reported based on the results of a clinical trial (Weedon and Chick, 1976). Screening of different parts of *Euphorbia peplus* using different, methods of extraction, animal and human *in vitro* systems and mouse *in vivo* systems using different detection methods are discussed in the following sections under screening of *Euphorbia peplus*.

Figure 5 A mature plant of *Euphorbia peplus*

1.7.1.1 Plant parts of *Euphorbia peplus* and their therapeutic effects

The literature references citing therapeutic use of different plant parts of *Euphorbia peplus* are given in Table 7. As shown in Table 7, the application of sap or latex of *Euphorbia peplus* has been reported in many cases.

Although the reported cure of basal cell carcinoma of the chest of a patient involved use of latex or sap of the plant, the use of whole plant was reported in treating cancers of stomach, liver, and uterus (Shimans'ka, 1961). Also the use of dried aerial parts of the plant was reported to inhibit spindle formation in the C-mitosis of onion seed tips (Barnard, 1949) and stems and leaves have been used in folk remedy to treat warts and corns (Fernie, 1914; Heidt, 1940-1943).

In addition to the above, the NCI results showed some activity in the extracts made from the whole plant (Table 7). Hence the use of whole dried plant is more appropriate in an investigation taking an approach entailing a broad spectrum of anticancer-related biological tests.

Table 7 Reported uses and effects of different plant parts of *Euphorbia peplus*

Plant Part	Preparation	Disease or Effect	Reference
Sap of ST, LF	Hesse-Nassau folk remedy	Warts and corns	Fernie (1914), Heidt (1940-43)
Sap (Germany and Czechoslovakia)	Folk medicine	Warts	Madaus (1938)
Whole PL	95% EtOH extract	Inhibited spindle formation in C-mitosis of onion seed	Barnard (1949)
PL, Ukraine		Treating cancers of stomach, liver, & uterus	Shimans'ka (1961)
Latex/sap (Australia (NSW,1976)	Water extract	Treating basal cell carcinoma	Weedon and Chick (1976)
Latex (Australia (NSW, 1960)		Treating sun (skin) cancer	Hartwell (1990), NCI central files.
Dried aerial PL, or Latex	Fodder contaminant	Inflammation on mouse skin. Promote Burkitt's lymphoma and oesophageal cancer in goat	Nawito *et al* (1998)

*Legend for plant parts used: leaves (LF), stem (ST), flowers (FL), whole plant (PL).

1.7.1.2 Previous screening results of NCI on *Euphorbia peplus*

1.7.1.2.1 *In vitro* screening of *Euphorbia peplus*

A summary of results for the *in vitro* anticancer activity of *Euphorbia peplus* extracts obtained by the NCI's plant screening program during 1962-1977 is given in Table 8.

The *in vitro* screening of the extracts has been carried out against human epidermoid carcinoma of the nasopharynx (KB) and the results were given as measurements of protein synthesis according to Smith *et al* (1959) and Schepartz *et al* (1967). The ED_{50} value defined as the dose in μg/ml that inhibits cell growth to 50% of the control growth. The ED_{50} parameter has been selected, because relative reduction in tumour burden was considered a more meaningful measure of drug effectiveness than the absolute number of cells killed (Instruction 14 (Rev.9/84) page 13, DTP-NCI).

Table 8 NCI's *in vitro* screening results of *Euphorbia peplus* extracts against KB cells**

NCI code	Month of Collection	Location of Collection	Part used	Extraction Solvent	ED_{50} (μg/ml)
B607198	Jul-1962	NSW	PL (M&Y)	AQ	>100
B616210	Jan-1967	Norfolk Island	LF, ST, FL RT	AQ/EtOH	>100
B626182	Oct-1966	VIC	PL (fresh)	AQ/EtOH	26*, >100
B626282	Jan-1967	VIC	PL	AQ/EtOH	24*, >100
B657198	Jul-1962	NSW	PL (M&Y)	EtOH	>100
B663785	Jan-1972	Australia	PL	AQ/EtOH	>100
B664411	Jan-1976	NSW	Latex sample	-	>100
B664412	Jan-1976	NSW	Latex	-	25*, 21
B664413	Jan-1976	NSW	Latex comp.	-	N/A
B664450	Jun1976	NSW	PL	EtOH	27*, 27
B664451	Jun-1976	NSW	PL	$CHCl_3$	36
B665805	Apr-1975	Israel	PX	AQ	N/A
B665806	Apr-1975	Israel	PX	AQ/MeOH	N/A
B668481	Sep-1969	Chile	RT, FL, ST LF	AQ/EtOH	>100
B862238	Feb-1974	Panama	„ + FR	CH_2Cl_2	N/A

**Extracted from the screening data summary of the Development Therapeutics Programme of the NCI (USA). *Passed stage I of NCI's sequential screen. N/A= data not available to public. Legend for abbreviations used: Victoria (VIC), New South Wales (NSW), leaves (LF), stem (ST), flowers (FL), roots (RT), fruit (FR), latex and young (Y) and mature (M) whole plant (PL), aqueous (AQ).

The plants collected from different geographical locations were extracted into different solvents. The extraction procedure used was a one step solvent extraction at room

temperature with one or two solvents (Table 8). The extracts have been tested at concentrations of 0.1, 1, 10 and 100 μg/ml.

According to Table 8, although the extracts, B626182 and B616282 made from the whole plants showed higher inhibition potential (ED_{50}) of 26 and 27 μg/ml, respectively, in the first screen, the results obtained in the repeat screen were >100 for both extracts. Hence a 5-fold variation of results was shown.

The extracts of *Euphorbia peplus*, that gave an ED_{50} value of more than 100 μg/ml in the first stage of NCI's *in vitro* screening programme have been discontinued from further testing as they did not show a significant inhibition of KB cells.

However, the latex sample, B664412 showed relatively higher ED_{50} of 25 and 21 μg/ml respectively in both the first stage of the screening. Similar consistency, in duplicate measurements, was seen in the results of the extract, B664450. Furthermore, $CHCl_3$ extract, B664451 gave an ED_{50} of 36 μg/ml. Although these extracts passed the first stage of the screening program, they did not enter the subsequent stages of the program because they failed to show significant inhibition of KB cells ($ED_{50} \leq 6$ μg/ml). Hence, all of the extracts described in Table 8 were considered non-cytotoxic and inactive in the NCI's *in vitro* screening. In the early stage of the NCI's screening programme, plant extracts have been tested in both *in vitro* and *in vivo* screens simultaneously, even if the results of the *in vitro* screen were negative.

1.7.1.2.2 *In vivo* screening of *Euphorbia peplus* at the NCI

The summary of results for the *in vivo* anticancer activity of the *Euphorbia peplus* extracts described in Table 8, carried out by the NCI's plant screening program during 1962-77 is given in Table 9.

The extracts have been screened against implanted mouse cancers or tumours, and the antitumour activity of the extracts was measured by the survival time of the test mice (T) compared to the control mice (C) and expressed as (T/C%).

Table 9 NCI's *in vivo* screening results* of *Euphorbia peplus* extracts against animal tumour systems

NCI Code	Test System	T/C%	Test Status
B607198	LE LL SA	48 and 104	Non-toxic Inactive
B616210	LE WA AL	94 to 108	Inactive
B626182	LE WA	81 to 121	Inactive
B626282	LE WA	86 to 96	Inactive
B657198	LE LL SA	55 to 102	Inactive
B663785	PS	90 to 133	Inactive
B664411	PS	89 to 116	Activity failed
B664412	PS	87 to 117	Activity failed
B664413	PS	89 to 98	Activity failed
B664450	PS	91	Non-toxic Inactive
B664451	PS	119 to 128	**Activity passed**
B665850	N/A	N/A	N/A
B665806	N/A	N/A	N/A
B668481	LE **PS**	86-125	**Passed Stage I**
B862238	N/A	N/A	N/A

*Extracted from the screening data summary of the Development Therapeutics Program of the NCI (USA). Legend for cell lines used: AL=alkaloid assay. N/A= data not available to public. Abbreviations for tumours are L1210 leukaemia (LE), P388 leukaemia (PS), Lewis lung carcinoma (LL), sarcoma 180 (SA), and rat Walker carcinosarcoma 256 (WA).

In most of the *in vivo* screens the extracts showed deaths of the tumour bearing test animals while the tumour bearing control animals survived during the test period showing toxicity of the extract. Also there was no inhibition of the tumours in most of the cases.

However, as shown in Table 9, the CH_2Cl_2 extract of the sample, B664451 showed a marginal activity by extending the survival time of the test animal implanted with Leukaemia P388 (PS) by 128 % which is slightly above the lower limit of the test criterion, 125%. If the T/C% (ratio of test (T) to control (C)) or survival of test animal exceeds 125%, the extract is considered active in the inhibition assay. Same sample extract gave 36 µg/ml in the NCI's *in vitro* screening with KB cell line, as shown in Table 9.

Although the aqueous/EtOH extract B668481 did not show a considerable inhibition (ED_{50} >100 µg/ml) of *in vitro* protein synthesis of KB cells, it showed a marginal *in vivo* inhibitory activity (T/C=125%) against the P388 leukaemia (PS) implanted mouse, that

qualified its entry into the second stage of NCI *in vivo* screening programme. This could be due to the differential inhibitory effect of the extract against *in vitro* KB cells and *in vivo* PS leukaemia in mice. It could well be due to the indirect inhibitory activity of the extract against the PS *in vivo.*

In addition, the extract, B668481 showed a marginal activity slightly higher than that shown by B664451. Since the repeat experiment gave a reproducible results, B668481 was entered into the stage 2 of the screening program but failed due to lack of sufficient inhibitory activity on the progression of the implanted P388 leukaemia (PS).

Results of previous investigations of *Euphorbia peplus* extracts in both *the in vitro* and *in vivo* anticancer screening programs of the NCI have not shown considerable anticancer activity. Hence, further investigations of these *Euphorbia peplus* extracts had not been carried out by the NCI.

1.7.2 *Viola odorata* L.

Viola odorata (Synonym: Sweet Violet) belongs to the family: *Violaceae. V. odorata* has been widely distributed in Victoria (Ewart, 1939) and this plant was claimed to be effective in the treatment of cancer according to Neil (1889).

A root infusion of *Viola odorata* has been used in the treatment of uterine cancers (Hartwell, 1982) whereas use of leaves as well as the whole plant is reported in treating various cancers including throat cancers. Hence sequential extracts of both aerial parts and roots were used in the investigation of *Viola odorata.* Figure 6, shows a fragment of *Viola odorata* creeper grown in a home garden in Victoria, Australia.

According to Kapoor (1990), the attributes of *Viola odorata* are described in Ayurveda as follows:

Rasa- Madhur, Tikta; **Guna**-Laghu, Snigdha; **Veerya**-Sheeta; **Vipaka**-Madhur; **Action and Uses**- *vata pitta samak kapha nisark, daah samak, rakta rodhak, sothahar anuloman, virachan, rakta pitta samak, jawarghana.*

According to Susruta (vol. 3, p 246-264), the aetiology of tumours is described as Vataja-Gulma (due to aggravation of Vatu), Pittaja Gulma (due to aggravation of Pitta), and

Kaphaja Gulma (due to aggravation of Kapha). Hence according to the principles of Ayurveda, attributes of *Viola odorata,* including its Madhu and Tikta Rasa, may enable it to counteract the tumour condition caused by Vata and Pitta aggravation. However, it may not be effective in alleviating tumour conditions caused by Kapha aggravation. The leaves, stem, flower, roots and the whole plants have been used in preparation of decoctions or a topical application.

Figure 6 Mature plant of *Viola odorata* L.

1.7.2.1 Reported uses and effects of *Viola odorata*

The literature references citing the therapeutic uses of different plant parts of *Viola odorata.* are given in Table 10. As shown in Table 10, root infusions of *Viola odorata* have been used in the treatment of uterine cancers (Hartwell, 1982) whereas use of leaves as well as the whole plant is reported in treating various cancers including throat. The flowers, petals, roots, leaves and whole plant have been used medicinally (Ladwa and Dutta, 1969; Kapoor, 1990; Grieve, 1992).

Table 10 Reported uses and effects of plant parts of *Viola odorata*

Plant Part*	Preparation	Disease or Effect	Reference
PL, RT, LF,	Decoction, infusion, syrup, tincture	Uterine, throat, tongue, intestine, breast & stomach cancer, skin tumours	Hartwell (1982)
PL	Decoction	For various diseases in Unani and Ayurveda systems	Bamber (1916), Hakim (1904)
PL, FL, RT	Decoction	Use in indigenous medicine	Chopra *et al* (1969)
PL, LF, ST FL	Ayurvedic decoction with boiling water	Reported cure of throat cancer. Treating throat, tongue cancers	Fernie (1914). Kapoor (1990)
PL, FL	Aqueous extract	Treating cough, coryza, pharyngitis, tonsillitis, cheek spells, nerve disorders and swollen bladder	Sajjad and Zaidi (1984)
LF, RT	Water extract	*In vivo* anti-inflammatory activity	Kroutil, & Kroutilova (1968)
PL	Decoction	Emetic, expectorant, diuretic and for rheumatism	Korbelar & Endris (1958), Blazek & Kucera (1953)

*Legend for plant parts used: leaves (LF), stem (ST), flowers (FL), roots (RT), whole plant (PL).

1.7.2.2 Previous screening results of NCI on *Viola odorata*

The results of previous investigations by NCI using direct solvent extracts of *Viola odorata* for *in vitro* and *in vivo* anticancer activity are discussed below.

1.7.2.2.1 *In vitro* screening of *Viola odorata*

A summary of previous *in vitro* screening on human epidermoid carcinoma of the nasopharynx (KB) carried out and results obtained by the NCI's plant screening program for *Viola odorata* is given in Table 11.

As shown in Table 11, samples of fresh and dried parts and the whole plant of *Viola odorata* collected during 1962-1968 from different locations extracted in water (AQ), water/EtOH, EtOH/$CHCl_3$, EtOH, acetone (AC) and $CHCl_3$ have been tested in the NCI's plant screening program during 1962-1968.

Although the *in vitro* screening of EtOH/$CHCl_3$ extracts of the samples, B659191 and B659192 against KB cells gave, concentrations causing inhibition of protein synthesis to 50% of the control growth (ED_{50}) values of 35 and 22 μg/ml respectively, EtOH/$CHCl_3$ extracts of the samples B601438 and B814498 showed ED_{50} >100 μg/ml. Such a difference in results can be attributed to seasonal, geographical variation or due to localisation of activity in leaves, roots and stem; provided viability and sensitivity of the cell line remained stable throughout the experiment.

The sample, B659192 was rated passed in the stage 1 of NCI's sequential screen, because the slope indicated that the 10-fold dilution in dose would place the results below the selection criterion of ≤ 6μg/ml or subsequently ≤ 4 μg/ml. In order to pass the second stage the extract must have an arithmetic mean from the first two tests of ≤ 4μg/ml (Schepartz *et al*, 1967). However subsequent repeats (at 50 μg/ml) were not reproducible or ≤ 4μg/ml, hence B659192 was rated non-cytotoxic and inactive.

1.7.2.2.2 *In vivo* screening of *Viola odorata*

The *in vivo* screening results of the extracts of *Viola odorata* carried out by NCI are summarised in Table 12. According to Table 12, sample B601438 of *Viola odorata L* in AQ/EtOH extract administered at a dosage of 125 mg/kg gave 41% (<42%) tumour inhibition ratio of test (T) to control (C) of SA tumour implanted in mice and showed toxicity on the mice bearing CA, LE and SA tumours. Sample B659192 showed marginal inhibition (49%) of solid FV. Sample B814498 at doses of 6 – 50 mg/kg showed an increase in the survival time up to 137%, of mice implanted with PS tumours. However the subsequent experiments did not produce results conform to the selection criteria. Hence, these extracts were rated as inactive.

Table 11 NCI's *in vitro* screening results* of *Viola odorata* extracts against KB cells

NCI code	Date of Collection	Location of Collection	Part used	Extraction Solvent	ED_{50} µg/ml
B601438	N/A	Arizona	LF ST (fresh)	EtOH/$CHCl_3$	>100
B601503	N/A	Arizona	LF (fresh)	AQ	>100
B607770	Sep-1964	South Africa	LF	AQ/EtOH	>100
B609191	Feb-1963	Arizona	LF (fresh)	AC	>100
B626388	Feb-1967	South Africa	PL	AQ/EtOH	-N/A
B629607	Jul-1968	India	LF	AQ/EtOH	-N/A
B644707	N/A	India	LF ST FL	AQ/EtOH	>100
B659191	Feb-1963	Arizona	LF (fresh)	EtOH/$CHCl_3$	35
B659192	Feb-1962	Arizona	RT ST (fresh)	EtOH/$CHCl_3$	**≤22*,** >50
B814498	Feb-1963	India	LF ST FL	EtOH/$CHCl_3$	>100
B814899	N/A	India	LF ST FL	$CHCl_3$	>100

*Extracted from the screening data summary of the Development Therapeutics Programme of the NCI (USA). *Passed stage I of NCI's sequential screen. N/A= data not available to public. Legend for plant parts used: leaves (LF), stem (ST), flowers (FL), roots (RT), whole plant (PL), water (AQ), acetone (AC). However subsequent repeats (= 50 µg/ml) was not reproducible or ≤ 4µg/ml, hence B659192 was rated non-cytotoxic and inactive.

Although the samples B601438 and B 814498 gave ED_{50} values >100 µg/ml which were not considered by the NCI test criteria as active against *in vitro* KB cells, both of these sample extracts gave considerable inhibition of *in vivo* SA tumour growth and increased survival time of PS tumour bearing mice respectively.

The AQ, AQ/EtOH, AC and $CHCl_3$ extracts gave ED_{50} values >100 µg/ml against *in vitro* KB cell line and did not show considerable inhibition of *in vivo* inhibition of tumour growth or increase of the survival time of tumour bearing mice. However, EtOH/$CHCl_3$ extracts showed some activity in either *in vitro* or *in vivo* or in both systems.

Table 12 NCI's *in vivo* screening results of *Viola odorata* extracts against animal tumour systems**

NCI code	Test System	T/C%; inhibition[1] or Survival Time	Test Status
B601438	CA, LE, SA	41[1]	**Passed Stage I**
B601503	CA, LE, SA	94-117	Inactive
B607770	LE, SA, WA	67-95	Inactive
B609191	FV, LE, SA, H1	69-109	Inactive
B626388	LE	97	Inactive
B629607	LE	N/A	Inactive
B644707	PS	90-100	Inactive
B659191	FV, LE, SA, WA, H1	76, 109	Toxic inactive
B659192	FV, LE, SA, WA, H1	49-51 (FV)[1], 100	**Passed Stage I**
B814498	PS	99, 137	**Passed Stage I**
B814899	PS	103-112	Inactive

**Extracted from the screening data summary of the Development Therapeutics Program of the NCI (USA). The activity threshold, T/C ≤ 42%, for tumour inhibition model and T/C ≥ 125%, for animal survival time model. Legend used: AL=alkaloid assay. N/A= data not available to public, leukaemia L1210 (LE), P388 leukaemia (PS), adenocarcinoma (CA), sarcoma 180 (SA), rat Walker carcinosarcoma 256 (subcutaneous)(WA), Friend virus leukaemia (solid) (FV), and HS1 human sarcoma (egg) (H1).

Results obtained for the *Viola odorata* extracts using both the *in vitro* and *in vivo* anticancer screening programs of the NCI did not show anticancer activity according to the test criteria. Hence, further investigation of these *Viola odorata* extracts was not undertaken by NCI.

As indicated in Table 11 and 12, none of the samples of *Viola odorata* analysed in the previous NCI investigation were from Australia. In addition, the extraction procedure used in the previous investigation involved a cold solvent percolation followed by a water extract, which would not have been as efficient as the boiling water extract administered according to the indigenous methods. However, sequential soxhlet extraction with PE (40-60^{0}C), $CHCl_3$, $CHCl_3$-MeOH (1:1) followed by milder 70% EtOH/water, alkali and

acid extracts did provide a much greater fractionation procedure based on the increasing polarity of the compounds.

1.8 The general aim of the project

The general aim of the project was to investigate whether a combination of antioxidant, immunomodulatory and cytotoxicity tests can be used to identify, isolate and characterise anticancer compounds from the putative anticancer plants *Euphorbia peplus* and *Viola odorata*.

1.8.1 Specific aims of the project

The specific aims of the research project are;

1. To investigate the *in vitro* anticancer activity of the sequential extracts of *Euphorbia peplus* (Chapter 3) and *Viola odorata* (Chapter 4) against the NCI's 60 cell-line human panel using the sulforhodamine B method.

2. To investigate the *in vitro* anticancer activity using the [^{3}H]-thymidine uptake and MTS assays against CEM leukaemia and LOVO colon cancer cells and to compare the results using these assay systems and with the brine shrimp lethality assay (Chapter 3 and 4).

3. To determine the antioxidant activity of *Euphorbia peplus* and *Viola odorata* using the DPPH free radical scavenging assay, the ferric TPTZ reducing assay and the inhibition of linoleic peroxidation using TBARS and FTC methods (Chapter 5).

4. To determine immunomodulatory activities of *Euphorbia peplus* and *Viola odorata* extracts using *in vitro* anti-complement, anti-platelet aggregation and inhibition of COX-2 activity (Chapter 6).

5. To study the chemical nature of the active extracts following activity-guided fractionation using spectroscopic techniques (Chapter 7).

The overview of the experimental strategy taken to achieve the above aims is shown in Figure 7.

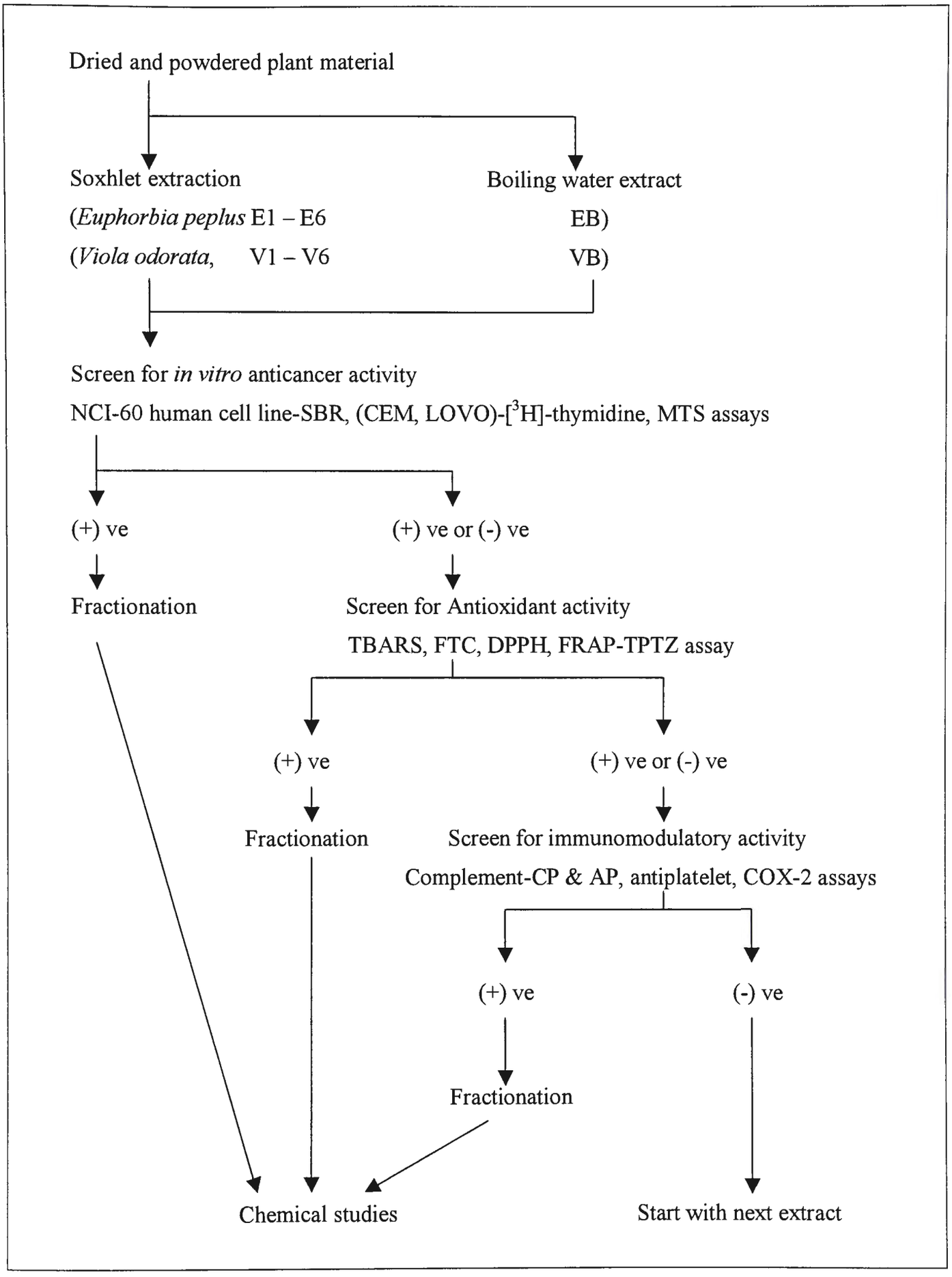


Figure 7 Overview of the experimental strategy

CHAPTER2 MATERIALS AND METHODS

2.1 Collection of plants for investigation

2.1.1 *Euphorbia peplus* L.

The whole plants of *Euphorbia peplus* L. was collected from home gardens in Baldwin, Hoppers Crossing, Werribee, in July 1997, and the lot collected from the city of Melbourne, Australia, in August 1997 was used in the experiments.

2.1.2 *Viola odorata* L.

The whole plants of *Viola odorata* L. was collected from home gardens in Werribee, Bentleigh and Hoppers Crossing and the lot collected in September 1997 from Dandenong ranges of Victoria, Australia with a permission from the Park Victoria, was used in the experiments described in this thesis

2.2 Identification of plants

Both plants were identified by Dr. Forman in the Herbarium of the Botanicals Gardens of Melbourne, Victoria, Australia. Fresh plant was dried and a voucher specimen was lodged in the herbarium of Victoria University of Technology.

2.3 Processing of plant materials

Plants collected were washed and contaminated weeds removed before being dried at 40^{0}C in a drying oven (Memmet, U.K) for 2/3 days. The dried plants were ground using Cereal Mill 6000 (Newport Scientific, U.K.) equipped with a 425 μm sieve. Heating during grinding was minimized by frequent passing of cold air around the grinding chamber. Finely powdered plant materials were sealed in double-layered polythene bags, labelled and stored at –20^{0}C in a cold room.

2.4 Method of extraction

2.4.1 Sequential solvent extraction

Sequential Soxhlet extraction of 100 g of dried, ground whole plants of *E. peplus* and *V. odorata* respectively, were carried out for 18 hours using 700ml each of petroleum spirit (b.p. 40 –60°C), $CHCl_3$, $CHCl_3$-MeOH (1:1 v/v) (BDH, U.K) to yield the extracts E1, E2, E3 and V1, V2, V3 from the two plants respectively. The residue was removed from the Soxhlet apparatus and extracted sequentially with 70% aqueous EtOH at 50°C, 10 %

NH_4OH (at room temperature) and 1 N HCl (BDH) (pH 2; room temperature) overnight to yield extracts, E4, E5, E6, and V4, V5, V6 respectively of the two plants. The extracts were concentrated using a rotary evaporator. The concentrated petroleum spirit, $CHCl_3$, and $CHCl_3$-MeOH extracts were evaporated to dryness using a nitrogen concentrator with a jet of nitrogen at 40°C. The concentrated residues were freeze dried using Dynavac freeze dryer.

Sequential soxhlet extracts, PE (40-60) (E1), $CHCl_3$ (E2), $CHCl_3$-MeOH (V3) followed by 70% EtOH-water (V4), 10% NH_4OH (V5) and 1 N HCl (V6) separately from the aerial parts and root of *Viola odorata* collected from Victoria, Australia during September 1998, were subjected to *in vitro* screening using different assays.

2.4.2 Aqueous extraction:

EB and VB were prepared by boiling 100g of the powdered plants with 700 ml of Milli Q water in a 2 L Erlenmeyer flask with stirring using a magnet bar on a heater/magnetic stirrer.

2.5 Screening for cytotoxic activity

2.5.1 Brine shrimp lethality Test

Hatching Brine Shrimp:

The brine shrimp (*Artemia salina* Leach) eggs purchased from Marine USA, were hatched in a rectangular glass tank (22 x 25 x 15 cm) containing artificial sea water prepared by dissolving 40 g of sea salt (Sigma Chemical Co. St. Louis, MO, USA) in 1 L of Milli Q water supplemented with 6 mg of dried yeast. A perforated plastic divider with 2 mm holes was inserted and clamped into the tank to make two unequal compartments. Approximately 50 mg of eggs were sprinkled into the larger compartment, which was covered with a black paper. while the smaller compartment was illuminated from its opened side using a 25W electric bulb. The artificial seawater in the tank was oxygenated using an aquarium pump. After 48 hours of incubation in a warm room (22-27°C), the phototropic nauplii were collected in 100 µl of seawater using a pipette from the illuminated side of the tank. The phototropic nauplii were separated from their shells by the divider.

Samples were prepared by dissolving 50 mg of the plant extracts in 50 µl of DMSO followed by the addition of seawater up to 25 ml. Further dilutions were made using each stock solution of the samples. A 1 ml aliquot each of the stock solution was diluted to 10 ml and 100 ml respectively with seawater.

Bioassay:
The brine shrimp lethality test was adopted from the modified method of Meyer, *et al* (1982) by using flat-bottom microtitreplates according to Solis *et al* (1993) instead of using vials with filter discs as the test vessels as described by Meyer *et al* (1982).

Three sample concentrations (i.e. 2000, 200 and 20 µg/ml), prepared by serial dilution of samples were dispensed in the wells of the flat-bottom 96-well microtitre plates (ICN Flow, U.K.) in quadruplicate using a volume of 100 µl seawater. Then, 200 µl of each sample stock solution were added in quadruplicate to column 1A to 4A, 5A to 8A and 9A to 12A respectively. Seawater (100 µl) was added to the wells of row B to F of each microtitre plate. A set volume (100 µl) from each well of the row A were then transferred to the subsequent well in row B using a pipette and mixed well by siphoning up and down thrice and thereafter continuing the dilution across the plate to row F. After final dilution on row F, a 100 µl portion of the diluted solution was discarded from each well of the row F. Dilutions (1:2) were made across the 6 rows (A to F) of each microtitre plate. Control wells containing DMSO and seawater were included in each experiment. A 100 µl suspension of nauplii containing 10-15 organisms in seawater was then added to each well. The plates were covered with their lids and incubated at 22-29 °C for 24 hours. Plates were then examined under a binocular microscope (x12.5) and the number of non-motile nauplii in each well was counted. The total number of nauplii in each well was counted 15 min after addition of 100 µl of methanol into each well. The percent deaths at each dose and the controls were determined. The LC_{50} values were calculated by probit analysis according to Finney (1964).

2.5.2 Anticancer activity

2.5.2.1 *In vitro* anticancer activity according to NCI's protocol using KB cell line

The NCI's anticancer screening programme began under the jurisdiction of the Cancer Chemotherapy National Service Center (CCNSC) in 1955 and the procedure used by the NCI in the earlier screening programme is described below in order understand the results discussed in Chapter 1.

Extraction Method

The plant material was air-dried at elevated temperature (40^0 C) and placed into cloth bags. These bags were kept at -20^0 C until processing. The dried plant material was ground using a Wiley mill with a 4 μm screen. The finely ground plant material was emptied into a borosilicate percolator, 10 cm diameter x variable length, and sufficient solvent or solvent mixture was added to cover the material. After steeping overnight at room temperature, the solvent extract was removed. The solvent extract was concentrated using a rotary evaporator and dried using a freeze dryer (Cragg, 1999, NCI Protocol, personal communication).

Cell culture

The stock cultures of mouse nasopharynx (KB) cell line on glass were cultivated on Eagle's basal medium containing 10% calf or human serum medium. The cells were maintained in a state of rapid growth by frequent subculture every 3 or 4 days. The cultures were re-fed 24 hours before use on test.

Test Procedure

The cells were removed from glass either with trypsin or Versene, or by mechanical scraping. Dispersed cells were centrifuged and resuspended in the medium. About 50, 000 cells in 3.9 ml of medium were implanted in a series of replicate 15-mm screw-cap culture tubes and the tubes were incubated at a 10^0 angle at 30^0C. After 24 hours the medium was removed and fresh medium containing 0.1 ml of the plant extract in respective solvent was added. Control tubes containing all the above except the sample were prepared. After 1 or 2 days, the protein content was determined according to the method of Oyama and Eagle (1956).

Routine testing of the plant extracts was done at concentrations of 1, 10 and 100 μg/ml (3 doses at 1.0 log intervals). The dose (ED_{50}), in μg/ml, that inhibits growth to 50% of control growth has been determined for all the samples testes. A sample with an ED_{50} less than 1 μg/ml has been retested at lower concentrations; one with an ED_{50} above 100 μg/ml has been considered inactive and has not been retested. In some cases, 5 dose levels at close intervals (0.3 log intervals) have been used to obtain a more precise end point.

Determination of Cytotoxicity

The determination of cytotoxicity has been based on the inhibition of cell protein synthesis. Measurements included the initial protein content per tube (C_0) using an aliquot of inoculum from the control tubes at the time of sample addition, the final protein content in control tubes (C) and the final protein content in the sample-treated tube (T).

The equivalent of a T/C value or $(T- C_0)/(C- C_0)$, was determined for each dose level.. An assumption was made that the response varies linearly with the log of the concentration within defined limits of response. An estimated ED_{50} value or the dose which, inhibits protein synthesis to 50% of controls, has been calculated using IBM 1410 computer program. A slope representing the change in response for 10-fold change in concentration has also been calculated.

Samples have been tested simultaneously at 3-5 dose levels against a single set of controls. Each dose level has been run in duplicate tubes, and the number of controls were equal to $2\sqrt{n}$, where n= number of sample replicates.

Performance of the *in vitro* screen is judged by estimating reproducibility of the ED_{50} value based on tests of positive control material carried out at 4 laboratories in 10 to 47 replicates, at 5 dose levels at 0.3 log intervals.

2.5.2.2 *In vivo* anticancer activity on mice

The *in vivo* screening of the plant extracts have been carried out at NCI, Frederick, MD, USA, against implanted mouse cancers and tumours such as, L1210 leukaemia (LE), P388 leukaemia (PS), Lewis lung carcinoma (LL), sarcoma 180 (SA), and rat Walker carcinosarcoma 256 (WA). The test procedure used by the NCI during 1962-1976, to determine the *in vivo* anticancer activity of the plant extracts described by Schepartz *et al* (1967) is given below.

Establishment of Test and Control Groups

Relative reproducibility of each test system was used to establish reasonable test and control groups. Ten animals each have been assigned to a treated group and a control group for LE and SA, and 6 animals for LL. The animals were assigned to the control and

experimental groups by a randomisation technique using Kenda II-Smith Tables of random numbers.

2.5.2.3 *In vitro* anticancer activity against CEM and LOVO cell lines

Extraction of plants

The extracts prepared as described in section 2.4.1 at dose levels of ≤100 μg/ml, were used in the *in vitro* screening against human leukaemia (CEM) and human colon cancer (LOVO) cell lines. The cancer cell lines were maintained in the following minimum essential medium (MEM) 50 ml HIFCS (Fetal Calf serum), 10 ml sterile glutamine, 10 ml sterile PIS, 0.5 ml sterile 5 μM, 2-ME (2-mercaptoethanol), 10 ml of 1M, HEPES, 500 ml RPMI 1640 medium (CSL Ltd, Melbourne) were added and mixed thoroughly. Medium was stored at 4°C in a cold room and dispensed in a lamina flow bench using sterile techniques.

Cell count: The optimum number of cells used in experiments was 1 x 10^6cells/ml or 1 x 10^5cells/100 μl. However, in order to obtain a linear response, 1 x 10^4 cells per well were maintained for each MTS assay.

Before each day's experiment, the number of cells maintained in 20 ml portions of medium in polystyrene tissue culture bottles (Falcon Co.) was counted to determine how many plates could be run depending on the available number of cells in the culture.

Counting Procedure:

Cell cultures were transferred under sterile technique into a 50 ml PP centrifuge tube. A 10 μl aliquot of the culture was transferred into an Eppendorff tube and 10 μl of trypan blue (TB) was added and mixed with the same pipette tip. A 10 μl aliquot of the mixture was placed on a haemocytometer and examined under a Leica microscope set to magnification x 400. The number of cells in the four fields was counted and averaged.

Cell count = Average field count/2 X 10^4 cells/ml

The culture was centrifuged at 300g (1400 rpm) for 5 min using a Beckman CS-15 centrifuge. The supernatant was decanted and the pellets were resuspended in a portion of

RPMI medium, which was adjusted to give cell concentrations of 1 x 10^5/100 µl. or 1 x 10^6/ml.

Preparation of Sample Solutions

About 5 mg of the sample extracts were weighed into 1.5 ml-Eppendorf tubes and dissolved either in 1 ml of DMSO or in MQ-water by vortexing for 1-2 min. The solutions were filtered through sterile 0.22 µm Millex GV filter cartridge (Millipore, MA, USA) and dispensed into 1 ml sterile graduated PP sample tubes. Sample solutions were dispensed into 4 x 6-well tissue culture plates (ICN Flow, U.K.). A 100 µl aliquots of samples were transferred into each well of the microtitre plate.

Aliquots (100 µl) of filtered and diluted sample solutions were added to the first well. The PBS was filtered using a 0.22 µm Millipore filter and the sample solutions were further diluted with 900 ml of PBS. In all other cases, 50 µl of the solution were transferred using 200 µl pipette into the first well of the sample plate, then a 250 µl aliquots of filtered PBS were added to the subsequent wells (well 2 through to well 12).

Then, 250 µl of the content of the first well was transferred into the second well mixed 4 times using the same pipette up to well 12 and the last 250 µl portion was discarded from the 12th well.

Subsequently, 100 µl from each well (except well 12) of the sample plate was transferred, in duplicate for each experiment into the microtitre plates containing 100 µl of 1 x 10^5 cells in RPMI. The 12th column of the microtitre plate was used as a control. Finally, a 100 µl each of the filtered PBS was added to each well of the 12th column containing 100 µl of 1 x 10^5 cells in RPMI. The microtitre plates were incubated in humidified 5% CO_2 incubator at 37°C for 20 hours.

2.5.2.4 Sulforhodamine B (SBR) assay using NCI's panel of 60 human cancer cell lines

Screening procedure: The NCI screening procedure (Monks *et al*, 1991) was used to screen the sequential extracts of *Euphorbia peplus* and *Viola odorata*. An aliquot of cell suspensions (100 µl) that were diluted according to the particular cell type and the expected target cell density (5000-40,000 cells per well based on cell growth characteristics of each cell line)

were added into 96-well microtiter plates by a pipette. Inoculates were allowed a pre-incubation period of 24 hours at 37^{0}C for stabilization. Dilution at twice the intended test concentration was added at time zero in 100 µl aliquots to the micrititer plate wells. Usually, test compounds were evaluated at five 10-fold dilutions. The highest well concentration of the crude plant extracts used in the routine testing has been 100 µg/ml, but for the standard agents the highest well concentration used dependent on the agent, which is usually 10E-4M. Incubations lasted for 48 hours in 5% CO_2 atmosphere and 100% humidity. The cells were assayed at the NCI using the sulfarhodamine B (SBR) assay described below, according to Rubinstein *et al* (1990) and Skehan *et al* (1990).

Sulforhodamine B Assay

The extracts have been screened against NCI's human 60 cell-panel at concentrations of 0.01, 0.1, 1, 10 and 100 µg/ml using sulforhodamine B assay according to Monks *et al* (1991).

The cell cultures were fixed with trichloroacetic acid and stained for 30 min, with 0.4% (w/v) SRB dissolved in 1% acetic acid. At the end of the staining period, SRB was removed and cultures were quickly rinsed four times with 1% acetic acid to remove unbound dye. The acetic acid was poured directly into the culture wells from a beaker. Residual wash solution was removed by sharply flicking plates over a sink for complete removal of rinsing solution. After being rinsed the cultures were air dried until no standing moisture was visible. Bound dye was visualised with 10 mM unbuffered Tris base (pH 10.5) for 5 minutes ona gyratory shaker.

OD was read in either an Uvmax microtiter plate reader (Molecular Devices, Menlo Park, CA) or a Beckman DU-70 spectrophotometer. For maximum sensitivity, OD was measured at 564 nm. Because readings were linear with dye concentrations only below 1.8 OD units, however, sub-optimal wavelengths were generally used, so that all samples in an experiment remained within the linear OD range. With most cell lines, wavelengths of approximately 490-530 nm, according to Skehan *et al* (1990) has worked well for this purpose. A plate reader was used to read the optical densities, and a microcomputer processed the optical densities into the special concentration parameters defined below.

Special concentration parameters (GI50, TGI and LC50)

The previously used ED_{50} parameter has been renamed by the NCI with the incorporation of the COMPARE program into its screen.

The GI50 value, the concentration that causes 50% growth inhibition, has been introduced to emphasize the correction for cell count at time zero. Thus GI50 is the concentration of test drug or extract where100 x (T-T0)/(C-T0)= 50 (Boyd, *et al* 1992, and Monks *et al*, 1991) where T is the optical density at time zero, and C is the optical density of the control. The GI50 is a T/C-like parameter that can have values from +100 to –100. GI50 measures the growth inhibitory power of the test agent. The TGI is the concentration of the test drug or extract where 100 x (T-T0)/(C-T0) = 0. Thus, the TGI signifies a cytostatic effect. The LC50 which signifies a cytotoxic effect, is the concentration of sample where 100 x (T-T0)/T0 = -50. The control OD is not used in the calculation of LC50. For mean graph and COMPARE purposes, the value assumed for the GI50 in such a case is the highest concentration tested (HICONC). Similar approximations are made when GI50 cannot be calculated because the GI50 does not go as high as 50 or above. In such a case, the lowest concentration tested is used for the GI50. Corresponding approximations are made for the TGI and for the LC50.

Mean Graphs and Concentration parameters (GI50, TGI and LC50)

A "mean graph" is a pattern created by plotting positive and negative values generated from a set of GI50, TGI and LC50 values. The positive and negative values are plotted along a vertical line that represents the mean response of all the cell lines in the panel to the test sample. Positive values project to the right of the vertical line and represent cellular sensitivities to the test sample that exceed the mean. Negative values project to the left and represent cell line sensitivities to the test sample that are less than the average value.

The positive and negative values, called deltas, are generated from the GI50 data (or TGI or LC50 data) by a three-step calculation. The GI50 value for each cell line tested against a test sample is converted to its $\log_{10}$ GI50 value. The $\log_{10}$ GI50 values are averaged. Each $\log_{10}$ GI50 value is subtracted from the average to create the delta. Thus, a bar projecting 3 units to the right denotes that the GI50 (or TGI or LC50) for that cell line occurs at a

concentration 1000 times less than the average concentration required for all the cell lines used in the experiment.

Despite being interpolated values, these concentration parameters provide useful information about the sub-panel selective cytotoxicity of the test sample. In an extreme case where a sample is essentially inert and the GI50s are all represented by the high concentration approximation (greater than the highest concentration used), the mean graph becomes a flat vertical line (the mean line) and COMPARE has no pattern to correlate. The opposite extreme case is where a sample is so potent that the lowest concentration tested is used to approximate all of the GI50s. In this case, the mean graph is also a flat vertical line and COMPARE has nothing to correlate. The difference in the two extreme cases is in the retests that are done. Although the inert compound would not be retested, the potent compound would be retested at a more appropriate concentration range.

The GI50, TGI and LC50 mean graphs presents the in vitro results of Development Therapeutic Programme (DTP) of the NCI which emphasize the differential effects of test sample on various human tumour cell lines. The complete presentation and organization of the mean graph data were to optimise the disease-type-sub panel specific effects of the test sample (Boyd and Paull 1995).

2.5.2.5 [^{3}H]-Thymidine uptake assay using human CEM and LOVO cancer cell lines

The ^{3}H-thymidine uptake assay was carried out according to Pietersz *et al (*1988). ^{3}H-thymidine (100 μCi/100 μl) (product No. 6005412) purchased from Amersham Chemical. Co, UK in 5 ml of RPMI solution was used in the assay. After 20 hrs of incubation 1 μCi/μl of thymidine was added to each well and further incubated for 4 hrs.

After incubating the pulsed cells in a humidified 5% CO_2 incubator at 37^{0}C, the cells were harvested using Cell harvester (Packard Co.).

A glass fibre filter RG (Packard Co.) was placed on the filter holder with smooth side down and the two guide holes fitting into the notches of the harvester base. Filter holder was placed on the filter paper and the lever was lowered tightened and locked using the left lever. The filter was washed thrice with distilled water by pressing knobs labelled

"Water " and "Vacuum" thrice. The assay plate with cells was placed on the bottom tray and tray handle was lifted. While holding the tray handle "Hot vacuum" knob was pressed in order for the cells to be sucked on to the filter. "Water" knob was then pressed to rinse the assay plate with water taking care not to let the plate overflowed with water. The washing the plate was repeated thrice. Both "Cold" and "Hot" vacuum knobs were pressed and locked and the knob "Water" was pressed 10-15 times to wash the cells further. While keeping the suction on, the filter holder lever was opened so that the filter was stuck by suction on the filter holder. The assay plate was removed from the bottom tray and EtOH was added and rinsed under suction. The filter with cells on the filter holder was allowed to dry at 65^{0}C for 20 min. in a drying oven.

Table 13 Protocol for the measurement of radioactivity

```
 Data Mode:                CPM
 Radionuclide:             3H-MicroScint        Scintillator: Liq/Plast
Range: Low                  Efficiency Mode: Normal
                                               Region A:       2.90-35.00
B:       2.90-256.00
 Count Time (min):         1.00
 Count Termination:        no
 Count Delay (min):         0.00
 Additional Reading:       depletion
 Target Temperature:       18.00  (68.2F)
 Plate Orientation:        Inverted
 Background Subtract:      none
 Half Life Correction:     no
 Sample Screening:         no
 Quench Indicator:         tSIS
 Data File Name:           3-H.032              Drive & Path: a:\
 Save Each Plate to a File: yes

 Platemap blank subtraction = No
 Cross talk reduction: Off
 Data file path invalid. Saved data to: C:\$TCDATA$\3-H.032.
```

The plastic template (Packard Co.) with 96-holes was aligned with the 96 circles of the filter containing dried cells and placed on the dried filter still on the filter holder and the outer edges of the filter paper was cut using a knife. The cut filter was placed on the template holder and the template was pressed on it. Then 30 µl of micro Scint-O cocktail from Packard Co. was added to each circle of cells (representing each well of the microtitre assay plat) on the filter and the template was sealed with a transparent adhesive seal purchased from Packard Co. The covered template with the filter was placed on the sample changer of the Top Count (Packard) scintillation counter and the radioactivity was measured using the protocol (#2) given in Table 13.

2.5.2.6 MTS assay using murine CEM and LOVO cancer cell lines

The MTS assay was carried out according to the procedure given in the Promega Technical Bulletin (TB169, 1994). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium, inner salt (MTS) and phenazine methosulphate (PMS) PMS solutions purchased from Promega, WI USA that have been kept at –20^{0}C were transferred to a water bath maintained at 37^{0}C and allowed to thaw for 10 minutes. An aliquot (2.0 ml) of MTS solution was removed from its amber reagent bottle using aseptic technique and transferred to a test tube. Then, 100 µl of PMS solution was added to the 2.0 ml of MTS solution immediately before addition to the culture plate containing cells. The test tube was swirled gently to ensure complete mixing of the combined MTS/PMS solution.

Before the addition of the MTS/PMS mixture, absorbance measurement of the 96 well assay plate containing the sample and the cells in culture medium which has been incubated at 37^{0}C in a humidified 5% CO_2 incubator for 20 hours was made at 490 nm using EL 312e ELISA plate reader (Biotech Instruments Inc.).

Addition of the combined MTS/PMS solution was substituted for the pulse of ^{3}H-thymidine in the thymidine incorporation assay at the time point when the pulse of radioactive thymidine is usually added.

Using a multi-channel pipette, 20 µl of combined MTS/PMS solution were pipetted into each well of the 96 well assay plate. The plate was gently shaken to mix the solutions homogeneously and incubated at 37^{0}C in a humidified 5% CO_2 incubator for 1-4 hours. After 1 hour the absorbance of each well of the assay plate was recorded at 490 nm using the ELISA plate reader.

The absorbance values corrected for the controls verses log concentration of the extract were plotted and the IC_{50} value corresponding to one-half the difference between the maximum (plateau) and minimum (no extract control) absorbance values were calculated. The IC_{50} value is the concentration of the extract necessary to give 50% of the maximum response.

2.6 Screening for antioxidant activity

Extraction of plant material was carried out using a Soxhlet apparatus according to Sroka *et al* (1994). The assay of antioxidant activity of the crude extracts was carried out employing linoleic acid system according to Sroka *et al* (1994), Kikuzaki and Nakatani (1993) and Tsuda *et al* (1994a), and the formation of malondialdehyde (MDA) was measured using thiobarbituric acid reducing substances (TBARS) method (Buege and Aust, 1978). Results were compared against antioxidant activities of α-tocopherol and α-tocopherol according to Hong (1994).

2.6.1 Rapid TBARS method

The rapid TBARS method was carried out according to Sroka *et al* (1994) using linoleic acid instead of using OP (*Oenothera paradoxa*) oil, according to Kikuzaki and Nakatani's (1994) method described in Section 2.6.3. After 5 min incubation at 25^0C, 100 µl of freshly prepared 37.8 mM ammonium ferrous sulphate (AR, BDH) was added to 500 µl of incubate followed by 2 ml of TBARS reagent and proceeded according to the TBARS method (Section 2.6.3.2).

2.6.2 TPTZ method development

The Fe^{3+}-TPTZ (ferric 2, 4, 6-tripyridyl-*S*-triazine) method of Benzie and Strain(1996) was modified slightly by increasing the concentration of the reagent and increasing the reaction volume to 825 µl using 1.5 ml cuvettes. A Varian Cary 1E spectrophotometer, equipped with a multicell chamber, was used to measure absorbance. The sequence of adding the reagent was also modified by adding the sample and the distilled water to the cuvette followed by the addition of the reagent mixture as described by Liu *et al*, 1982). This method did not lead to the formation of air bubbles.

Reagents;

Acetate buffer, 0.3 mM/L, pH 3.6, 3.1 g of Sodium acetate trihydrate (BDH, A. R., U.K.) and 16 ml of glacial acetic acid (BDH) were dissolved in 800 ml of Milli-Q water. The pH was adjusted to 3.6 and made up to 1L with Milli-Q water.

TPTZ solution, 8.0 mM/L, 624 mg of TPTZ (Sigma Chemical Co., Australia) was dissolved in 250 ml of 36 mM/l HCl (BDH).

Ferric chloride solution, 20 mM/L, 540 mg of $FeCl_3$.6H2O (Sigma Chemical Co.) was dissolved in 100 ml of Milli-Q water.

Fe^{2+} standard. Aqueous solutions of $FeSO_4.7H_2O$ (BDH A. R.) were used as Fe^{2+} standards mentioned above.

Plant extracts: The freeze-dried residues of the extracts, E1 to E6 & EB and V1 to V6 & VB were dissolved in DMSO (10%) (Sigma) and diluted with 99.5% EtOH were used in the assays. Antioxidant standards, trolox, DL-α-tocopherol, and quercetin were purchased from the Sigma Chemical Company, St. Louis, USA and $FeCl_2$ from BDH and were also prepared in the same solutions as described for the samples.

The reaction mixture was prepared immediately before starting the measurements. Then, 25 ml of acetate buffer, 3 ml of TPTZ solution and 2 ml of the $FeCl_3$ solutions were mixed. Aliquots of the samples (50 µl) and 25 µl of distilled water were added to the cuvette and incubated for 15 min at 37°C in the multicell chamber of the spectrophotometer. A 750 µl of the reagent mixture, which was kept in a water bath at 37°C, was then pipetted into the cuvette. At 5 min. after starting incubation, the absorbance was measured at 593 nm against the reagent blank. The absorbance of the reagent blank was monitored throughout the experiment in order to check the stability of the reagent.

A dose response curve was obtained using a series of concentrations of each sample and standard. The gradient of the dose response curve was used to determine the antioxidant activity provided it was within its linear range.

The 200 µg/ml solutions of the extracts, E1 to E6 of *E. peplus*, and V1 to V6 of *V. odorata*, were used to determine the distribution of antioxidant activity among the extracts. The TPTZ reaction kinetics was used to provide a direct measurement of the antioxidant activity of the sample. Hence, the results are given as a relative percent activity based on the slope of the linear trend line in relation to that of Fe-II standards.

The relative ferric reducing antioxidant activity (RFRAOA) of the samples tested were calculated by dividing the absorbance value of the extract by the absorbance value of the Fe^{+2} standard at a concentration of 200 mg/L. For all other measurements the RFRAOP

were calculated by dividing the gradient of the linear trend line of Fe^{2+} standards from the gradients of the trend lines obtained for the dilution series of each sample

2.6.3 Inhibition of lipid peroxidation

The lipid peroxidation method of Kikuzaki and Nakatani (1993) was adopted with modifications. Auto-oxidation of linoleic acid in the water-alcohol system was carried out using the method of Osawa and Namiki (1981).

A mixture of 4.0 ml sample solution, 4.1 ml of 2.51% linoleic acid (Aldrich Chemical Co Inc. Milwoukee, WI, USA) in 99.5% EtOH, 8 ml of 0.05M phosphate buffer (pH 7.0) and 3.9 ml of distilled water was added to amber bottles with screw caps and incubated in an oven at 40^0C in the dark. Known volumes, 100 µl and 250 µl of the incubates, were used for the FTC and TBARS tests respectively. Sample volumes were drawn from the incubates, daily, up to 15-20 days for determining antioxidant activity.

2.6.3.1 Ferric thiocyanate (FTC) method

The FTC method of Mitsuda *et al* (1967) and Osawa and Namiki (1981) modified by Kikuzaki and Nakatani (1993) was carried out. To 100 µl of the incubate in 10 ml tube, 9.7 ml of redistilled alcohol, 100 µl of 30% NH_4SCN solution in MQ water and 100 µl of 20 mM $FeCl_2.4H_2O$ were added and mixed thoroughly using a vortex mixer. At 3 min. after addition of the $FeCl_2$, the absorbance at 500 nm was measured against a reagent blank. The measurement was made daily until the absorbance of the control reached the maximum as determined by the lower absorbance value on the following day. The percent inhibition of linoleic acid peroxidation, 100-[(Absorbance increase of sample/Absorbance increase of control) x100] was calculated and expressed as the antioxidant activity

2.6.3.2 Thiobarbituric acid reacting substances (TBARS) method

The method of Ottolenghi (1959) and Kikuzaki and Nakatini (1993), was modified by reducing the sample size and replacing TBARS reagent with TBA (thiobarbituric acid)-TCA (trichloroacetic acid)-HCl reagent as suggested by Beuge and Aust (1978). The TBA-TCA-HCl reagent was made using 30% w/v aqueous TCA, 0.75% w/v aq. TBA, and 0.25 N HCl. To 250 µl of the incubate in a screw cap centrifuge tube, 500 µl of the TCA-TBA-HCl reagent was added, mixed thoroughly on a vortex mixer, and placed in a boiling water bath for 10 min. and cooled and then centrifuged at 3000 g for 20 min. The absorbance of

the supernatant was measured at 532 nm against a reagent blank. The antioxidant activity was calculated as described for the FTC method.

2.6.4 DPPH free radical scavenging activity method

The methods adopted by Sassa *et al* (1990) and Lee *et al* (1996) were modified by using 500 μM methanolic solution of DPPH (1,1-diphenyl-2-picryl-hydrazyl) (Sigma) and 100 mM acetate buffer. The pH of the acetate buffer was made up to 8.8 in order to maintain stable DPPH radicals as alkaline pH stabilizes the free radicals (Bors and Saran, 1987). The maximum absorbance of the alkaline DPPH at pH 8.8 was found to be at 530 nm. All measurements were, therefore, made at 530 nm.

The reaction mixture was prepared by adding 25 ml of the 500 μM of DPPH, 50 ml of 100 mM acetate buffer pH 8.8, and 50 ml of MeOH in an Erlenmeyer flask.

Before the measurements were made, the absorbance maxima of the DPPH incubation mixture was determined after obtaining the visible spectrum of the DPPH incubation mixture using Varian Cary UV-VIS spectrophotometer in double beam mode. The absorbance maxima of the DPPH incubation mixture was found to be at 530 nm.

After adding 50 μl of the sample solution to a 950 μl of the DPPH solution in a 1.5 ml cuvette, and standing for 10 min at room temperature (20^{0}C), the absorbance was measured at 530 nm.

FRSA values were calculated from an average of six replicate determinations, taking the percent inhibition of the free radicals by the control as 0%. The FRSA values were estimated using the equation given for inhibition of lipid peroxidation.

2.6.5 Comparison of TPTZ, DPPH-FRSA, TBARS and FTC methods

The TPTTZ results obtained for 200 mg/L solutions of the samples were used to compare different methods. The FRSA results were calculated as the percent inhibition of free radicals in the reagent blank using a concentration of sample at 200 mg/L. The corresponding TBARS and FTC values are the percent inhibition of linoleic peroxidation by the active sample at 200 mg/L.

Statistical analysis. Student's t-test for significance of difference of means was calculated using SAS statistical software package. The Coefficient of Variation (CV) values were calculated by dividing the standard deviation by mean and expressed as a percentage.

2.7 Screening for humoural immunomodulatory activity

In this research, the complement assay method developed by Klerx *et al* (1983) and developed by Kroes (1996) was adopted. The principles of the assay entail incubation of human serum at different concentrations with rabbit erythrocytes (alternative pathway) or with antigen-labelled sheep erythrocytes (classical pathway). Lysis effected by the complement from serum results in release of different amounts of haemoglobin from the erythrocytes, optical density of which is measured in the supernatant. The inhibition of complement activity is given as percent inhibition compared to the identical controls without the inhibitor. These values are obtained for a serum blank and the serum blank of the rabbit erythrocytes and for identically tested serum containing the plant extracts. The absorbance due to plant extract and autolysis of erythrocytes are corrected by measuring the absorbance of heat-deactivated serum.

The sequential extracts (E1 to E6) as well as the boiling water extract (EB) of the plant, *Euphorbia peplus* were screened for *in vitro* inhibition of complement activity or "anticomplement activity" via classical and alternative pathways.

Due to the limited availability of resources only the extracts of *Euphorbia peplus* and *Viola odorata* that showed a considerably high antioxidant activity were investigated for *in vitro* inhibition of arachidonic acid induced platelet aggregation activity in whole blood using the impedance method.

In this research, selected sequential extracts of *Viola odorata* and *Euphorbia peplus* were investigated for inhibition of COX-2 enzyme using a TLC separation of the reaction mixture followed by measurement of the radioactive arachidonic acid and/or its metabolites was carried out according to Mitchell *et al* (1994). However, the solvent system used for TLC separation was that used by Saeed *et al* (1995). The very low sensitivity of the radioactive detection by the Tracemaster-20 analyser was further improved by using a Fuji screen. The Fuji radiotracer was used to scan the radioactive zones and the quantification was performed using Fuji Image Gauge software.

2.7.1 The complement activation assay

In vitro Alternative and Classical pathway activities were carried out using human serum (Red Cross blood Bank, Victoria) according to microtitre assay (Kroes, 1996) with normal rabbit and sensitised sheep erythrocytes respectively as target cells in the presence of the plant extracts. inulin and dextran sulphate were used as standards.

The Alternative and Classical pathway activities were expressed as AP50 and CH50 units per ml according to the definitions as described by Mayer (1961) and Takada et al (1978).

Complement tests were carried out using the haemolytic assay procedure (Mayer, 1961; Takada *et al*, 1978; Van Dijk *et al*, 1980) modified to micro scale according to Klerx *et al* (1980) and Kroes (1995).

Reagents and buffers;

VSB-5x Stock [25 mM]

NaCl (20.75 g) and Na-Veronal (2.55 g) were dissolved in 400 ml of MQ water and the pH was adjusted to 7.35 using HCl and made up to 500 ml with MQ water.

Stock Ca/Mg solution: $MgCl_2.6\ H_2O$ (10.17 g) and $CaCl_2.2\ H_2O$ (2.21 g) were dissolved in 100 ml of MQ water.

EGTA-Mg Stock (Platts-Mills, 1974)

EGTA (3.804 g) was dissolved in 45 ml of MQ water by adding a minimum quantity of solid NaOH., and 3.075 g of $MgSO_4.7H_2O$ was dissolved in 45 ml of MQ-water and the two solutions were combined. The pH of the solution was adjusted to 7.35 with citric acid (10%) and NaOH and made up to 100 ml with MQ-water. The solution was sterilized by passing through a 0.22 µ Millipore filter.

VSB^{++}

An aliquot (200 ml) of VSB x5 stock was mixed with 10 ml of the Ca/Mg stock solution. Approximately 600 ml of MQ-water was added and the pH was adjusted to 7.35 with citric and Na_3-citrate and made up to 1000 ml with MQ-water.

Saline solution: NaCl (9 g) was dissolved in 1L of MQ-water and filtered through a 0.22 µ Millipore filter.

Alsevere's Solution; **G**lucose (2.05 g), Na-citrate.$2H_2O$ (0.8 g) and NaCl (0.42 g) were dissolved in 50 ml of MQ-water, and the pH was adjusted to 6.1 using 10% sodium citrate solution, made up to 100 ml and sterilized at 121^{0}C for 15 min.

Sheep Blood (Defibrinated) (ShE) for CP

Sheep blood was purchased from Ameadua, Melbourne in 20 ml bottles and diluted 1:1 in Alsever's solution.

Rabbit Blood (RaE) (Defibrinated) for AP

Rabbit blood was purchased from Ameadua Company (Melbourne) in 10 ml bottles. The blood was preserved in 1:1 Alsever's solution. An amount (4 ml) of the RaE in Alsevere solution was mixed with 6 ml of the 0.9% saline solution in a 20 ml plastic tube and centrifuged at 1250 x g for 5 min. The supernatant was removed and the pellets were resuspended in 10 ml saline solution and centrifuged at 1250 x g for 5 min. This procedure was repeated twice and the pellet of packed cells was kept on ice. A 400 µl portion of the packed cells was suspended in 7.1 ml of EGTA-VB in a 50 ml plastic tube, and 100 µl of this suspension was mixed with 6.4 ml of MQ-water in a 10 ml tube. The optical density (OD) of this solution was measured at 414 nm. An OD_{414} of 1.230 corresponded to a RaE suspension of 1.15 x 10^8 cells/ml. The RaE concentration was adjusted to 1.15 x 10^8 cells/ml using the following formula:

Volume of additional EGTA-VB required = (initial volume- 100 µl) x (OD measured-1.230)/ 1.230

Human Blood (without anticoagulant)

Human blood, screened and found negative for infectious diseases, was collected from the Red Cross Blood Bank, Victoria. Prior to the collection of blood, permission to use human blood in this research, was obtained from the School of Life Sciences and Technology, Faculty of Engineering & Science, Victoria University of Technology. A maximum of 10 ml was collected in 50 ml Griener tubes and transported on dry ice to the Laboratory. The blood was thawed at room temperature of 25^{0}C then kept at 0^{0}C prior to centrifugation at 4^{0}C for 15 min at 1000g. This procedure was repeated three times and the serum supernatant (approximately 1.0 ml) was separated using a sterilized Pasteur

pipette into Eppendorf tubes (1.5 ml) and individual sera or pool of 5 donors in small aliquots kept at -70^{0}C.

Rabbit Anti-Sheep Red Blood Cell Stroma, IgG Fraction (S-8014)

Rabbit anti-sheep red blood cell stroma IgG fraction was purchased from Sigma. The freeze-dried cell stroma was reconstituted by adding 2.0 ml of MQ-water to the content of the vial and by mixing on a vortex. The turbid suspension was centrifuged and the supernatant was stored in aliquots at –20^0 C. Prior to the experiment, the supernatant was diluted (1:100) with GV^{++} buffer (gelatin veronal).

Sensitization of ShE for Human CP

Washing

ShE (2 ml) in 1:1 Alserver's solution was mixed with 8 ml of saline solution in a 50 ml plastic tube and centrifuged at 1250 x g for 5 min. The supernatant was discarded and the pellet was suspended in 10 ml of saline solution and centrifuged at 1250 g for 5 min. This procedure was repeated twice and the pellet of packed cells was kept on ice.

Preparation of ShE suspension

Packed ShE (200 µl) was suspended in 9.8 ml of VSB^{++} in a 50 ml plastic tube, and 100 µl of the suspension was mixed with 4.9 ml of MQ-water in a 50 ml tube and the OD_{414} was measured using a spectrophotometer. An OD_{414} of 0.522 corresponded to a ShE suspension of 4x 10^8 cells/ml The ShE concentration was adjusted to 4x 10^8 cells/ml using the following formula;

Volume of additional VSB^{++} required = (initial volume- 100 µl) x [(OD measured-0.522)/ 0.522]. The ShE suspension was kept on ice.

Sensitizing of ShE

The ShE were sensitised by adding an equal volume of heat inactivated antisera (amboceptor) diluted in 1:100 in VSB^{++} constantly shaking for 10 min at room temperature. The mixture was centrifuged at 1250 x g for 5 min. and the supernatant was discarded. The pellet of packed cells was resuspended in 16 ml of VSB^{++} to the final ShE concentration of 4x 10^8 cells/ml. The optimum dilution of antiserum required to sensitise

ShE was determined by the MTP assay using serially diluted antiserum and washed ShEs (Meyer, 1961). The optimum dilution was found 1:100 in VSB^{++}.

Plant Extracts

Sequential Soxhlet extracts in PE, $CHCl_3$, MeOH- $CHCl_3$, EtOH, NH_4OH and HCl were used for screening immunomodulatory activity. Samples were prepared at concentrations ranging from 0.1 to 1000 µg/ml. In some cases the extracts were dissolved in 10% DMSO, and then the solution was diluted with the buffer, VSB^{++}. For coloured solutions, the absorbance at 414 nm of the drug solution diluted in VBSG, with no serum or erythrocytes, was determined and subtracted from the sample values.

The solvent used to dissolute the extracts were tested by itself too. Values from 4 to 6 separate determinations were averaged i.e. mean ± SEM of four values. SEM was determined for each plasma dilution and drug concentration.

2.7.1.1 The alternative pathway

The assay was performed in EGTA-VB (7.35±0.05) and in the presence of different concentration of plant extracts. The concentrations tested were 1000, 500 and 100 µg dissolved in 300 µl of DMSO. The serum volume used was between 10 and 30 µl. The target cells were 100 µl of RaE. Heat inactivated serum (45 min. at 56^0 C) was used as a control against each active serum test sample. This control consisted of a complement free identical serum media and was used for correcting against any haemoglobin present in the active human serum sample.

Sample dilution in U-well microtitre plates

EGTA-VB (100 µl) was added to the wells of row B up to G. Sample solutions (200 µl) were added in triplicate to row A which gave a 1:2 sample dilution. Subsequently, 100 µl of the solution in row A was transferred to row B and after mixing thoroughly using the pipette, 100 µl of the solution was transferred to the next row. This procedure was continued up to row G and a 100 µl from the final mixture in row G was discarded.

Preparation of Controls

EGTA-VB (100 µl) was added to the well H1 to H6 (H1 to H3 for the determination of the activity of the serum; H4 to H6 are the serum blank). EGTA-VB (125 µl) was added to the well H7 to H9 (0% haemolysis control), and MQ-water (125 µl) was added to H10 to H12 (100% haemolysis control).

Preparation of serum dilution and pre-incubation

The reagent blank was prepared by mixing 500 µl of heat-inactivated serum with 500 µl of EGTA-VB. A 25 µl portion of this solution was added to A3 to G3, A6 to G6, A9 to G9, A12 to G12 and H4 to H6. Human pooled serum (1.00 ml) was mixed with 1. 00 ml of EGTA-VB and used immediately. A 25 µl aliquot of this solution was added to A1 to H1, A2 to H2, H3, A4 to G4, A5 to G5, A7 to G7, A8 to G8 A10 to G10, and A11 to G11. The microtitre plate was then covered with its lid and pre-incubated at 37^{0}C for 30 min.

Incubation

After pre-incubation, 25 µl of the RaE suspension were added to each well of the microtitre plate and incubated at 37^{0}C for 30 min.

Measurement of haemolysis

After incubation, the microtitre plates were centrifuged in a SORVAL centrifuge at 1000 x g for 2 min. and 50 µl of the supernatant of each well was transferred to each corresponding well of a F-well (flat bottom) microtitre plate containing 200 µl of MQ-water. The OD_{414} was measured using an ELISA reader. The serum activity was calculated using the following formula;

Serum Activity as % lysis = [(Mean OD_{414} (H1, H2, H3) - Mean OD_{414} (H4, H5, H6))/(Mean OD_{414} (H10, H11, H12) - Mean OD_{414} (H7, H8, H9)] x 100%

The inhibitory activity of the samples against the control was calculated using the following formula;

% Inhibition = 100 – [(Mean OD_{414} (A1, A2)- OD_{414} (A3)/(Mean OD_{414} (H1, H2, H3)- Mean OD_{414} (H4, H5, H6)] x 100%

The concentration in the test system giving 50% inhibition of the haemolytic activity (IC_{50}) was calculated using the following formula;

IC_{50} = (50 –b)/ a

Where; a = [Inhibition (conc. 2) – Inhibition (conc. 1)]/ [conc. 2 – conc. 1]

b = Inhibition (conc. 1) – a * conc. 1

Inhibition = a * (sample concentration) + b

conc. 1: highest sample concentration giving less than 50% activity of control. Conc. 2: lowest sample concentration giving more than 50% activity of control.

2.7.1.2 The classical pathway

Sample dilution on U-well microtitre plates

An aliquot of VSB^{++} (50 µl) was added to wells in row B continuing up to G. For a 1:2 dilution, 100 µl of sample solutions were added in triplicate to row A. Subsequently, 50 µl of the solution in row A was transferred to row B and after mixing with the pipette, 50 µl of the mixture was transferred to the next row. This procedure was repeated up to row G. An aliquot of 50 µl of the final mixture in row G was discarded. To achieve 1:3 dilution of the sample solution, 75 µl of the sample solution was added to row A and 25 µl was transferred to the subsequent rows up to row G.

Preparation of controls

VSB^{++} (50 µl) was added to H1 to H6 (H1 to H3 for the determination of the activity of the serum; H4 to H6 are the serum blank). Then, 100 µl of VSB^{++} were added to H7 to H9 (0% haemolysis control), with 100 µl of MQ-water added to H10 to H12 (100% haemolysis).

Preparation of serum dilution and pre-incubation

The reagent blank was prepared by mixing 63 µl of heat-inactivated serum with 5.00 ml of VSB^{++}. This solution (50 µl) was added to A3 to G3, A6 to G6, A9 to G9, A12 to G12 and H4 to H6. Human pooled serum (125 µl) was mixed with 10 ml of VSB^{++}. This solution was used immediately after preparation. 50 µl of this solution was added to A1 to H1, A2 to H2, H3, A4 to G4, A5 to G5, A7 to G7, A8 to G8, A10 to G10 and A11 to G11. The plate was covered with its lid and pre-incubated at 37^0C for 30 min. After the pre-incubation, 50 µl of the ShEA-suspension was added to each well of the microtitre plate and incubated at 37^0C for 60 min.

Measurement of haemolysis

The plates were centrifuged at 1000 x g in Sorval centrifuge for 2 min. An aliquot of the supernatant of each well (50 µl) was transferred to each corresponding well of a F-well (flat bottom) microtitre plate containing 200 µl of MQ-water and mixed well using the pipette. The optical density was measured at 414nm using an ELISA reader.

Calculations

The results for the activity of the classical pathway was calculated as described for alternative pathway and given as CH50 units. Comparison of percent CH50 values obtained in the presence of plant extracts with those of blanks enabled the calculation of percentage activation from the plant extracts.

2.7.2 Assay of inhibition of platelet aggregation

Reagents:

tri-Sodium citrate: 3.7% w/v: 3.8g of tri-Sodium citrate.2 H_2O [99%, Fw=294.1] (UNILAB Laboratory Reagent from Ajax Laboratory Chemicals, NSW Australia) was dissolved in 100 ml MQ water.

Phosphate Buffer, 0.2 M NaH_2PO_4: (50 mM, pH 7.4); 6.24 g of 98.0% NaH_2PO_4.2 H_2O (BDH, GR, Fw=156.01) was dissolved in 200 ml MQ water, and 0.2 M Na_2HPO_4 5.678 g of 99.0% anhydrous Na_2HPO_4 (BDH, AR, Fw=141.96) was dissolved in 200 ml of MQ water by heating to 60C in a water bath.

19 ml of 0.2 M NaH_2PO_4 was mixed with 81 ml of 0.2 M Na_2HPO_4 and 3.6 g of NaCL (BDH) was added and made up to 400 ml with MQ water, after adjusting pH with NaOH and HCl.

Arachidonic acid [5,8,11,14-Eicosatetraenoic acid] (AA) (0.8 mM): Na_2CO_3 (0.2 g) was dissolved in 100 ml of MQ-water and the solution was deaerated by passing nitrogen gas. An ampule containing 10 mg AA [Sigma A9673, free acid 99%, Fw=304.5] was opened using forceps. Absolute EtOH (20 µl) and 730 µl of 0.2% Na_2CO_3 were added and mixed thoroughly using a vortex mixer. The mixture was transferred to two Eppendorf tubes and stored under nitrogen at –20 C. This solution can be kept for three months. The Eppendorf tubes were kept in an icebox while carrying out the experiment. Around 20-25µl of the standard solution was used.

Saline; 9 g of NaCl (BDH) was dissolved in 1 L of MQ-Water and passed through a 0.22 µm sterile filter and stored in stoppered polypropylene bottles.

Plant Extracts: Freeze-dried residues were used; 0.01 g were dissolved in 10 ml of Phosphate buffer and 20-50 µl were used.

Assay Procedure:

Collection of Blood:

3.8% w/v tri-Sodium citrate solution was added up to the 1 ml mark of 10 ml graduated glass centrifugal tubes. Human blood, collected via venepuncture, was transferred straight into the tubes from the syringe up to the 10 ml mark soon after collection. The tubes were inverted thrice and placed in an icebox.

Human blood, tested and found negative for hepatitis, IDS and VD antibodies, collected from healthy volunteers reported to be free of medication for 7 days, through the Red Cross Blood Bank, Victoria, was used in the experiments.

Platelet count was measured using the Coulter T-540E counter and the Coulter Blood counter at the Mercy Hospital, Werribee.

Count was [198 x 10^9 platelets/ litre (NR 150-400).] = 1.98 x 10^8 /ml in 9:1 solution. At 95% CI.

Required number of platelet rich plasma (PRP) was 2.5 to 3.0 x 10^8 /ml.

Blood samples were mixed in 3.8 % w/v sodium citrate solution (9:1) and centrifuged in a Sorval centrifuge at 260 x g at 20^0 C for 15 min. Supernatants containing PRP were transferred to glass tubes, stoppered, and placed in an icebox. The remainder of the blood was centrifuged at 1200 x g at 20^0 C for 10 min. The supernatant containing platelet poor plasma (PPP) was transferred to a tube labelled "PPP" and placed in an icebox. All aggregation studies were carried out at 37^0 C using PRP, having a platelet count between 2.5 and 3.0 x 10^8 /ml of plasma.

2.7.2.1 Measurement of optical aggregation

The method used by Saeed *et al* (1995) was used with slight modifications with the available accessories of the aggregometer.

Aggregation was monitored using aggregometer Model 550-VS (Chrono-log Corporation, U.S.A.). The Red & Black leads of the cable from Optical slot [R&B] of the second channel were connected to the appropriate pen sockets [R&B] of the chart recorder [Pharmacia]. Appropriate sensitivity was achieved by turning the Range and Zero depression knobs of the recorder and baseline knob of the aggremeter. The temperature of test wells of the aggregometer is permanently set to 37^0C. The light transmission was adjusted to 0 and 100% using PRP and PPP, respectively. Platelet aggregation was induced by adding a 20 µl of AA to the cuvette containing PRP. The % light transmission was measured for 4-6 minutes. Inhibition of platelet aggregation was tested by incubating 750 µl of PRP, a magnet bar, 20 µl of the extract in a cuvette for 1 min. and subsequent addition of 20 µl of AA and by recording the percentage transmission for 4-6 minutes. The bottom of the PRP cuvette was observed for aggregation. Absence of an aggregate indicated that the extract inhibited the platelet aggregation induced by AA.

The transmission effect of the extract solution on PRP was determined by adding the same volume of the extract solution to the PPP cuvette before starting the experiment. The resulting aggregation was recorded and expressed as percentage inhibition compared with control at 4 min after addition of AA. After finishing the experiment, the used cuvettes were dipped in detergent solution and the magnet bars were placed in a beaker containing MQ-water for washing and then they were rinsed and wiped with a tissue for re-use.

Calculation of Results

Optical aggregation results were expressed as a percentage of aggregation at a given time interval starting from the time of reagent addition. Aggregation was defined as the difference between the 0% (PRP) baseline and the 100% (PPP) baseline.

2.7.2.2 Measurement of impedance aggregation

In addition to the measurement of optical aggregation, impedance aggregation was measured to determine the effect of plant extracts on AA induced platelet aggregation. The impedance technique is rapid, labour efficient and requires only a small quantity of blood.

Impedance measurement using whole blood was carried out according to Ingerman-Wojonski and Silver (1984).

Whole blood;

Human blood, with permission obtained from the VUT, was collected through the Red Cross Blood Bank, Victoria according to the established procedure, in a 10 ml siliconized glass centrifugal tube containing 1 ml of 3.8% w/v tri-sodium citrate up to the mark. The tube was stoppered and inverted thrice and kept on ice. In order to maintain the consistency of blood dilutions, 25 ml of whole blood-citrate mixture was diluted with 25 ml of 0.9% saline solution at the beginning of the experiment.

Measurement of Impedance Aggregation

The aggregometer was switched on and allowed to stabilize for 30 min. The chart recorder was connected to the aggregometer. Impedance gain was set so that the travelling distance of the chart paper by each large square indicates impedance of 5 ohms and each small square indicates 0.5 ohms.

Two siliconized plastic disposable cuvettes with disposable magnet bars were placed in the outer incubation wells of the aggregometer and prewarmed for 10 min. Then 1 ml of diluted citrated whole blood solution was pipetted into each cuvette and incubated for 5 min. The cuvettes were then transferred to the inner test wells of the aggregometer. The

stirrer speed was set to 10 (10,000 rpm). The electrode probe assembly jacks were plugged into each receptacle on the side of the heater wall of the two test wells. The connected electrode probe assemblies were inserted firmly into the cuvettes in the sample wells. The electrode probe assembly wires were tucked down behind the probes and the heater block cover was closed completely. Chart recorder pens were lowered and the chart drive was activated. The lower red pen was placed at 10% from the initial left-hand mark on the chart using the Impedance Zero knob on the aggregometer. The chart paper was allowed to run for 2 min. and the chart baseline was monitored for stability.

While depressing the calibration button on the aggregometer and turning the impedance gain knob bring the pen to the line marked 50 on the chart. This calibrated the instrument to move 40 small chart divisions for a 20-ohm change in impedance. The heater block cover was then opened and 10 µl of the diluted AA solution was added to the bottom of each cuvette using a 10 µl microsyringe. While keeping the plunger depressed, the syringe was dipped in MQ-water in a beaker and the plunger was released. The heater block cover was then closed. The recorder was activated and the chart paper was allowed to run for 6 min. At the end of the desired recording time, the recorder pens were raised and the chart drive was switched off. Heater block covers were opened and the electrode probe assemblies were removed and washed in a beaker of MQ-water using a whisking motion in order to lyse the red cells and remove the aggregate. The electrode probe assemblies were then washed with saline and touched dried using Kimwipes tissue paper and insert into the next sample to be tested.

Two incubation wells were used to hold two clean cuvettes containing 1 ml of saline solution, into which the cleaned probe assemblies, were placed when not in use to maintain them at 37^{0}C. Testing time was shortened by placing new cuvettes with stir bars in the incubation wells, as they became available in order to maintain a supply of prewarmed cuvettes.

Calculation of Results

Results were determined by direct inspection of the chart recording and reported expressed as either the aggregation ohms at a given time interval from reagent addition or the maximum aggregation in ohms to the nearest ohm.

2.7.3 Determination of COX-2 inhibitory activity

The inhibition of COX-2 enzyme using a TLC separation of the reaction mixture followed by measurement of the radioactive arachidonic acid and/or its metabolites was carried out according to Mitchell *et al* (1994). However, the solvent system used for TLC separation was that used by Saeed *et al* (1995). The very low sensitivity of the radioactive detection by the Tracemaster-20 analyser was further improved by using a Fuji screen. Fuji radio tracer was used to scan the radioactive zones and the quantification was performed using Fuji Image Guage software.

Reagents:

Citric acid, citric acid (BDH) (2.1 g) was dissolved in 10 ml Milli Q water. The solution was prepared fresh. 400 µl were used for stopping the reaction.

Phosphate Buffer; 0.2 M NaH_2PO_4: (50 mM, pH 7.4); Amount (6.24 g) of 98.0% $NaH_2PO_4.2H_2O$ (BDH, GR, Fw=156.01) was dissolved in 200 ml MQ water. For 0.2M Na_2HPO_4, 5.678 g of 99.0% anhydrous Na_2HPO_4 (BDH, AR, Fw=141.96) was dissolved in 200 ml of MQ water by heating to 60C in a water bath.

A volume (19 ml) of 0.2 M NaH_2PO_4 was mixed with 81 ml of 0.2 M Na_2HPO_4 and 3.6g of NaCl (BDH) was added and made up to 400 ml with MQ water, after adjusting pH with NaOH and HCl.

Arachidonic acid [5,8,11,14-Eicosatetraenoic acid] (AA) (0.8 mM): 0.2 g of Na_2CO_3 was dissolved in 100 ml of Milli Q-water and the solution was deaerated by passing nitrogen gas. An ampule containing 10 mg AA [Sigma A9673, free acid 99%, Fw=304.5] was opened using forceps. 20 µl of absolute EtOH and 730 µl of 0.2% Na_2CO_3 were added and mixed thoroughly using a vortex mixer. The mixture was transferred to two screw capped vials and stored under nitrogen at -20^0C to avoid oxidation. This solution could be kept for three months. The Eppendorf tubes were kept in an ice box while carrying out the experiment. Approximately 20-25 µl of the standard solution was used in the tests.

[1-^{14}C] Arachidonic acid [5,8,11,14-Eicosatetraenoic acid] (AA*)

[1-^{14}C] Arachidonic acid (AA*) having a radioactive concentration of 50 μCi/ml was purchased from Amersham Life Science (UK). An aliquot (40 μl) of AA* was transferred to a screw capped vial and concentrated to solid 10 μl by passing a stream of nitrogen gas through the solution and the solids were redissolved using 10 μl of EtOH. Into the AA* concentrate, 190 μl of unlabelled AA and 900 μl of phosphate buffer were added and mixed using a vortex.

COX-2 [Cyclooxygenase-2 or Prostaglandin H Synthase 2 (ovine), COX-2, 1K unit in 80 mM Tris-HCl, pH 8.0; was purchased from Cayman Chemicals (USA) and stored at –80^{0}C.

Glutathione (GSH) reduced form, Glutathione (γ-Glu-Cys [Succinic acid]-Gly] 97% w/w, (FW= 307.3), [D2804] was purchased from Sigma Chemical Co., and a 2.5 mg was dissolved in 1 ml of 0.9% NaCl solution.

Hematin, Hematin from bovine blood (FW = 633.5), [H3505] was purchased from Sigma Chemical Company, Australia. Hematin solution was prepared by dissolving 0.634 g of hematin in 1 ml of 0.9% NaCl solution.

Epinephrine; (-)- Epinephrine (+) bitartrate, 85% w/w (FW=333.3), [E4375] was purchased from Sigma Chemical Co., Australia. Epinephrine solution was prepared by dissolving 2 mg of epinephrine in 1 ml of 0.9% NaCl solution.

Plant Extracts: Freeze-dried extracts in up to 30 % DMSO were used. 0.01 g was dissolved in 10 ml of phosphate buffer and 100 μl was used. Solvent system for TLC of cyclo-oxygenase products:

Eluent for TLC:

EtOAc-isooctane-HOAc-water 90:50:20:100 ml. The solvent mixture was continuously stirred overnight at 4^{0}C. The solvent mixture was allowed to separate into two layers in a separating funnel and the upper organic layer was used as the eluent. The eluent was prepared fresh before use.

Assay Procedure:

The COX-2 enzyme activity was measured by the conversion of AA* to its metabolites by thin layer chromatography (TLC). Sample solution (100 µl) was transferred to a sliconized 10 ml screw capped vial. Into the vial containing sample solution, 100 µl of the AA*-AA-Tris Buffer mixture, 10 µl of hematin, 100 µl of GSH, 100 µl of epinephrine, 10 µl of COX-2, and 580 µl of tris-buffer were added mixed gently using a vortex. A control vial containing all the above solutions except the sample was also prepared. All vials containing samples and controls were incubated at 37^{0}C a water bath equipped with a shaker. After 10 min of incubating, 400 µl of citric acid was added and mixed to stop the reaction. Also, 7 ml of EtOAc was added and mixed thoroughly using a vortex for 2 min. to extract the organic compounds. The solution was then centrifuged at 575 g (1000 rpm in a Sorval centrifuge) 0^{0}C for 10 min. The upper organic layer free from the sediments (6 ml), was collected and transferred into a siliconized vial. The solvent was evaporated at 37^{0}C to dryness using a stream of nitrogen gas and 40 µl of EtOH was added to the vial containing dry residue. After mixing thoroughly using a vortex, 35 µl of the solution was applied on a silica gel G TLC plate (20 x 20 cm) (Analtech, Delaware, USA). The TLC plate was developed using the eluent prepared as described under *Eluent for TLC.* Radioactive zones were located and quantified by using 1) Tracemaster-20 automatic TLC-linear analyser and 1-D Chroma ver 7.25 chromatography data system (Model LB285, Berthold, West Germany). 2) Using Fuji screens. Fuji radiotracer was used to scan the radioactive zones and the quantification was performed using Fuji Image Gauge software.

Percent inhibition of COX-2 activity was calculated using following formula;

$$[C\text{-}T/C] \times 100 = \%\ \text{Inhibition}$$

Where; C and T are the radioactivity (counts per min.) shown by the control and the test chromatograms respectively. Differences between control and test measurements were assessed by Student's t- test using the SPSS (ver. 9) statistical package.

2.8 High performance liquid chromatography (HPLC)

HPLC was performed with a Varian (Varian, Australia) high-performance liquid chromatograph equipped with a Varian 9100 auto sampler equipped with a Rhyodyne

injector assembly with a 20 µl sample loop, a Varian 9012 solvent delivery system, and a Varian 9065 Polychrom photodiode array detector. The extracts were separated on a Vydac 218TP54 silica C_{18} semi-preparative column (25 cm x 4.6 mm i.d.) having a particle size of 5 µm from Vydac, CA, USA. Vydac analytical guard cartridge (25x 4.6 mm i.d.) having a particle size of 5 µm, was used between the injector and the sample.

The eluents used for HPLC separation of the sequential extracts consisted of 0.1% trifluoroacetic acid (AR, BDH, U.K.) in deaerated MQ water (A) and 100% acetonitrile (BDH-Hypersol) (B). The gradient was 100-0% A in 60 min at a flow rate of 0.5 ml/min. UV spectra were recorded from 200-367 nm at a rate of 1.00 spectrum(s) and a resolution of 1.2 nm.

2.9 Fractionation of crude extracts

Selected sequential extract were subjected to fractionation using a semi-preparative Vydac, C_{18} reverse phase column (250 x 46 mm i.d.), which contained packing material of 5 µm particle size (Vydac, U.S.A) protected by a C_{18} Alltech guard column on HPLC. The column end was connected to a Foxy Jr. fraction collector. The mobile phase used for the fractionation of E4 extract consisted 95% of 0.1% trifluoroacetic acid (AR, BDH, U.K.) in deaerated MQ water (A) and 5% acetonitrile (BDH-Hypersol) (B) eluted isocratically for 30 min. For the separation of individual compounds the mobile phase consisted of A and B using the following gradient over a total run time of 45 min: 100% A for 6 min, 90% A in 10 min, 80% A in 10 min, 30% A in 10 min, 20% A in 10 min, and 0% A until completion of the run. The flow rate of the mobile phase was 0.5 ml/min.

The fraction collector was programmed to collect 8 fractions at a flow rate of 0.5 ml/min in 100 ml Erlenmeyer flasks. Each fraction was collected for 7.0 min. The HPLC system was automated for repeated collection of 26 fractions into each flask. The volume of sample injection was set to 50 µl using a 50 µl sample loop in the autosampler. The organic solvent fractions collected were evaporated using a rotatory evaporator and residues were obtained. The aqueous fractions were freeze-dried. The dry residues were re-dissolved using DMSO, or a suitable solvent as the case may be.

2.10 Instrumental analysis

2.10.1 HPLC analysis

HPLC chromatograms acquired using a Varian 9065, Polychromauto diode array detector were examined for peak purity using Varian Star Workstation version 5.3 software and the UV spectral studies were carried out using the polychrome software included in the Varian Star Workstation 5.3 software package. Compounds were separated on a Vydac 218TP54 silica C_{18} semi-preparative column (250 x 4.6 mm i.d.), having a particle size of 5 μm, (Vydac), A Vydac analytical guard cartridge (25 x 4.6 mm i.d.) having a particle size of 5 μm, was used between the injector and the sample.

2.10.2 Mass spectrometry (MS)

2.10.2.1Electrospray ionisation (ESI)-MS

Table 14 Typical values for SEI-MS using Micromass Platform II

Micromass Platform II		
	ESI +ve Mode	ESI –ve Mode
ES probe, Capillary (kV)	+3.5	-3.0
S-cone, Cone (V)	+25	-25

Electro spray ionisation (ESI) MS of the sample solutions were carried out on a Platform II instrument (Micromass, Cheshire, U.K.) equipped with ESI interface. The MS was optimised to achieve maximum sensitivity.

Typical ESI conditions: source voltage 4.78 kV, sheath gas flow-rate 5.3 bar, auxiliary gas flow 1.2 bar, capillary voltage –31 V and capillary temperature C. Full scan spectra from 200 1400 u in the positive and negative ion mode were obtained (scan time 1 s).

For each sample two separate injections were made. For the first set of injections the electrospray lenses and skimmers were adjusted to detect $[M-H]^-$ ions in a 'normal'

negative ion mode. For the second set of injections, adjustments were made to detect $[M+H]^+$ ions in positive ion mode.

In order to obtain the necessary voltages for the skimmers and lenses, quercetin was infused into the ESI-MS and the lenses and skimmers were adjusted to obtain maximum abundance for the aglycone of quercetin.

2.10.2.2GC-MS

GC-MS analyses were carried using a 30 m x 0.25 mm i.d. x 0.25 μm film thickness DB-5MS (5%-phenyl) methylpolysiloxane capillary column (J&W Scientific, CA, USA) installed in HP5980 (Hewlett Packard) GC instrument interfaced to a HP 5971 mass selective detector operated in scanning mode (m/z 40-400). GC-MS analysis were temperature programmed from 50^0 C (4min. solvent delay) to 280^0 C (1 min hold) at at 10^0 C/min. Samples (1 μl) were injected manually using a Hamilton 10 μl syringe on split/splitless mode. Helium (ultra pure from BOC Gas Supplies) was used as the carrier gas. Mass specral analysis was carried out using HP Chemstation software.

2.10.2.3 Derivatisation procedure for GC-MS analysis

The derivatisation of the possible acids, alcohols amines and phenols present in the extracts and residues were carried out using hexamethyldisilazane (HMDS) and the typical catalyst, trimethylchlorosilane (TMS) purchased from Sigma Cemical Co, Australia, according to the method of Pizzoferrato (1993). Silyl derivatisation was carried out using 1-10 mg dried sample and treating the sample with 100 μl of trymethylchlorosilane (TMS) and 100 μl of hexamethyldisilazane (HMDS) at 60 ^{0}C for 1 hour in a tightly closed amber vial. Both the TMS and HMDS were purchased from Sigma Chemical Co, Australia.

The sample extract was completely dried under a stream of nitrogen gas in a dried amber vial. Then 100 μl of fresh HMDS and 100 μl of fresh TMS added and the vial was tightly closed with a screw cap containing a Teflon inner liner and kept at 60 ^{0}C for 1 hour.

2.10.3 AAS analysis

The plant extracts, E4 and V3, were tested for the iron content using an Atomic Absorption Spectrometer (Varian Spectra) equipped with a Fe lamp, using an air-acetylene

flame. The concentrations of the calibration curve used were at a lower range: 0.00 to 0.30 ppm, whereas the high concentration range was 0.0 to 10.00. Five concentrations of the Fe standards were used in each range.

2.10.4 UV-VIS analysis

UV-VIS spectroscopy was carried out using Varian Cary 1E UV-VIS spectrophotometer in single beam mode. The spectrum of appropriately diluted samples in EtOH, MeOH and $CHCl_3$ were obtained between 400 and 800 nm. Varian Cary software.

2.11 TLC analysis

The thin layer chromatographic analysis was carried out using 5x20 cm, silica gel $60F_{254}$ – precoated TLC plates purchased from Merck, Darmstadt, Germany. Solvent systems used were, toluene-EtOAc (97:3) and EtOAc-MeOH-MQ water (100:13.5:10). Detection of the spots was carried out at UV 254 nm and 366 nm.

2.12 Statistical analysis

Statistical analysis was performed using paired Student's t test. A P-value of less than 0.05 was considered as significantly different. SPSS for Windows Release 9.0.1 from SPSS Inc. USA (1989-1999) and in some cases The SigmaPlot for Windows Version 3.02 from Jandel Corporation (1986-1995), software were used for the statistical analysis of results.

CHAPTER 3 INVESTIGATION OF ANTICANCER ACTIVITY OF *Euphorbia peplus* L.

ABSTRACT

Euphorbia peplus L. (petty spurge) has been used in traditional medical systems to treat cancer, warts and corns. This plant was purported to have used to treat skin, stomach, liver and uterine cancers and was reported to offer a cure for a chest basal cell carcinoma of a patient. However, extracts of *Euphorbia peplus*, have failed to show any significant growth inhibitory activity against *in vitro* human KB cells and *in vivo* mouse LE, PS, LL, SA and rat WA tumours when these extracts were tested in the screening programme of the US National Cancer Institute (NCI). *Euphorbia peplus* has not been tested on the NCI's latest disease-oriented 60 cell-line human panel.

Hence in this research project on *Euphorbia peplus*, we have employed a much more exhaustive sequential soxhlet extraction procedure than used previously. This was followed by: testing for *in vitro* anticancer activity using the NCI's disease oriented 60 cell-line human panel, which was based on the sulforhodamine B method. The results obtained for CEM cells were compared with [^{3}H]-thymidine uptake, MTS and brine shrimp lethality assays.

According to our results the petroleum ether (E1), $CHCl_3$ (E2) and $CHCl_3$-MeOH (E3) extracts obtained from whole plant of *Euphorbia peplus,* showed a selective but weak *in vitro* growth inhibition using the human leukaemia, non-small cell lung, and breast cancer cell lines. The highest inhibitory potency was shown by the E3 extract at a concentration of 0.15 μg/ml using a HOP-92 cell line from the non-small lung cancer subpanel. A total growth inhibition of A498 and UO-31 cell lines was demonstrated for the E1 extract at 26 and 28 μg/ml, respectively, whereas inhibition of the TK-10 cell line required E2 at a level of 37 μg/ml.

According to the results obtained for E2 extract, it may be inferred that the sensitivity of the assays decreases in the order, MTS> [^{3}H]-thymidine>sulforhodamine B when CEM cells were used as the test cell line, whilst the brine shrimp assay showed the least sensitivity.

3.1 Introduction

Preparations of various parts of the plant, *Euphorbia peplus* L. (Euphorbiaceae), have been used in traditional systems of medicine to treat cancers of the skin (Hartwell, 1982), stomach, liver, and uterus (Shimans'ka, 1961) and have reportedly cured a basal cell carcinoma (Weedon and Chick, 1976) (Table 7 of Chapter 1). The latex of this plant also has been used to treat corns and warts (Mas-Guindal, 1941 and Madaus, 1938). Although *E. peplus* has been used medicinally to treat asthma (Nelson, 1951), catarrh and has been used as a purgative, it is reported to induce painful vomiting and purgation in domestic animals (Sharaf, 1949) and toxic effects on livestock (Salah-Zayed *et al*, 1998; Nawito *et al*, 1998).

In an earlier screening programme, the 95% ethanol extract of *Euphorbia peplus* showed a marked contraction of chromosomes and inhibition of spindle formation in C-mitosis of onion root cells (Barnard, 1949). Although C-mitotic inhibitory activity of this plant has been reported, the compounds responsible for this activity have not been studied. According to Hasan and Abdel-Mallek (1994), complete inhibition of aflatoxin production of *Aspergillus flavus* (IMI 89717) was effected by a 4.0% aqueous extract of *Euphorbia peplus* leaves. This aflatoxin inhibitory property of *Euphorbia peplus* is suggested to be due to the interference of its constituents in the biosynthetic pathway of *A. flavus*.

However, as discussed in Chapter 1, section 1.7.1, the extracts of *Euphorbia peplus*, have failed to show any marked cytotoxic activity against human KB cells *in vitro* or antineoplastic activity in mouse LE, PS, LL, SA and rat WA tumours *in vivo* using the NCI's screening programme. This may be due to a number of factors including the mild extraction procedure used, the insensitivity of the tumour systems, and the method of detection and acceptance criteria used in the NCI's previous investigation during 1962-76, which was discussed in Chapter 1, section 1.4.8.

Mild extraction procedures are not thought to be effective in facilitating the extraction of most of the compounds bound to the plant matrix. In most of the traditional methods of administration of plants involved preparation of a decoction in boiling water and concentrating to $1/8^{th}$ of its original volume (Jayasinghe, 1986). According to Farnsworth (1994), saponins in a crude aqueous plant extract facilitate the dissolution of otherwise insoluble compounds such as fatty acids in water. Also, the complex mechanism of absorption of an orally administered mixture in the body facilitates intake of complex

insoluble and macromolecules mostly through the transportation across lipoprotein membranes. In addition to an effective extraction procedure, use of a sensitive *in vitro* test system and a valid method of detection as discussed earlier in Section 1.4.8 of Chapter 1, are thought to play an important role in investigating putative anticancer plants for anticancer activity.

In this study, extracts of the putative anticancer plant, *Euphorbia peplus,* were investigated for their effectiveness as inhibitors using *in vitro* "cytotoxic" anticancer assays.

3.2 The aim of the research project

The aim of the research project described in this chapter is to investigate whether the extracts of *Euphorbia peplus* possess any *in vitro* anticancer activity.

The specific aims of the study discussed in this Chapter are;

1. Test sequential extracts of *Euphorbia peplus* using the 60 cell-line human panel of the conventional *in vitro* anticancer screening program of the NCI, which utilises the sulforhodamine B assay system for evaluating cytotoxic anticancer activity.

2. To test the sequential extracts of *Euphorbia peplus* for *in vitro* anticancer activity utilising a cancer cell line (CEM leukaemia) and different methods of detection such as [^{3}H]-Thymidine incorporation and MTS assays.

3. To compare the results of these tests with the brine shrimp lethality test, which is widely used as a simple monitor for cytotoxic activity.

3.3 Results and discussion

3.3.1 *In vitro* screening of *Euphorbia peplus* against NCI's 60-human tumour panel

Due to constraints in accessing the screening facility at NCI, only the organic solvent extracts E1, E2, E3 and E4 were screened against the NCI's human 60-cell panel. The aqueous extracts E5, E6 and EB extracts have not been tested in the NCI's screen, as aqueous extracts were not been successful in previous screenings conducted by the NCI.

For each extract tested, the dose response curves of the cell lines of each subpanel are given in one set of plots and 9 such plots are given for nine sub panels. Cell lines that did not grow satisfactorily during the experiments were not presented. Hence results of 52-58 cell lines representing all types of human cancers are presented in the dose-response curves. In addition, mean graphs illustrating GI50, TGI and LC50 values and their log values are given for each extract tested. According to the norms of NCI, the GI50 may be viewed as a growth-inhibitory level of effect, the TGI signifies a "total growth inhibition" or cytostatic level of effect and the LC50 is the lethal concentration, "net-cell killing" or cytotoxicity parameter (Boyd and Paull, 1995).

The dose response curves indicating percentage growth versus log sample concentration and the mean graphs obtained for *in vitro* screening of the extracts E1, E2, E3 and E4 are given in Figures 8 and 9, 10 and 11, 12 and 13, and 14 and 15, respectively. The results given in Figure 9, 11, 13 and 15, for the 40-sensitive cell lines (GI50 < 50 μg/ml and TGI<100 μg/ml) are given in Table 15 (a) and (b). The GI50 and TGI values discussed in this thesis are the values given in Table 15(a) and (b) calculated from their corresponding log GI50 and log TGI values given in Figures 9, 11, 13 and 15.

The panel average GI50 values of the mean graphs calculated by the computer program for E1, E2, E3 and E4 were 26.3, 24.6, 21.4 and 50.1 μg/ml, respectively, (Figure 9, 11, 13, and 15). These GI50 values have been calculated after taking into account, the upper concentration limits of 100 and 50 μg/ml respectively for E1, E2 and E3, E4. Since the average GI50 values were more than the NCI's potency criterion of <0.1 μg/ml these extracts were not considered to be effective in inhibiting cancer growth.

Because of the greater number of "default" (> than the tested highest concentration) values involved in the calculation of the panel average values, the Mg_MID, Delta and Range values given in Figure 9, 11, 13 and 15 do not reflect any meaningful panel-averaged sensitivity of the cell lines to the given extract.

However, the individual highest potency found against HOP-92 cell line of lung (non-small cell) cancer subpanel by E1, E2, and E3 (0.42, 0.45 and 0.15 μg/ml) were close to the required potency level of 0.1 μg/ml. In addition, E1 and E2 showed a relatively high potency of 0.41 and 0.50 μg/ml, respectively, against the MDA-N cell line of the breast

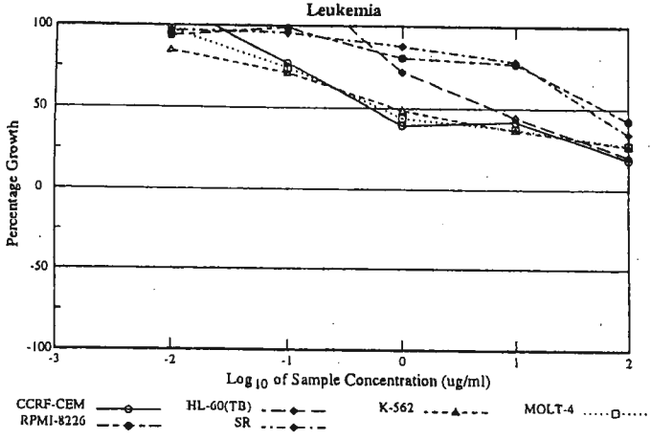
Figure 8 Dose response curves of E1 extract of *Euphorbia peplus* L., against cell lines of human leukaemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancer sub panels. These data for our extracts were provided by the Development Therapeutics Programme of the NCI (USA).

Dose Response Curves

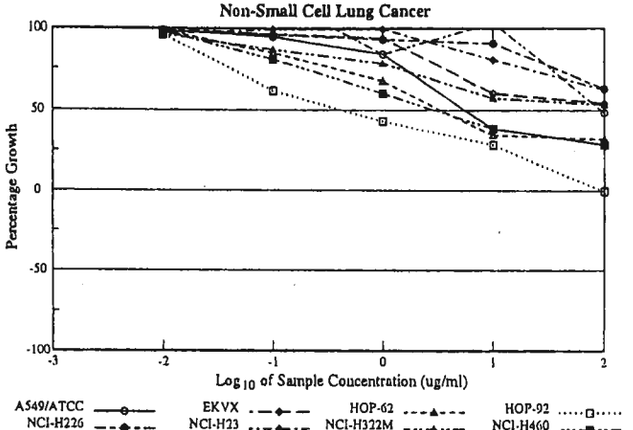
SF001

Report Date: May 22, 2000 | **Test Date:** May 9, 2000

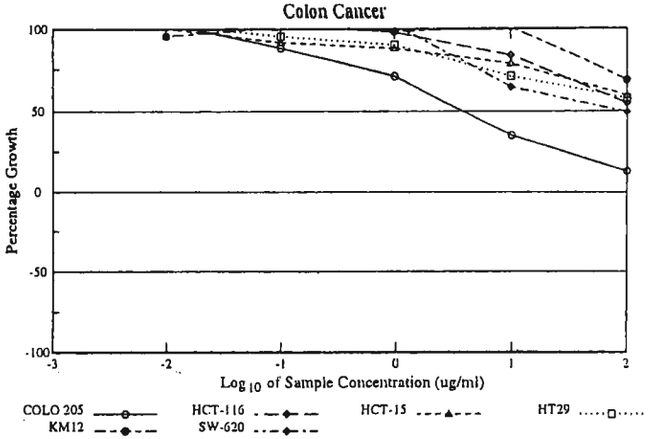
Leukemia



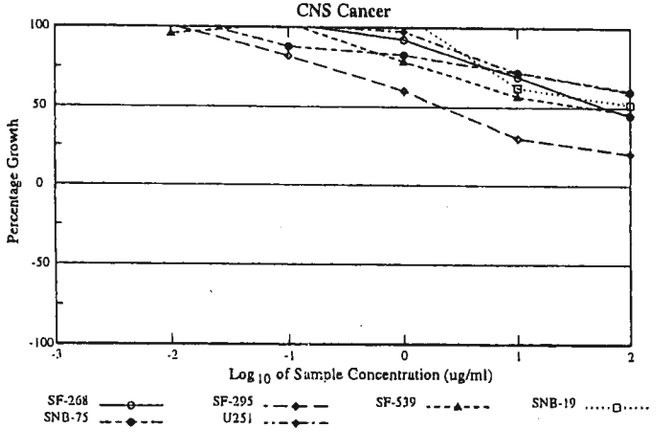
Non-Small Cell Lung Cancer



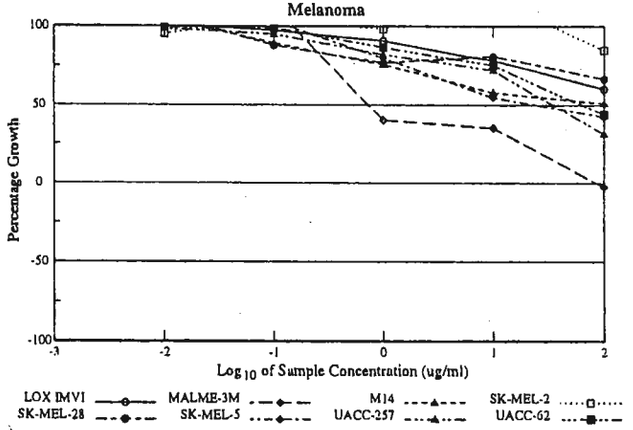
Colon Cancer



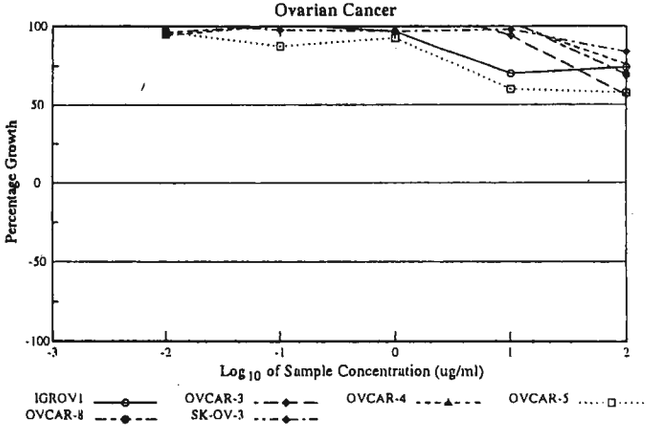
CNS Cancer



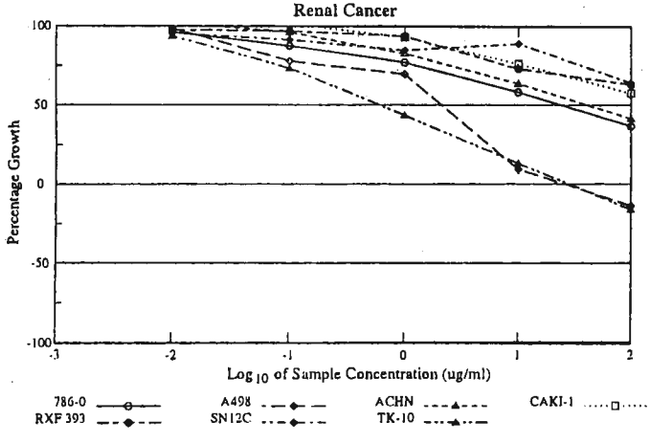
Melanoma



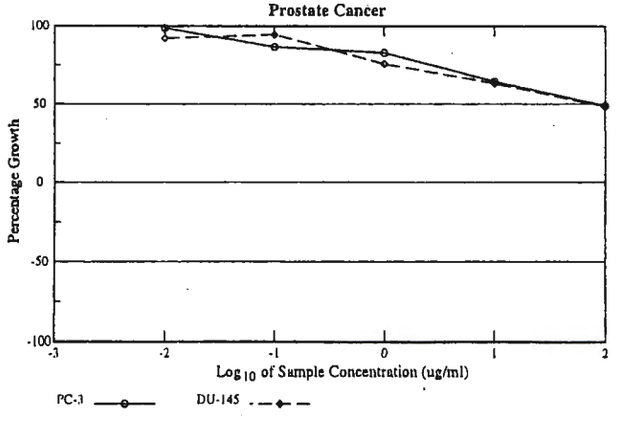
Ovarian Cancer



Renal Cancer



Prostate Cancer



Breast Cancer

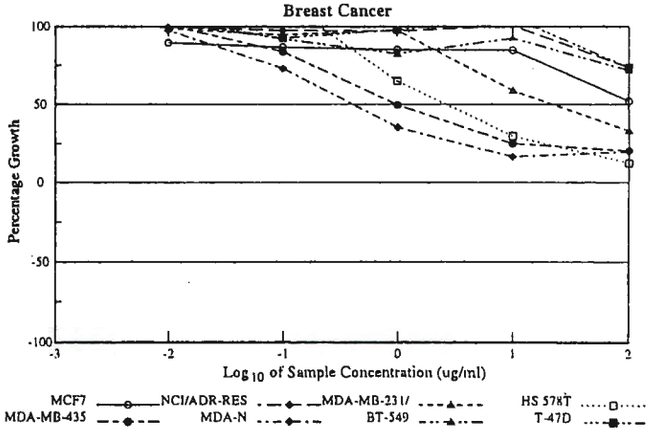
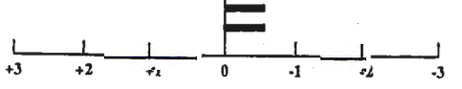


Figure 9 GI50, TGI, and LC50 Mean graphs of E1 extract of *Euphorbia peplus* L., derived from the dose-response data of Figure 7. The $\log_{10}$ GI50, $\log_{10}$ TGI and $\log_{10}$ LC_{50} values respectively are given for each cell lines. These data were provided by the Development Therapeutics Programme of the NCI (USA).

Mean Graphs

Report Date: May 22, 2000 | **Test Date:** May 9, 2000

Panel/Cell Line	Log_{10} GI50	GI50	Log_{10} TGI	TGI	Log_{10} LC50	LC50
Leukemia						
CCRF-CEM	-0.27		> 2.00		> 2.00	
HL-60(TB)	0.81		> 2.00		> 2.00	
K-562	-0.06		> 2.00		> 2.00	
MOLT-4	-0.19		> 2.00		> 2.00	
RPMI-8226	1.79		> 2.00		> 2.00	
SR	1.65		> 2.00		> 2.00	
Non-Small Cell Lung Cancer						
A549/ATCC	0.75		> 2.00		> 2.00	
EKVX	> 2.00		> 2.00		> 2.00	
HOP-62	0.53		> 2.00		> 2.00	
HOP-92	-0.38		1.99		> 2.00	
NCI-H226	> 2.00		> 2.00		> 2.00	
NCI-H23	> 2.00		> 2.00		> 2.00	
NCI-H322M	> 2.00		> 2.00		> 2.00	
NCI-H460	0.47		> 2.00		> 2.00	
NCI-H522	1.97		> 2.00		> 2.00	
Colon Cancer						
COLO 205	0.59		> 2.00		> 2.00	
HCT-116	> 2.00		> 2.00		> 2.00	
HCT-15	> 2.00		> 2.00		> 2.00	
HT29	> 2.00		> 2.00		> 2.00	
KM12	> 2.00		> 2.00		> 2.00	
SW-620	1.97		> 2.00		> 2.00	
CNS Cancer						
SF-268	1.78		> 2.00		> 2.00	
SF-295	0.33		> 2.00		> 2.00	
SF-539	1.64		> 2.00		> 2.00	
SNB-19	> 2.00		> 2.00		> 2.00	
SNB-75	> 2.00		> 2.00		> 2.00	
U251	> 2.00		> 2.00		> 2.00	
Melanoma						
LOX IMVI	> 2.00		> 2.00		> 2.00	
MALME-3M	-0.14		1.93		> 2.00	
M14	> 2.00		> 2.00		> 2.00	
SK-MEL-2	> 2.00		> 2.00		> 2.00	
SK-MEL-28	> 2.00		> 2.00		> 2.00	
SK-MEL-5	1.39		> 2.00		> 2.00	
UACC-257	1.54		> 2.00		> 2.00	
UACC-62	1.81		> 2.00		> 2.00	
Ovarian Cancer						
IGROV1	> 2.00		> 2.00		> 2.00	
OVCAR-3	> 2.00		> 2.00		> 2.00	
OVCAR-4	> 2.00		> 2.00		> 2.00	
OVCAR-5	> 2.00		> 2.00		> 2.00	
OVCAR-8	> 2.00		> 2.00		> 2.00	
SK-OV-3	> 2.00		> 2.00		> 2.00	
Renal Cancer						
786-0	1.37		> 2.00		> 2.00	
A498	0.32		1.41		> 2.00	
ACHN	1.61		> 2.00		> 2.00	
CAKI-1	> 2.00		> 2.00		> 2.00	
RXF 393	> 2.00		> 2.00		> 2.00	
SN12C	> 2.00		> 2.00		> 2.00	
TK-10	-0.22		1.45		> 2.00	
Prostate Cancer						
PC-3	1.93		> 2.00		> 2.00	
DU-145	1.89		> 2.00		> 2.00	
Breast Cancer						
MCF7	> 2.00		> 2.00		> 2.00	
NCI/ADR-RES	> 2.00		> 2.00		> 2.00	
MDA-MB-231/ATCC	1.34		> 2.00		> 2.00	
HS 578T	0.42		> 2.00		> 2.00	
MDA-MB-435	-0.01		> 2.00		> 2.00	
MDA-N	-0.39		> 2.00		> 2.00	
BT-549	> 2.00		> 2.00		> 2.00	
T-47D	> 2.00		> 2.00		> 2.00	
MG_MID	1.42		1.98		2.00	
Delta	1.81		0.57		0.00	
Range	2.39		0.59		0.00	



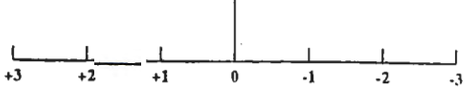


Figure 10 Dose response curves of E2 extract of *Euphorbia peplus* L., against cell lines of human leukaemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancer sub panels. These data were provided by the Development Therapeutics Programme of the NCI (USA).

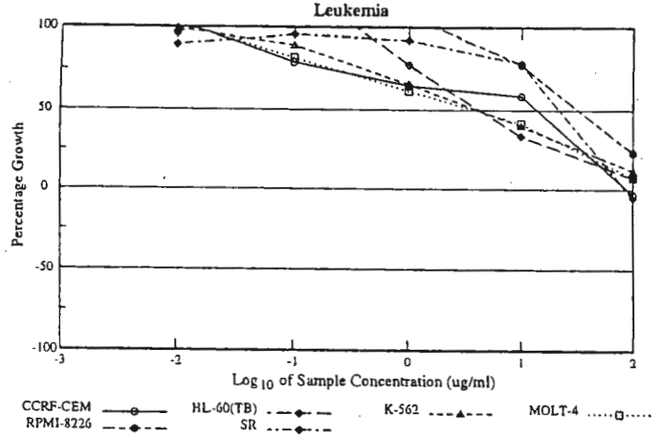
Dose Response Curves

SF002

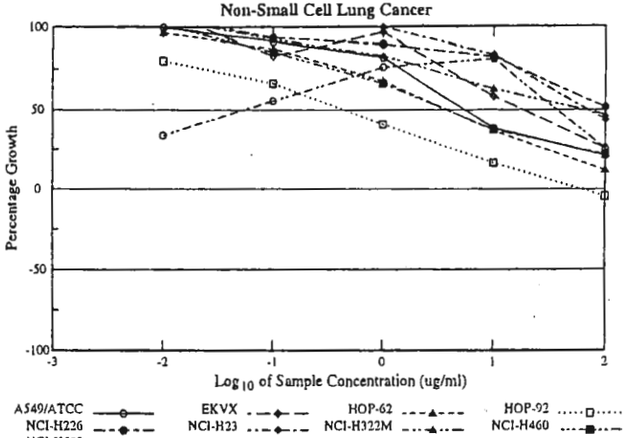
Report Date: May 22, 2000

Test Date: May 9, 2000

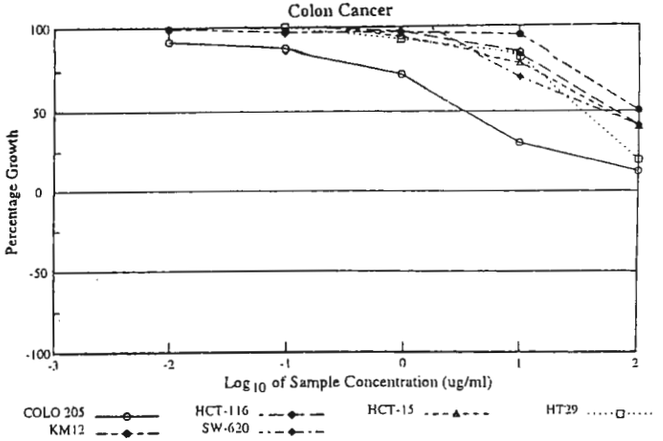
Leukemia



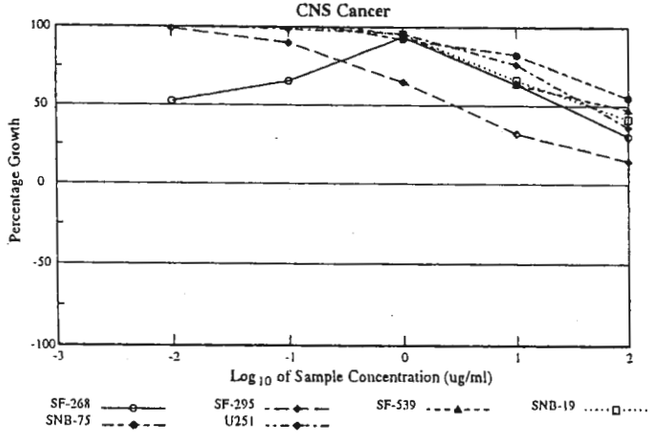
Non-Small Cell Lung Cancer



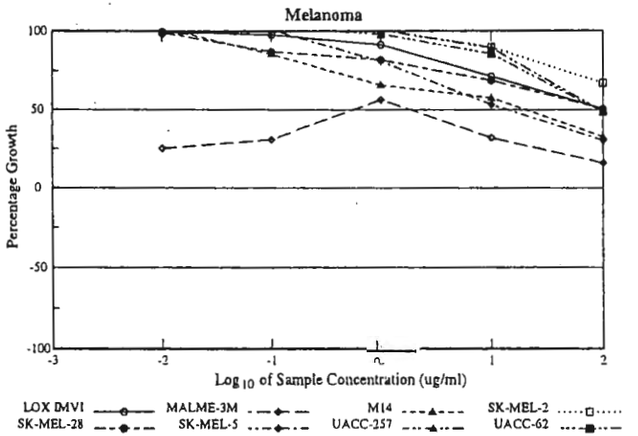
Colon Cancer



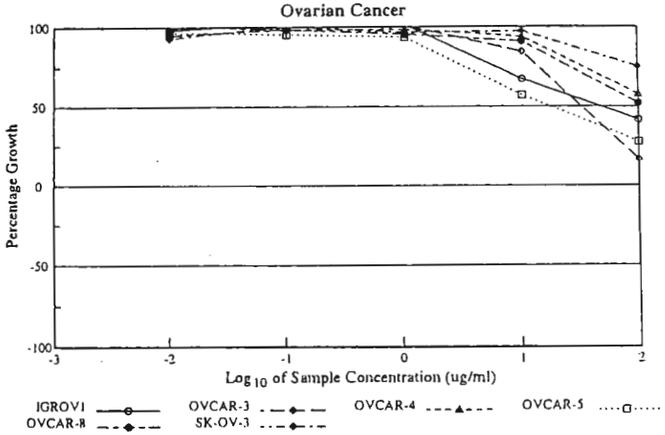
CNS Cancer



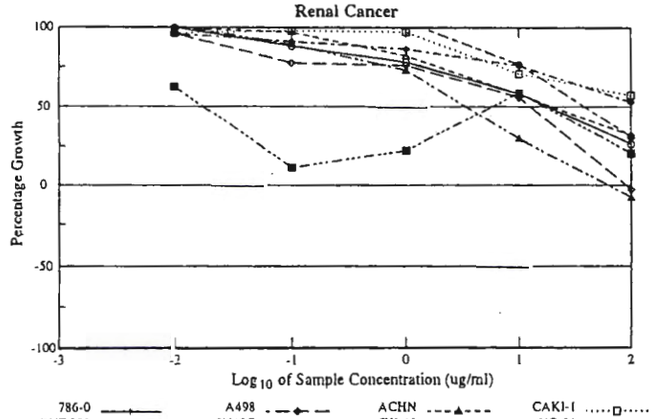
Melanoma



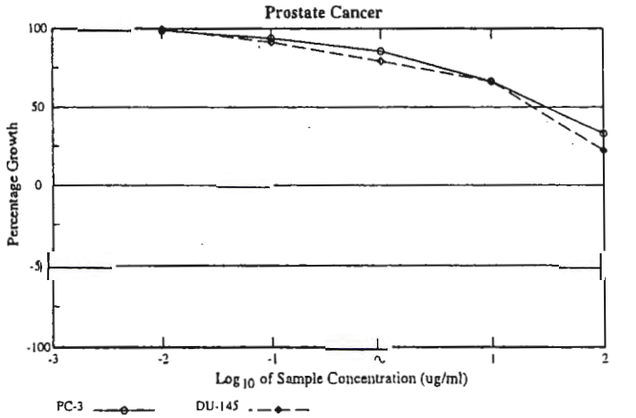
Ovarian Cancer



Renal Cancer



Prostate Cancer



Breast Cancer

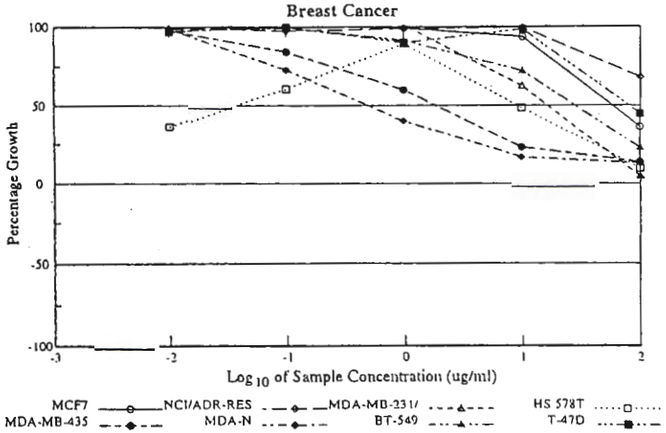
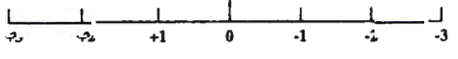
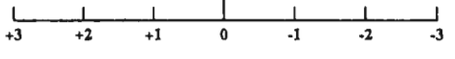


Figure 11 GI50, TGI, and LC50 Mean graphs of E2 extract of *Euphorbia peplus* L., derived from the dose-response data of Figure 9. The log_{10} GI50, log_{10} TGI and log_{10} LC_{50} values respectively are given for each cell lines. These data were provided by the Development Therapeutics Programme of the NCI (USA).

Panel/Cell Line	Log_{10} GI50	GI50	Log_{10} TGI	TGI	Log_{10} LC50	LC50
Leukemia						
CCRF-CEM	1.14		1.95		> 2.00	
HL-60(TB)	0.63		> 2.00		> 2.00	
K-562	0.62		> 2.00		> 2.00	
MOLT-4	0.59		> 2.00		> 2.00	
RPMI-8226	1.51		> 2.00		> 2.00	
SR	1.34		1.94		> 2.00	
Non-Small Cell Lung Cancer						
A549/ATCC	0.74		> 2.00		> 2.00	
EKVX	1.26		> 2.00		> 2.00	
HOP-62	0.58		> 2.00		> 2.00	
HOP-92	-0.35		1.80		> 2.00	
NCI-H226	> 2.00		> 2.00		> 2.00	
NCI-H23	1.85		> 2.00		> 2.00	
NCI-H322M	1.82		> 2.00		> 2.00	
NCI-H460	0.57		> 2.00		> 2.00	
NCI-H522			> 2.00		> 2.00	
Colon Cancer						
COLO 205	0.53		> 2.00		> 2.00	
HCT-116	1.80		> 2.00		> 2.00	
HCT-15	1.75		> 2.00		> 2.00	
HT29	1.52		> 2.00		> 2.00	
KM12	> 2.00		> 2.00		> 2.00	
SW-620	1.70		> 2.00		> 2.00	
CNS Cancer						
SF-268	1.41		> 2.00		> 2.00	
SF-295	0.46		> 2.00		> 2.00	
SF-539	1.84		> 2.00		> 2.00	
SNB-19	1.66		> 2.00		> 2.00	
SNB-75	> 2.00		> 2.00		> 2.00	
U251	1.66		> 2.00		> 2.00	
Melanoma						
LOX IMVI	> 2.00		> 2.00		> 2.00	
MALME-3M			> 2.00		> 2.00	
M14	1.31		> 2.00		> 2.00	
SK-MEL-2	> 2.00		> 2.00		> 2.00	
SK-MEL-28	> 2.00		> 2.00		> 2.00	
SK-MEL-5	1.15		> 2.00		> 2.00	
UACC-257	1.98		> 2.00		> 2.00	
UACC-62	1.96		> 2.00		> 2.00	
Ovarian Cancer						
IGROV1	1.67		> 2.00		> 2.00	
OVCAR-3	1.51		> 2.00		> 2.00	
OVCAR-4	> 2.00		> 2.00		> 2.00	
OVCAR-5	1.25		> 2.00		> 2.00	
OVCAR-8	> 2.00		> 2.00		> 2.00	
SK-OV-3	> 2.00		> 2.00		> 2.00	
Renal Cancer						
786-0	1.26		> 2.00		> 2.00	
A498	1.10		1.96		> 2.00	
ACHN	1.29		> 2.00		> 2.00	
CAKI-1	> 2.00		> 2.00		> 2.00	
RXF 393	1.58		> 2.00		> 2.00	
SN12C	> 2.00		> 2.00		> 2.00	
TK-10	0.53		1.80		> 2.00	
UO-31			> 2.00		> 2.00	
Prostate Cancer						
PC-3	1.49		> 2.00		> 2.00	
DU-145	1.37		> 2.00		> 2.00	
Breast Cancer						
MCF7	1.76		> 2.00		> 2.00	
NCI/ADR-RES	> 2.00		> 2.00		> 2.00	
MDA-MB-231/ATCC	1.21		> 2.00		> 2.00	
HS 578T			> 2.00		> 2.00	
MDA-MB-435	0.27		> 2.00		> 2.00	
MDA-N	-0.30		> 2.00		> 2.00	
BT-549	1.45		> 2.00		> 2.00	
T-47D	1.90		> 2.00		> 2.00	
MG_MID	1.39		1.99		2.00	
Delta	1.74		0.19		0.00	
Range	2.35		0.20		0.00	





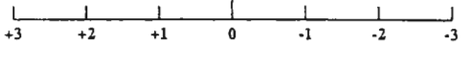
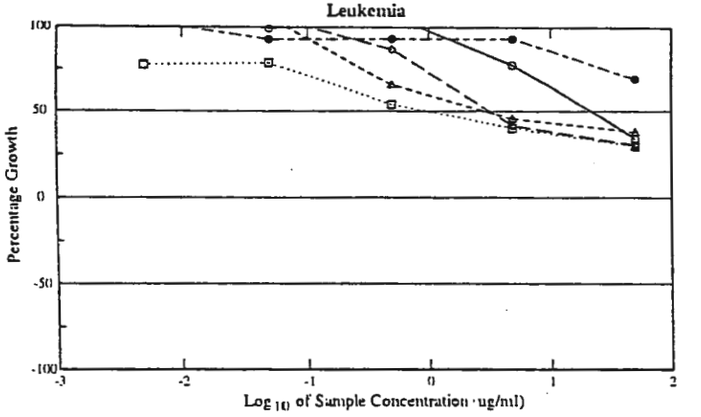
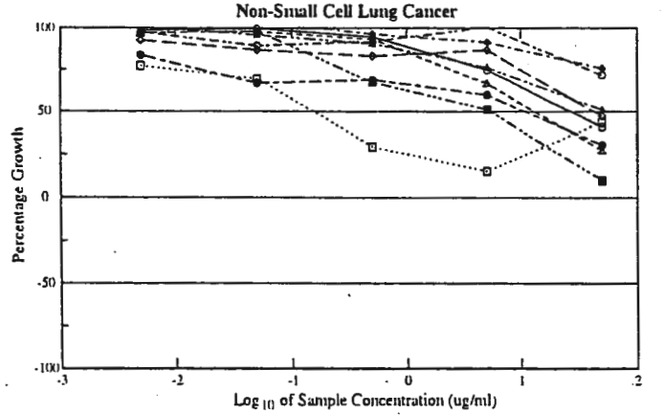


Figure 12 Dose response curves of E3 extract of *Euphorbia peplus* L., against cell lines of human leukaemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancer sub panels. These data were provided by the Development Therapeutics Programme of the NCI (USA).

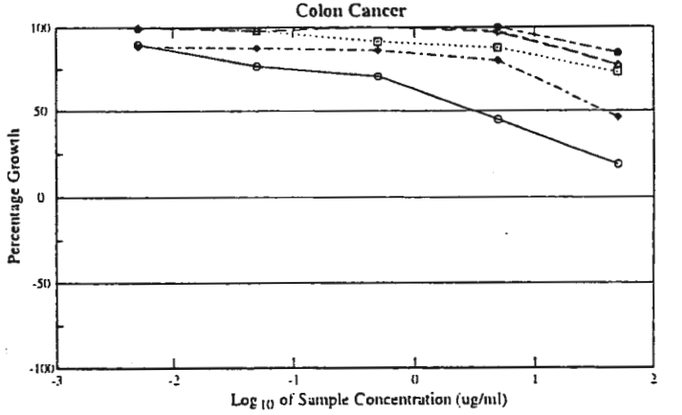
Leukemia



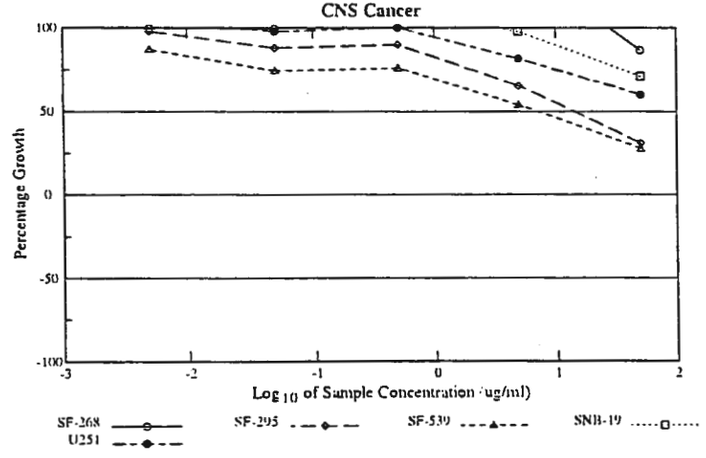
Non-Small Cell Lung Cancer



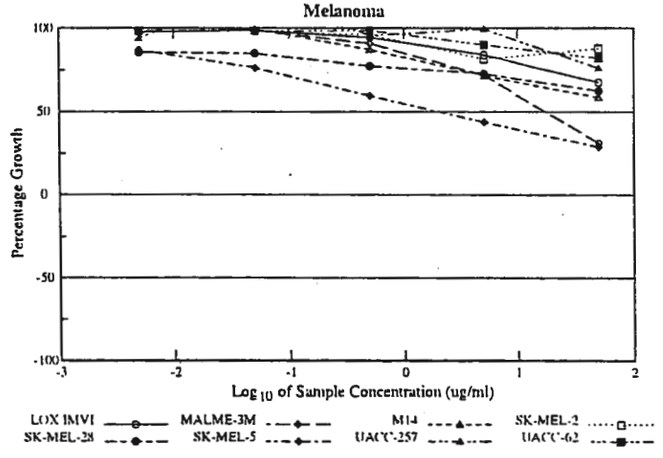
Colon Cancer



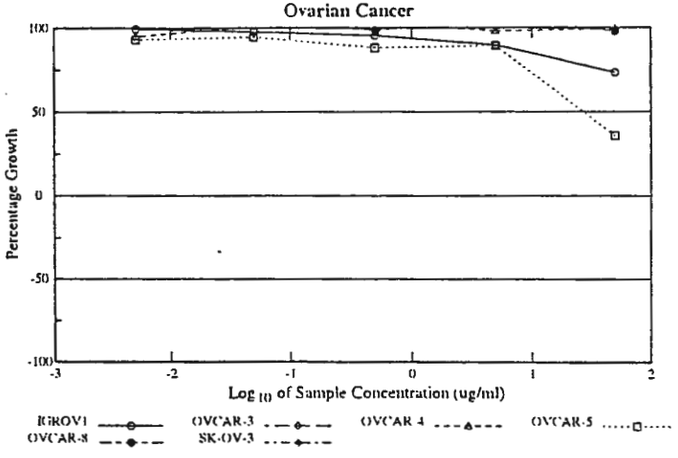
CNS Cancer



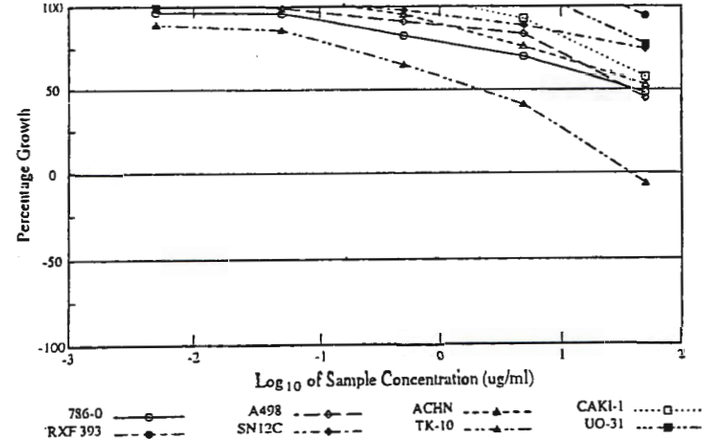
Melanoma



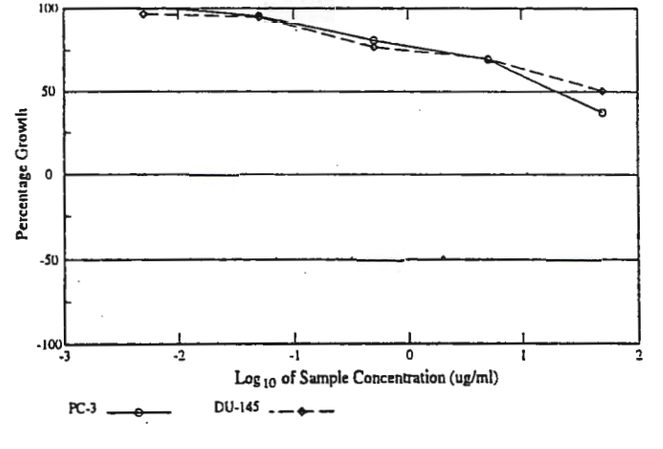
Ovarian Cancer



Renal Cancer



Prostate Cancer



Breast Cancer

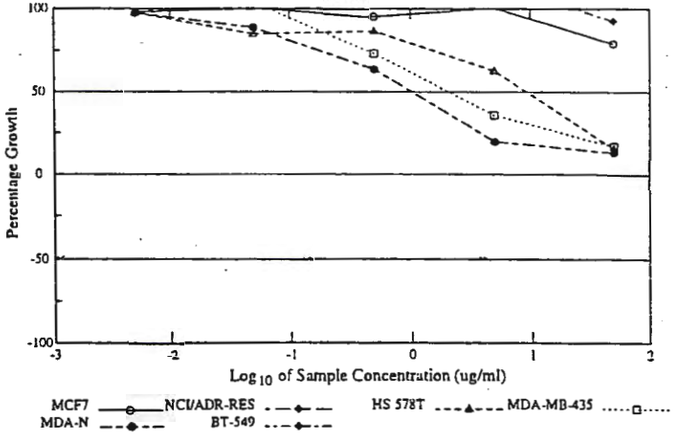


Figure 13 GI50, TGI, and LC50 Mean graphs of E3 extract of *Euphorbia peplus* L., derived from the dose-response data of Figure 11. The log_{10} GI50, log_{10} TGI and log_{10} LC_{50} values respectively are given for each cell lines. These data were provided by the Development Therapeutics Programme of the NCI (USA).

National Cancer Institute Developmental Therapeutics Program

Mean Graphs

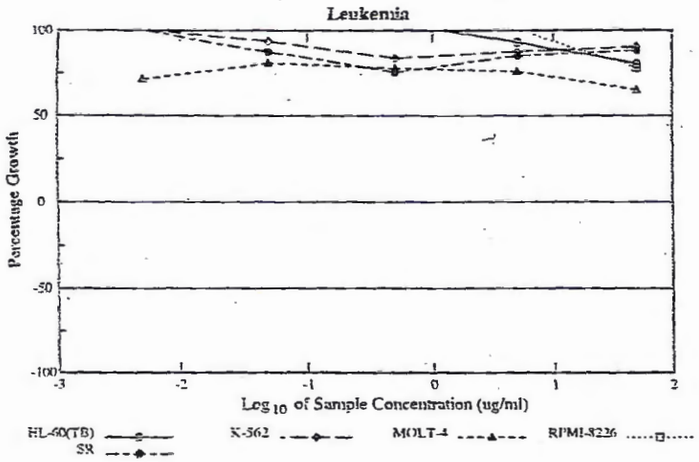
N186781-P
0H600001-N

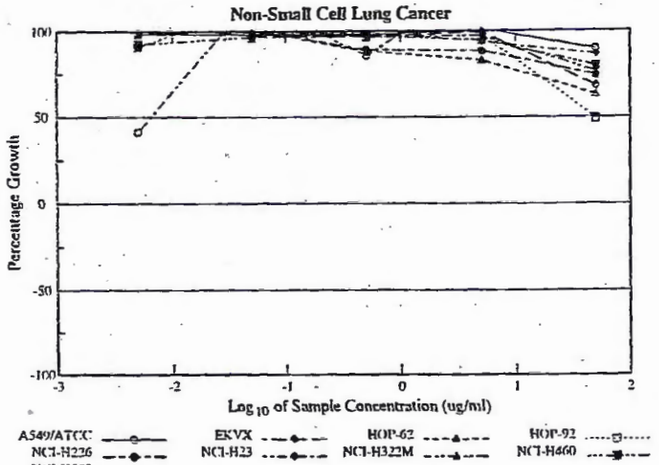
NSC: 0-P5574 -0 / 1	Units: ug/ml	SSPL:	Exp. ID:9910NS85-46
Report Date: October 28, 1999		Test Date: October 11, 1999	

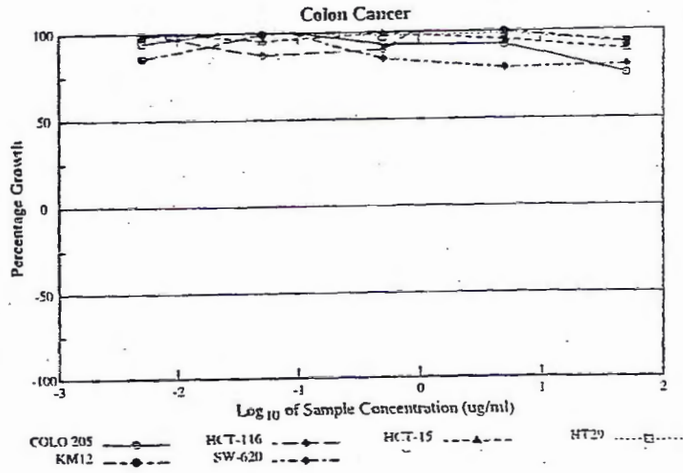
Panel/Cell Line	Log_{10} GI50	GI50	Log_{10} TGI	TGI	Log_{10} LC50	LC50
Leukemia						
HL-60(TB)	1.33		> 1.70		> 1.70	
K-562	0.51		> 1.70		> 1.70	
MOLT-4	0.47		> 1.70		> 1.70	
RPMI-8226	-0.02		> 1.70		> 1.70	
SR	> 1.70		> 1.70		> 1.70	
Non-Small Cell Lung Cancer						
A549/ATCC	1.42		> 1.70		> 1.70	
EKVX	1.63		> 1.70		> 1.70	
HOP-62	1.12		> 1.70		> 1.70	
HOP-92	-0.82		> 1.70		> 1.70	
NCI-H226	1.03		> 1.70		> 1.70	
NCI-H23	> 1.70		> 1.70		> 1.70	
NCI-H322M	> 1.70		> 1.70		> 1.70	
NCI-H460	0.73		> 1.70		> 1.70	
NCI-H522	> 1.70		> 1.70		> 1.70	
Colon Cancer						
COLO 205	0.49		> 1.70		> 1.70	
HCT-116	> 1.70		> 1.70		> 1.70	
HCT-15	> 1.70		> 1.70		> 1.70	
HT29	> 1.70		> 1.70		> 1.70	
KM12	> 1.70		> 1.70		> 1.70	
SW-620	1.58		> 1.70		> 1.70	
CNS Cancer						
SF-268	> 1.70		> 1.70		> 1.70	
SF-295	1.15		> 1.70		> 1.70	
SF-539	0.85		> 1.70		> 1.70	
SNB-19	> 1.70		> 1.70		> 1.70	
U251	> 1.70		> 1.70		> 1.70	
Melanoma						
LOX IMVI	> 1.70		> 1.70		> 1.70	
MALME-3M	1.24		> 1.70		> 1.70	
M14	> 1.70		> 1.70		> 1.70	
SK-MEL-2	> 1.70		> 1.70		> 1.70	
SK-MEL-28	> 1.70		> 1.70		> 1.70	
SK-MEL-5	0.30		> 1.70		> 1.70	
UACC-257	> 1.70		> 1.70		> 1.70	
UACC-62	> 1.70		> 1.70		> 1.70	
Ovarian Cancer						
IGROV1	> 1.70		> 1.70		> 1.70	
OVCAR-3	> 1.70		> 1.70		> 1.70	
OVCAR-4	> 1.70		> 1.70		> 1.70	
OVCAR-5	1.43		> 1.70		> 1.70	
OVCAR-8	> 1.70		> 1.70		> 1.70	
SK-OV-3	> 1.70		> 1.70		> 1.70	
Renal Cancer						
786-0	1.61		> 1.70		> 1.70	
A498	1.56		> 1.70		> 1.70	
ACHN	> 1.70		> 1.70		> 1.70	
CAKI-1	> 1.70		> 1.70		> 1.70	
RXF 393	> 1.70		> 1.70		> 1.70	
SN12C	> 1.70		> 1.70		> 1.70	
TK-10	0.32		1.57		> 1.70	
UO-31	> 1.70		> 1.70		> 1.70	
Prostate Cancer						
PC-3	1.30		> 1.70		> 1.70	
DU-145	> 1.70		> 1.70		> 1.70	
Breast Cancer						
MCF7	> 1.70		> 1.70		> 1.70	
NCI/ADR-RES	> 1.70		> 1.70		> 1.70	
HS 578T	0.97		> 1.70		> 1.70	
MDA-MB-435	0.32		> 1.70		> 1.70	
MDA-N	0.01		> 1.70		> 1.70	
BT-549	> 1.70		> 1.70		> 1.70	
MG_MID	1.33		1.70		1.70	
Delta	2.15		0.13		0.00	
Range	2.51		0.13		0.00	

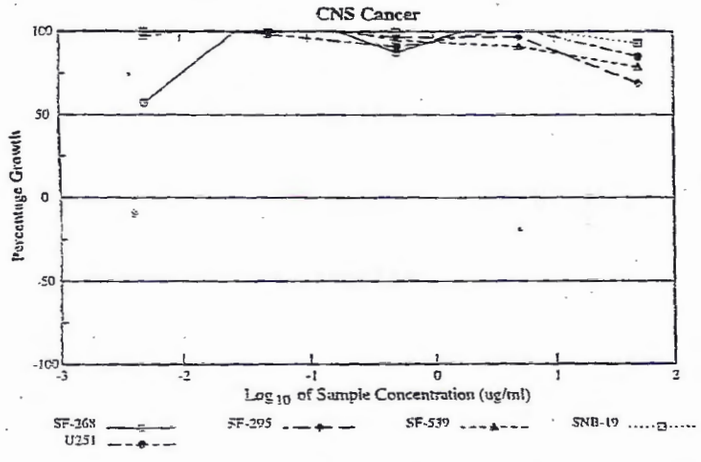
+3 +2 +1 0 -1 -2 -3

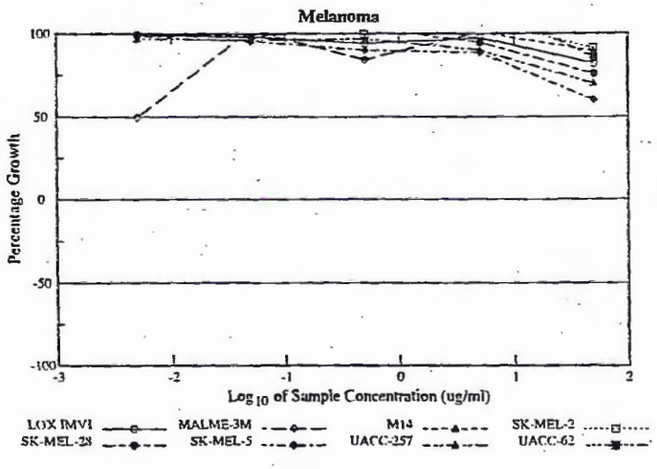
Figure 14 Dose response curves of E4 extract of *Euphorbia peplus* L., against cell lines of human leukaemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancer sub panels. These data were provided by the Development Therapeutics Programme of the NCI (USA).

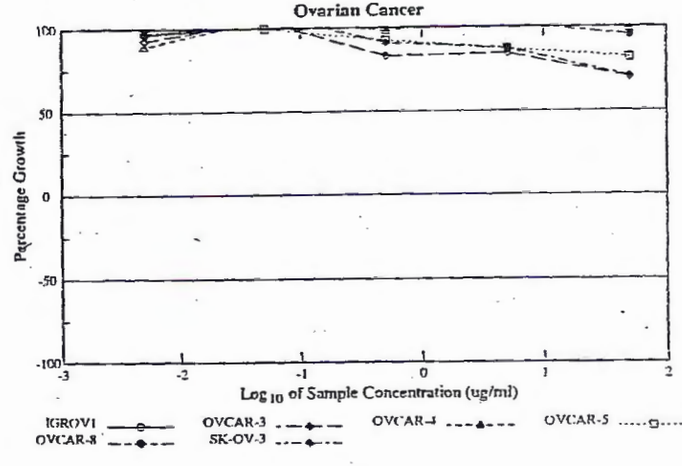

Leukemia
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
HL-60(TB)
K-562
MOLT-4
RPMI-8226
SR

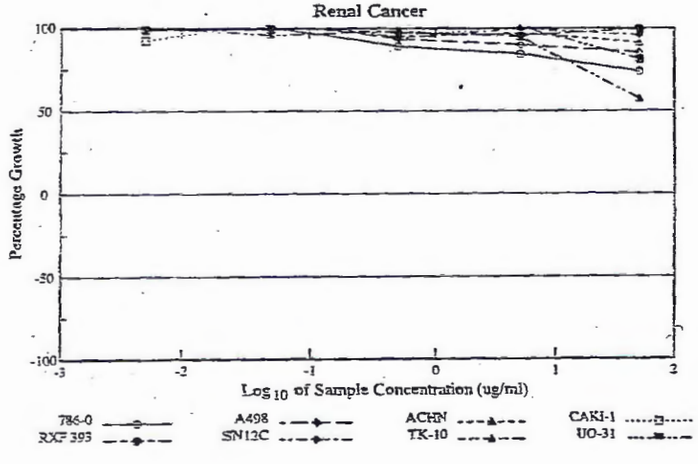

Non-Small Cell Lung Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
A549/ATCC
EKVX
HOP-62
HOP-92
NCI-H226
NCI-H23
NCI-H322M
NCI-H460
NCI-H522

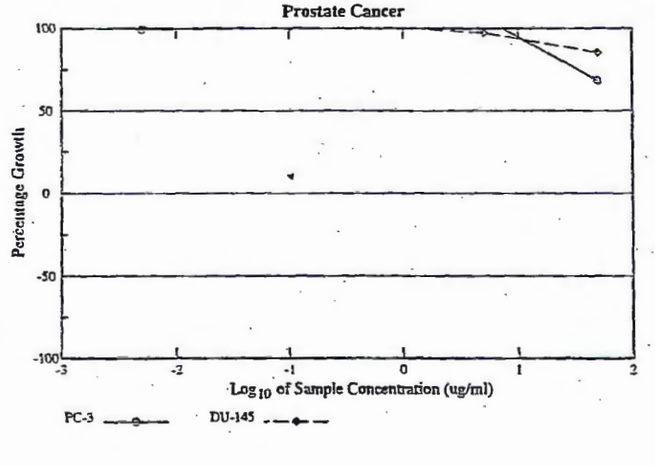

Colon Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
COLO 205
HCC-116
HCT-15
HT29
KM12
SW-620


CNS Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
SF-268
SF-295
SF-539
SNB-19
U251


Melanoma
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
LOX IMVI
MALME-3M
M14
SK-MEL-2
SK-MEL-28
SK-MEL-5
UACC-257
UACC-62


Ovarian Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
IGROV1
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
SK-OV-3


Renal Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
786-0
A498
ACHN
CAKI-1
RXF 393
SN12C
TK-10
UO-31


Prostate Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
PC-3
DU-145

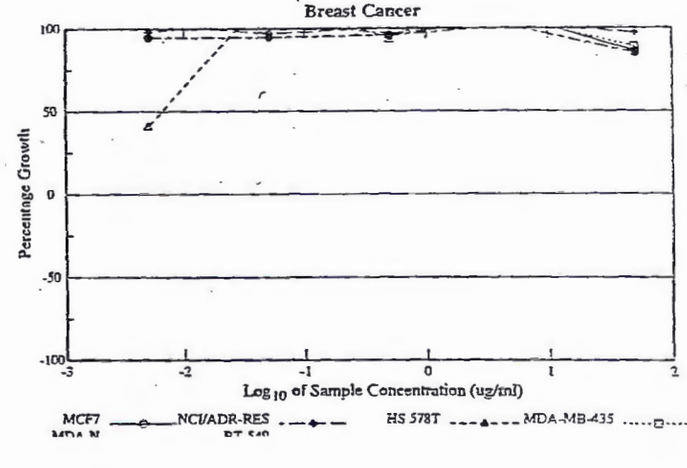

Breast Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
MCF7
NCI/ADR-RES
HS 578T
MDA-MB-435

Figure 15 GI50, TGI, and LC50 Mean graphs of E4 extract of *Euphorbia peplus* L. derived from the dose-response data of Figure 13. The $\log_{10}$ GI50, $\log_{10}$ TGI and $\log_{10}$ LC_{50} values respectively are given for each cell lines. These data for our extracts were provided by the Development Therapeutics Programme of the NCI (USA).

Mean Graphs

NSC 308847-D
Euphorbia peplus

Report Date: October 28, 1999

Test Date: October 11, 1999

Panel/Cell Line	Log_{10} GI50	GI50	Log_{10} TGI	TGI	Log_{10} LC50	LC50
Leukemia						
HL-60(TB)	> 1.70		> 1.70		> 1.70	
K-562	> 1.70		> 1.70		> 1.70	
MOLT-4	> 1.70		> 1.70		> 1.70	
RPMI-8226	> 1.70		> 1.70		> 1.70	
SR	> 1.70		> 1.70		> 1.70	
Non-Small Cell Lung Cancer						
A549/ATCC	> 1.70		> 1.70		> 1.70	
EKVX	> 1.70		> 1.70		> 1.70	
HOP-62	> 1.70		> 1.70		> 1.70	
HOP-92	1.68		> 1.70		> 1.70	
NCI-H226	> 1.70		> 1.70		> 1.70	
NCI-H23	> 1.70		> 1.70		> 1.70	
NCI-H322M	> 1.70		> 1.70		> 1.70	
NCI-H460	> 1.70		> 1.70		> 1.70	
NCI-H522			> 1.70		> 1.70	
Colon Cancer						
COLO 205	> 1.70		> 1.70		> 1.70	
HCT-116	> 1.70		> 1.70		> 1.70	
HCT-15	> 1.70		> 1.70		> 1.70	
HT29	> 1.70		> 1.70		> 1.70	
KM12	> 1.70		> 1.70		> 1.70	
SW-620	> 1.70		> 1.70		> 1.70	
CNS Cancer						
SF-268	> 1.70		> 1.70		> 1.70	
SF-295	> 1.70		> 1.70		> 1.70	
SF-539	> 1.70		> 1.70		> 1.70	
SNB-19	> 1.70		> 1.70		> 1.70	
U251	> 1.70		> 1.70		> 1.70	
Melanoma						
LOX IMVI	> 1.70		> 1.70		> 1.70	
MALME-3M			> 1.70		> 1.70	
M14	> 1.70		> 1.70		> 1.70	
SK-MEL-2	> 1.70		> 1.70		> 1.70	
SK-MEL-28	> 1.70		> 1.70		> 1.70	
SK-MEL-5	> 1.70		> 1.70		> 1.70	
UACC-257	> 1.70		> 1.70		> 1.70	
UACC-62	> 1.70		> 1.70		> 1.70	
Ovarian Cancer						
IGROV1	> 1.70		> 1.70		> 1.70	
OVCAR-3	> 1.70		> 1.70		> 1.70	
OVCAR-4	> 1.70		> 1.70		> 1.70	
OVCAR-5	> 1.70		> 1.70		> 1.70	
OVCAR-8	> 1.70		> 1.70		> 1.70	
SK-OV-3	> 1.70		> 1.70		> 1.70	
Renal Cancer						
786-0	> 1.70		> 1.70		> 1.70	
A498	> 1.70		> 1.70		> 1.70	
ACHN	> 1.70		> 1.70		> 1.70	
CAKI-1	> 1.70		> 1.70		> 1.70	
RXF 393	> 1.70		> 1.70		> 1.70	
SN12C	> 1.70		> 1.70		> 1.70	
TK-10	> 1.70		> 1.70		> 1.70	
UO-31	> 1.70		> 1.70		> 1.70	
Prostate Cancer						
PC-3	> 1.70		> 1.70		> 1.70	
DU-145	> 1.70		> 1.70		> 1.70	
Breast Cancer						
MCF7	> 1.70		> 1.70		> 1.70	
NCI/ADR-RES	> 1.70		> 1.70		> 1.70	
HS 578T			> 1.70		> 1.70	
MDA-MB-435	> 1.70		> 1.70		> 1.70	
MDA-N	> 1.70		> 1.70		> 1.70	
BT-549	> 1.70		> 1.70		> 1.70	
MG_MID	1.70		1.70		1.70	
Delta	0.02		0.00		0.00	
Range	0.02		0.00		0.00	

+3 +2 +1 0 -1 -2 -3

Table 15(a) Summary of results for sensitive cell lines without defaults

HUMAN PANEL		EXTRACT													
			E1				E2				E3				E4
Subpanel	Cell line	log_{10} GI50	GI50	log_{10} GI50	TGI	log_{10} GI50	GI50	log_{10} GI50	TGI	log_{10} GI50	GI50	log_{10} GI50	TGI	log_{10} GI50	GI50
Leukemia	CCRF-CEM	-0.3	0.5			1.1	13.8	2	89.1						
	HL-60(TB)	0.8	6.5			0.6	4.3			1.3	21.4				
	K-562	-0.1	0.9			0.6	4.2			0.5	3.2				
	MOLT-4	-0.2	0.7			0.6	3.9			0.5	3				
	RPMI-8226					1.5	32.4			0	0.9				
	SR	1.7	44.7			1.3	21.9	1.9	87.1						
Non-Small Cell	A549/ATCC	0.8	5.6			0.7	5.5			1.4	26.3				
lung cancer	EKVX					1.3	18.2			1.6	42.7				
	HOP-62	0.5	3.4			0.6	3.8			1.1	13.2				
	HOP-92	-0.38	0.42	2	97.7	-0.35	0.45	1.8	63.1	-0.8	0.15			1.68	47.9
	NCI-H226									1	10.7				
	NCI-H460	0.5	3			0.6	3.7			0.7	5.4				
Colon Cancer	COLO 205	0.6	3.9			0.5	3.4			0.5	3.1				
	HT-29					1.5	33.1								
	SW-620									1.6	38				
CNS Cancer	SF-268					1.4	25.7								
Glioma	SF-295	0.3	21.4			0.5	2.9			1.2	14.1				
	SF-539	1.6	43.7							0.9	7.1				
	SNB-19					1.7	45.7								
	U251					1.7	45.7								
Melanoma	MALME-3M	-0.1	0.7	1.9	85.1					1.2	17.4				
	M14					1.3	20.4								
	SK-MEL--5	1.4	24.6			1.2	14.1			0.3	2				
	UACC-257	1.5	34.7												

cancer subpanel. These GI50 values are 100 times lower than their respective panel averages.

Figures 8, 10 and 12 show that the dose response curves of E1, E2 and E3 extracts against most of the cell lines extended towards the 50% growth line and in some cases towards the 0% line, showing that growth is inhibited as the concentration of the extracts increases. However, the extract E4 did not show considerable growth inhibition below 50 µg/ml (Figure 14).

Table 15(b) Summary of results for sensitive cell lines without defaults

HUMAN PANEL		EXTRACT											
			E1				E2				E3		
Subpanel	Cell line	log_{10} GI50	GI50	log_{10} TGI	TGI	log_{10} GI50	GI50	log_{10} TGI	TGI	log_{10} GI50	GI50	log_{10} TGI	TGI
Ovarian Cancer	IGROVI					1.7	46.8						
	OVCAR-3					1.5	32.4						
	OVCAR-5					1.3	17.8			1.4	26.9		
Renal Cancer	786-0	1.4	23.4			1.3	18.2			1.6	40.7		
	A-498	0.3	2.1	1.4	25.7	1.1	12.6	2	91.2	1.6	36.3		
	ACHN	1.6	40.7			1.3	19.5						
	RXF393					1.6	38						
	TK-10					0.5	3.4	1.8	63.1	0.3	2.1	1.6	**37.2**
	UO-31	-0.2	**0.6**	1.5	28.2								
Prostate Cancer	PC-3					1.5	30.9			1	20		
	DU-145					1.4	23.4						
Breast Cancer	MDA-MB-231/	1.3	21.9			1.2	16.2						
	HS-578T	0.4	2.6							1	9.3		
	MDA-MB-435	-0.01	**1**			0.3	1.9			0.3	2.1		
	MDA-N	-0.4	**0.4**			-0.3	**0.5**			0.01	**1**		
	BT-549					1.5	28.2						
MG_MID		**0.6**	**12.5**	**1.7**	**59.2**	**1**	**18**	**1.9**	**78.7**	**0.8**	**14.5**	**1.6**	**37.2**

Figures 9, 11 and 13 of the extracts E1, E2 and E3 showed horizontal lines extending towards the right direction of the mean graphs for GI50 and TGI but not for LC50. These horizontal lines indicate the degree of growth inhibitory activity of the respective cell line compared to the panel average represented by the vertical line of each mean graph. However, the extract E4 did not show any horizontal lines in any of its mean graphs indicating an inactive response below 50 μg/ml (Figure 15).

The activity of the E1 extract against the four cell lines CCRF-CEM, HL-60 (TR), K-561 and MOLT-4 of the leukaemia subpanel gave GI50 values (0.54, 6.45, 0.87 and 0.65 μg/ml respectively) less than its panel average of 26.3 μg/ml. Also the GI50 values obtained for the E2 extract against five cell lines, CCRF-CEM, HL-60 (TB), K-562, MOLT-4 and SR out of the tested six cell lines of the leukaemia subpanel (13.8, 4.3, 4.2. 3.9 and 2.2 μg/ml respectively) were less than E2's panel average of 24.6 μg/ml. Similar selectivity was seen in the estimated GI50 results obtained for E3 activity against the K-562, MOLT-4 and RPMI-8226 cell lines of 3.24, 2.95 and 0.93 μg/ml, respectively

As for non-small cell lung cancer subpanel, extract E1 gave GI50 values of 5.6, 3.4, 0.42, 2.9 μg/ml, respectively, against the four cell lines, A549/ATCC, HOP-62, HOP-92, and NCI-H460 out of the nine cell lines tested. Somewhat similar responses were seen in Figure 11 for E2 against the non-small cell lung cancer sub panel, in which four horizontal lines were extended to the left of the vertical line representing the panel average (GI50=26.3 μg/ml). The estimated GI50 values, 5.5, 18, 3.8, 0.5 and 3.7 μg/ml, respectively, for E2 activity against the five cell lines, A549/ATCC, EKVX, HOP-62, HOP-92 and NCI-H460 were less than the panel average of 24.6 μg/ml. For E3 activity, GI50 values of 13.2, 0.15, 10.7, and 5.4 μg/ml were obtained, respectively, against the four cell lines, HOP-62, HOP-92, NCI-H226 and NCI-H460. The results obtained using the non-small cell lung cancer subpanel were less than the panel average of 21.4 μg/ml.

In addition, the activity of the E1 extract gave GI50 values of 21.9, 2.6, 0.98 and 0.41 μg/ml against the four cell lines, MDA-MB-231, HS-578T, MDA-MB-435 and MDA-N, respectively, of the breast cancer subpanel, which were lower than the panel GI50 average. Also the activity of the E2 extract gave GI50 values of 16.2, 1.86 and 0.5 μg/ml against the cell lines of MDA-MB-231, MDA-MB-435 and MDA-N, respectively, for the breast cancer subpanel whereas the panel GI50 average for E2 was 24.6 μg/ml. Extract E3 gave GI50 values of 9.3, 2.1, and 1.0 μg/ml against the cell lines HS-578T, MDA-MB-435 and MDA-N, respectively, whereas its panel GI50 average was 21.4 μg/ml. The dose response curves for the activity of E3 crossed below the 50% growth line as the concentration increases slightly above 10 μg/ml.

Thus on the basis of NCI's selection criteria discussed in the Section 1.6.11 of Chapter 1, the extracts E1 and E2 showed a subpanel specific growth inhibition towards leukaemia, non-small cell lung and breast cancer subpanels whereas E3 extract was selective towards the leukaemia and breast cancer sub panels. For the resistivity of the panel, all of the six cell lines of the ovarian cancer subpanel tested did not give 50% growth inhibition below 100 μg/ml concentration of E1 (Figure 8). At least one cell line of all the sub panels tested showed 50% growth inhibition to the extracts E1, E2 and E3.

In respect to the total growth inhibition activity (TGI) of the *Euphorbia peplus* extracts, the extract E1 (Figure 8) showed a total growth inhibition or 0% growth when the HOP-92 cell line (TGI=97.7 μg/ml) of non-small cell lung cancer subpanel was used. The

MALME-3M cell line (TGI= 85.1 μg/ml) of the melanoma sub panel and A498 (TGI=25.7 μg/ml) and UO-31 (TGI=28.2 μg/ml) cell lines of the renal cancer subpanel also showed strong inhibition whereas the other cell lines did not show a total growth inhibition within the maximum tested concentration of 100 μg/ml. Relatively lower TGI values of the A498 and UO-31 cell lines of the renal cancer sub panel indicate higher sensitivity of these renal cancer cells to E1 extract among the entire human panel (mean TGI = 95.5 μg/ml) tested.

The highest growth inhibition potential (minimum ED_{50} values) of 21 μg/ml (Table 8) given by mild solvent extracts of *Euphorbia peplus* against the NCI's *in vitro* human KB tumour cell line is comparable with the human cancer panel averages (GI50) of 26.3, 24.6 and 21.4 μg/ml obtained by the sequential extracts, E1, E2 and E3, respectively. However, the sensitivity of the HOP-92 cell line to the E1, E2, and E3 extracts of 0.42, 0.45 and 0.15μg/ml respectively was approximately 100-fold higher than the highest sensitivity shown by the KB cell line. In addition the E1, E2 and E3 extracts resulted in relatively low GI50 values, 0.4, 0.5 and 1.0 μg/ml against the MDA-N cell line of the breast cancer subpanel. It would appear therefore that the sequential organic solvent extracts, E1, E2, and E3 of *Euphorbia peplus* when tested against the *in vitro* HOP-92 and MDA-N cell lines of the human cancer panel did provide a more efficient screening method compared with using the crude extract against the KB cell line.

A differential growth inhibition effect by the *Euphorbia peplus* extracts, E1, E2 and E3 were prominent using sub panels that showed a selective inhibition. For example, the most sensitive CCRF-CEM cell line (GI50=0.54 μg/ml) in the leukaemia subpanel was about 100 times more sensitive than the least sensitive cell line RPMI-8226 (GI50=61.7 μg/ml) for the E1 extract.

These results indicate that the use of sequential extraction method against a number of different cancer cell lines increased the detectable inhibitory activity of *Euphorbia peplus*. Moreover, the use of number of disease oriented cell lines in a subpanel enabled the detection of selective inhibitory power as suggested previously by Boyd and Paull (1995), even though the selectivity among cancer cell lines did not directly utilise the selectivity observed between cancer cells and normal cells.

As discussed in section 1.6.1.1 of Chapter 1, the *in vitro* growth inhibitory activity criterion for first stage of the screening programme was ED50 ≤ 6 μg/ml for KB cell line and ED50 ≤ 1 μg/ml for the second stage (Schepartz *et al*, 1967). Although the above extracts show a weak growth inhibitory potential according to the current NCI's activity criteria, if the upper limit for activity criterion for GI50 was ≤ 4 μg/ml as proposed by Suh *et al* (1995), then the activity profile of the sequential extracts needs to increase to include 14 cell lines across the panel [Tables 15 (a) & (b)]. These results, when compared to the results obtained for paclitaxel (Taxol®), gave an ED50 value of <0.067 ng/ml against the NCI's human panel. It should be noted that the crude extracts from the plant *Taxus brevifolia* Nutt., from which taxol was extracted gave ED50 values ranging from 1-7 μg/ml against the KB cell line *in vitro* (Suffness & Wall, 1995).

NCI's acceptance criterion for crude plant extracts requires GI50 values of ≤ 0.1 μg/ml as in the case of a synthetic anticancer compound. Thus the extracts E1, E2 and E3 were considered inactive for further investigation by NCI. Lowering the acceptance limit of GI50 ≤ 1 μg/ml for crude plant extracts for *in vitro* assays would, however, render these sequential extracts active. If a higher criterion of ≤ 1 μg/ml is taken for the current results, then the activity profile of the active sequential extracts of *Euphorbia peplus* would give values as indicated in Table 16.

Table 16. Activity (GI50) profile of the sequential extracts of *Euphorbia peplus*

Sub panel	Cell line		Extract	
		E1	E2	E3
Leukaemia	CCRF-CEM	0.5	>1	>1
	K-562	0.9	>1	>1
	MOLT-4	0.7	>1	>1
	RPMI-8226	>1	>1	0.9
Lung (Non-Small Cell) Cancer	HOP-92	0.42	0.45	0.15
Melanoma	MALME-3M	0.7	>1	>1
Renal Cancer	UO-31	0.6	>1	>1
Breast Cancer	MDA-MB435	1	>1	>1
	MDA-N	0.4	0.5	1

According to Table 16, all three extracts can be regarded as active against the HOP-92 and MDA-N cell lines. The extracts, E1 and E2 showed relatively close growth inhibitory potentials whereas E3 gave a higher inhibitory potential against HOP-92 and a lower GI50 value for the MDA-N cell line. In addition, E3 exerted an inhibitory activity towards the RPMI-8226 cell line, which was not prominent for the E1 and E2 extracts. A comparison of the overall GI50 results showed a higher growth inhibitory activity for the E1 extract when compared with the other extracts.

Since it was difficult to maintain an *in vitro* cell line of basal cell carcinoma against which, an extract of *Euphorbia peplus* had an inhibitory effect (Weedon and Chick, 1976), the NCI human panel did not include a cell line from basal cell carcinoma. Hence, the IG50 values for the melanoma sub panel were studied for inhibitory activity. From the results given in Figures 9 and 13, E1 and E3 extracts gave GI50 values of 0.72 and 2.0 μg/ml (Table 15a), for the MALME-3M and SK-MEL-5 cell lines of the melanoma subpanel, at least 10 times less than their panel average. In fact, E1 exerted a total growth inhibition of MALME-3M cell line at its concentration of 85.1 μg/ml (Figure 9). Although the *Euphorbia peplus* extracts did not show subpanel specific growth inhibition of the melanoma subpanel, the E1 and E3 extracts did inhibit the growth of these two skin cancer cell lines.

In 1999, the NCI resorted to the use of "more sensitive" cell lines including MCH-7; NCI-H460 and SF268 as a prescreen prior to the testing on 60 human cell-line panel. However, the results given in Figures 9, 11, and 13, show that only the NCI-H460 cell line indicated a 50% growth inhibition (GI50) by the extracts E1, E2, and E3 at their concentrations of 3, 3.7, 5.4 μg/ml, respectively, and the 50% growth inhibition of MCF-7 cell lines occurred at concentrations of >50 μg/ml. Had the *Euphorbia peplus*. extracts been tested only against the latest NCI panel of the "sensitive" 3-cell lines including the MCF-7 (breast carcinoma), NCI-H460 (lung carcinoma), and SF-268 (glioma), the selective growth inhibitory activity inferred against the leukaemia, non-small cell lung and breast cancer sub panels would not have been detected.

3.3.2 *In vitro* screening of *Euphorbia peplus* extracts against the human leukaemia CEM system

In order to determine whether the sensitivity of the *in vitro* anticancer activity test could be increased by using a different method, extracts of *Euphorbia peplus* were assayed using a

against sensitive human CEM leukaemia cell line using a [^{3}H]-thymidine uptake by a human CEM cell line was carried out. The CEM cell line was selected because of previous results showed that this cell line showed high sensitivity to anticancer agents at low concentrations.

3.3.2.1 [^{3}H]-thymidine uptake assay

In order to assess the performance of the *in vitro* CEM-[^{3}H]-thymidine assay system, growth inhibition of the CEM cells were tested using two standard anticancer agents, doxorubicin and idarubicin. The results of this experiment are given in Figure 16.

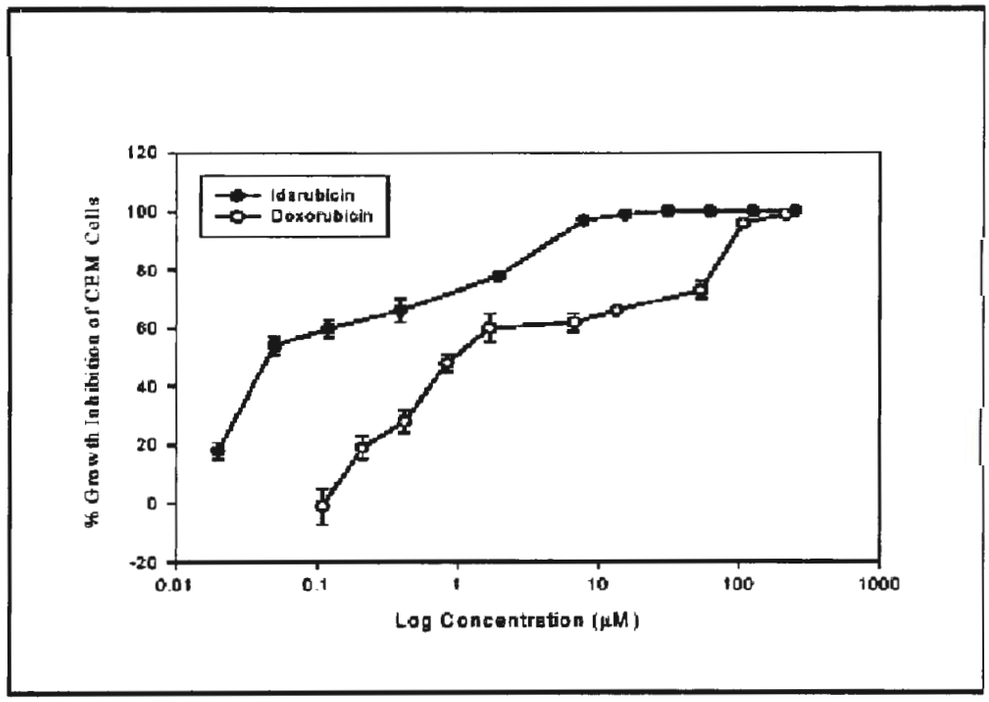


Figure 16 Percent growth inhibition of CEM leukaemia cells at different log concentrations of the standard anticancer agents, idarubicin and doxorubicin using [^{3}H]-thymidine uptake assay. Each data point is represented by the mean ± S.E of three independent replicate determinations.

According to Figure 16, idarubicin showed a 10-fold higher growth inhibition ($P<0.05$) against the CEM cells than that of doxorubicin. From the dose response curves given in

Figure 16, the GI50 values estimated for idarubicin and doxorubicin were 0.05-0.09 and 0.75-0.87 μM (0.02-0.05 and 0.44-0.5 μg/ml), respectively.

Fridborg *et al* (1995) found a concentration range of 0.085-0.16 and 0.13-0.76 μg/ml respectively for idarubicin and doxorubicin using fluorometry. The GI50 results of doxorubicin (adriamycin), as reported by Demetzos *et al* (1994) using flow cytometry for the sensitive cell lines, KB-3-1, S1, L1210 and DC-3F, fell within the range, 0.008–0.3 μM whereas Pietersz *et al* (1988) obtained GI50 results of 0.10-0.22 μM on CEM cell for idarubicin using [^{3}H]-thymidine uptake assay. Our results for idarubicin, were very close to those obtained by Pietersz *et al* (1988).

Table 17 Comparison of GI50 values for standard anticancer agents

Reference	Method	Cell line	Idarubicin	Doxorubicin
			μM	μM
Our results	[3H]-thymidine	CEM	0.05-0.09	0.75-0.87
Pietersz *et al* (1988)	[3H]-thymidine	CEM	0.10-0.22	N/D
NCI Webpage, (2001)	SRB	Human panel	N/D	0.005-1.58
Fridborg *et al* (1995)	fluorometry	ALL, CLL	0.085-0.16	0.13-0.76
Demetzos *et al* (1994)	flowcytometry	KB-3-1, L1210	N/D	0.008–0.3

(ND; Not Done)

Hence the GI50 values obtained for idarubicin (0.05-0.09 μM) and doxorubicin (0.75-0.87 μM) fell within a narrow range thus showing a good response by the CEM cells for both standards, under the test conditions. A very low GI50 given by both standards was evidence of the high sensitivity of the CEM cell line to standard anticancer agents as detected by the [^{3}H]-thymidine uptake assay. These data therefore showed that the CEM cell line used in these experiments had not developed drug resistance to idarubicin and doxorubicin.

The results of the anticancer activity of the extracts of the plant *Euphorbia peplus*, when screened against human leukaemia cell line (CEM), are given in Figure 17. As illustrated in

Figure 17, the E2 extract showed a significantly higher ($P<0.05$) percent inhibition of *in vitro* CEM cell growth compared with E1, E3, E4, E5, E6 and EB extracts at concentrations higher than 6.5 μg/ml. No significant differences ($P>0.05$) in inhibition of *in vitro* CEM cell growth was found for the aqueous extracts, E5, E6 and EB (Figure 17) as anticipated by the NCI.

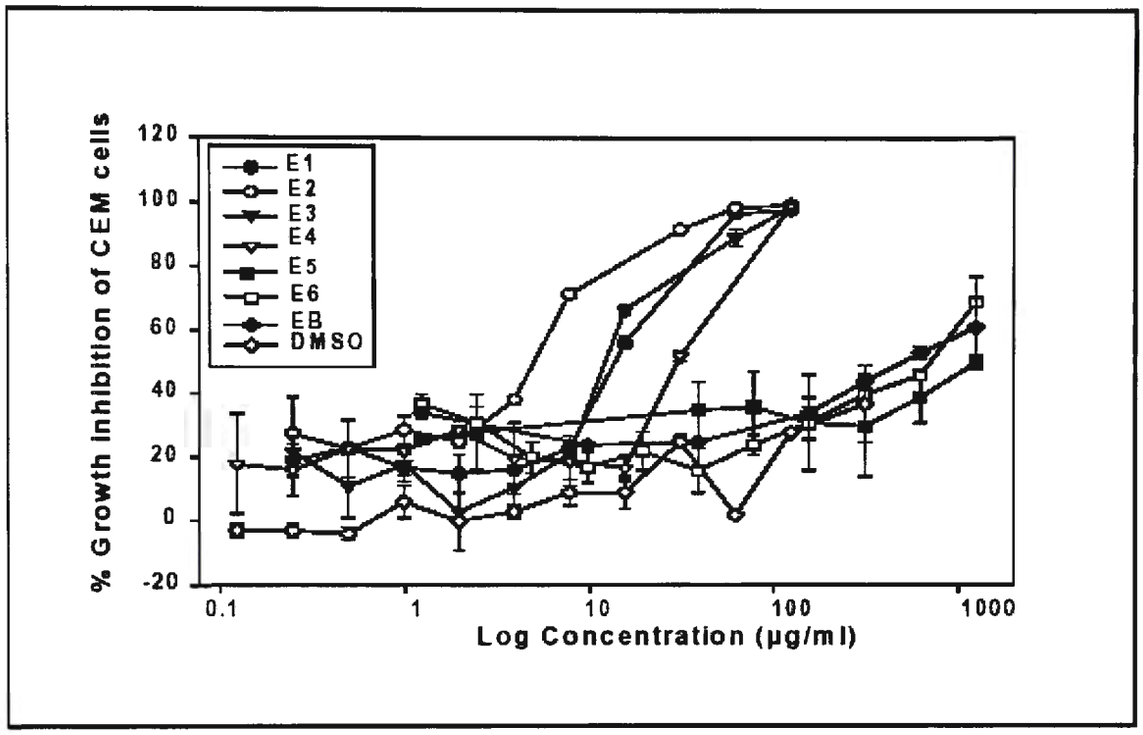


Figure 17 Percent growth inhibition of CEM leukaemia cells at different log concentrations of the *Euphorbia peplus* extracts, E1, E2, E3, E4, E5, E6 and EB compared with DMSO, measured by [^{3}H]-thymidine uptake assay. Each data point is represented by the mean ± S.E of three independent replicate determinations.

The GI50 estimated values from the dose response curves for the extracts (Figure 17) and that of the standards (Figure 16) are given in Table 18. As shown in Table 18, the GI50 value of the extract E2 (5.3 ± 0.2 μg/ml) is approximately three times lower than that of the extract E1 (15.5 ± 1.9 μg/ml) and the extract E3 (15.2 ± 3.7 μg/ml). No significant difference was shown between GI50 values of E1 and E3 ($P>0.05$). The GI50 value of E4 (39.2 ± 11.4) was found to be approximately eight times higher than that of E2. Hence

As indicated in Table 18, very high GI50 values were shown for the aqueous extracts, E5 (955±259), E6 (795±152) and EB (471±129) compared with much lower values obtained for the E1, E2, E3 and E4 extracts (GI50 values < 39.2±11.4). The GI50 values of both E5 and E6 extracts were approximately twice as much as that of the EB extract (Table 18).

According to the above results CEM cell growth inhibitory activity of the sequential solvent extracts of the plant *Euphorbia peplus* was more prominent in the organic extracts such as $CHCl_3$ (E2), petroleum spirit (40-60°C), $CHCl_3$-MeOH (E3) and 70% EtOH. (E4).

Table 18 Comparison of GI50 values ± SEM (standard deviation from the mean) obtained from the log growth curves of the extracts E1, E2, E3, E4, E5, EB and the standards doxorubicin and idarubicin measured by [^{3}H]-thymidine uptake assay.

Sample	GI50 Value (µg/ml)	±SEM
E1	15.5	1.9
E2	5.3	0.2
E3	15.2	3.7
E4	39.2	11.4
E5	955	259
E6	795	152
EB	471	129
Idarubicin	0.02	0.01
Doxorubicin	0.44	.06

The aqueous extracts, 10% aqueous NH_4OH (E5) and 2N HCl (E6) did not show a significant inhibitory activity (GI50 values> 500 µg/ml). However, the boiling water extract (EB) did show very low inhibitory activity (GI50 value=471±129), which was higher than the aqueous extracts.

The lower inhibitory activity of E4 (Figure 14 and 15) against cancer cells, compared with E3 (Figure 12 and 13) was found using the sulforhodamine B assay as well as the [^{3}H]-thymidine uptake assay. However, E4 did not show a 50% growth inhibition against any of the leukaemia cell lines used in the screen below the upper concentration limit (100 μg/ml), when assayed using the sulforhodamine B test. The CEM cell [^{3}H]-thymidine uptake assay gave a 50% growth inhibition at concentrations of 39.2 ±11.4 μg/ml and a 100% inhibition of CEM cells between concentrations of 125-156 μg/ml. For all three organic solvent extracts, there was a 100% inhibition of CEM cell growth between 125-156 μg/ml. The aqueous extracts did not show complete growth inhibition of at least 100% within the tested concentration range.

The observed inhibitory activity of the extracts may be due to cytostatic activity whereas the extract inhibits the proliferation of the cells but it does not actually kill the cells. On the other hand the cytotoxic effect kills the cells, which prevents their proliferation and uptake of [^{3}H]-thymidine at the stage of DNA replication. Hence the results of the [^{3}H]-thymidine uptake assay do not indicate whether the cell growth inhibitory effect is due to a cytostatic or a cytotoxic action of the extract. In order to determine the effect of extracts on dehydrogenase enzymes in metabolically active cancer cells that reduces the MTS assay was carried out using a CEM cell line.

3.3.2.2. MTS Assay

The results of the cytotoxic effect of the E1, E2, E3, E4 and EB extracts using the MTS assay and the CEM cell line as described in section 2.6.2.3. of Chapter 2, are illustrated in Figure 18.

The MTS assay was carried out with sample concentrations below 125 μg/ml as concentrations higher than that showed interference of pigment absorbance with the OD measurement. The data points at the concentrations, 100 and 50 μg/ml of E3 and E4 showed very low percent inhibition of MTS reduction compared with lower concentrations. This discrepancy may be due to the presence of plant pigments that interfere with the assay measurement at 490 nm. This is supported by the results of [^{3}H]-thymidine uptake assay, which does not show interference from plant pigments due to the measurement of radioactivity.

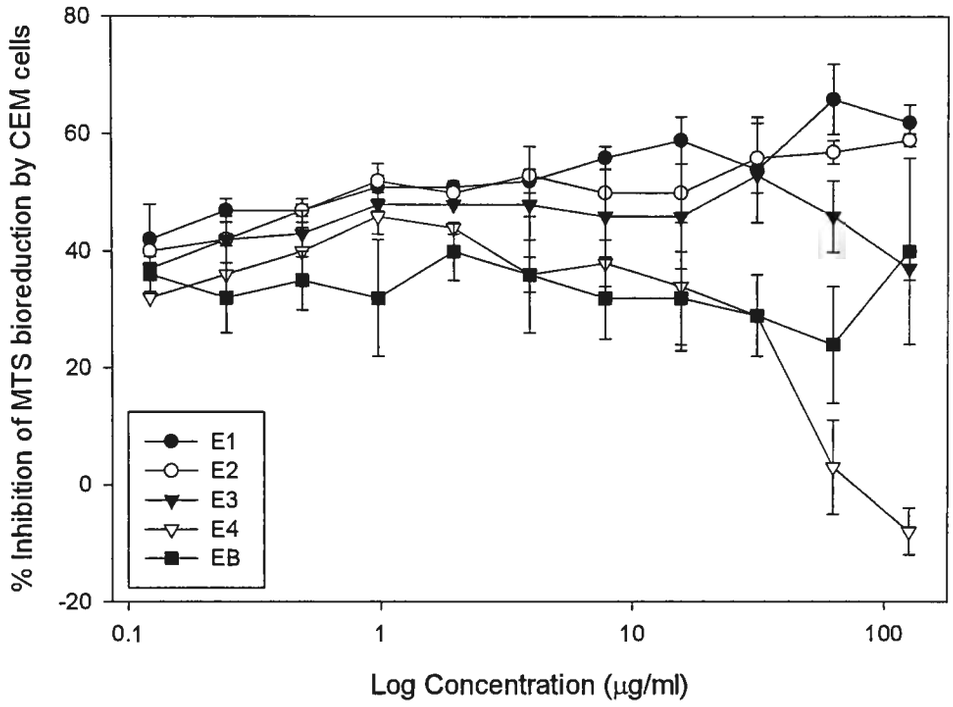


Figure 18 Comparison of % inhibition of bioreduction of MTS by CEM cells with increasing log concentration of E1, E2, E3, E4 and EB. Each data point is represented by the mean ± S.E of three replicate determinations adjusted for the absorbance of each extract, culture medium and cells. (E5 and E6 extracts were not used in the assay).

The GI50 values for MTS reduction by the extracts E1, E2, and E3 were 0.74±0.22, 0.73±0.21, and 23.2±6.3 µg/ml respectively. However, a comparison of the GI50 values between E1 and E2 did not show a significant difference (P= 0.500>0.05). Interestingly, the GI50 values between E1 or E2 and E3 did not show a significant difference (P=0.168 > 0.05) either, due to the wide variation among the results obtained for E3. The lack of a correlation was probably due to a wide variation for the E3 GI50 results. Although there was a significant difference (P=0.820>0.05) between the E1 and E3 results of the ^{3}H-thymidine uptake assay, the MTS assay did not yield results with a significant difference (P=0.168 > 0.05) between E1 and E3.

The highest inhibition results found for the E1 and E2 extracts at 62.5 μg/ml using [^{3}H]-thymidine uptake assay and MTS assay against CEM cells were 96.5%, 98.3% and 62%, 57% respectively. Thus, E1 and E2 showed 62% and 57% of CEM cell killing. The difference between the results of growth inhibition measured by the [^{3}H]-thymidine uptake assay and the MTS assay may be attributed to the % cytostatic activity of E1 (35%) and E2 (41%). The results of both assays indicate that the inhibition of thymidine uptake of CEM cells is mainly due to the cell killing effect of the *Euphorbia peplus* extracts.

3.3.3. Screening of *Euphorbia peplus* against Brine Shrimp

Results obtained for the sequential extracts of *Euphorbia peplus* using brine shrimp assay are given in Figure 19. As shown in Figure 19, E1 and E2 resulted in the highest number of deaths of brine shrimp nauplii when used at concentrations above 50 μg/ml concentration. The LC50 (dose causing death of 50% of the nauplii) values of E1, E2 and E3 were 13.5±3.1 and 42.3±1.2 163±92 μg/ml, respectively, whereas E6 and EB showed LC50>1000. μg/ml. Furthermore, there was a significant difference ($P=0.04< 0.05$) between the E1 and E2 results.

According to McLaughlin *et al* (1993) a positive correlation between brine shrimp toxicity and *in vitro* 9KB cytotoxicity ($P=0.036$ and kappa=0.56) was found for number of compounds screened using both tests. However, because of number of false positive results given by the brine shrimp assay, it was treated as a general toxicity assay at the time it was first used in conjunction with the more specific potato disc antitumour assay, to eliminate compounds with toxic effects that are not antitumour (McLaughlin *et al*, 1993). However, in the present study, the brine shrimp assay ($P=0.033$) was shown to be superior, or similar the potato disc antitumour assay at predicting *in vivo* activity as cytotoxicities in a series of human solid tumour cell lines ($P=0.033$-0.334), which is similar to the results reported by McLaughlin *et al* (1993).

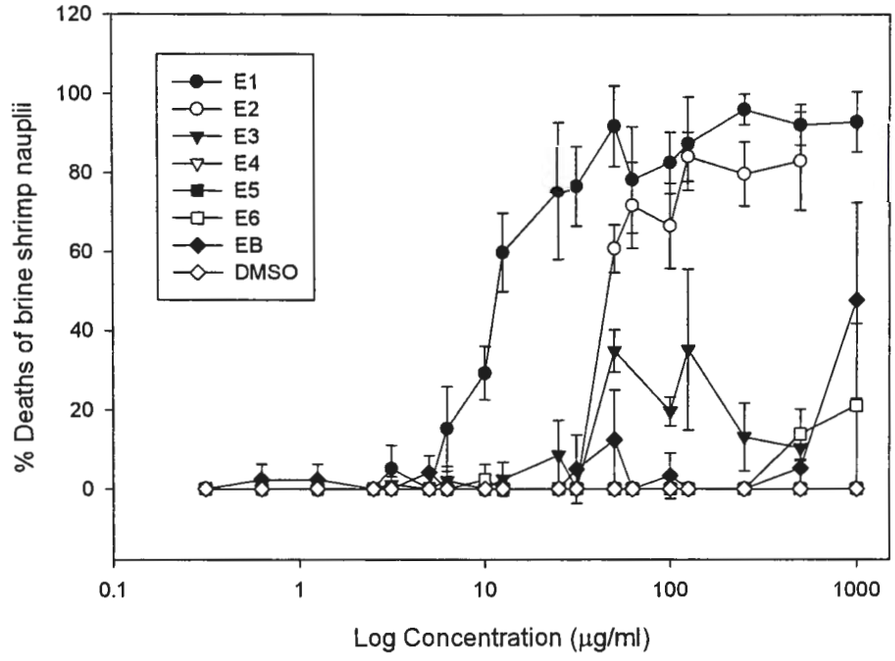


Figure 19 % Deaths of brine shrimp larvae at log concentrations of *Euphorbia peplus* extracts E1, E2, E3, and E6. Each data point is represented by the mean ± S.E of four independent replicate determinations. Results of E4, E5 and DMSO are not included, as they did not show deaths of brine shrimp nauplii within the tested concentration range.

3.3.4 Comparison of assays

A comparison of different assay results obtained for *Euphorbia peplus* extracts against the CEM cell line with the results of brine shrimp assay is given in Table 18.

As illustrated in Table 18, there was a significant difference ($P<0.05$) between the results for the extracts, E1 and E2 when tested using the sulforhodamine B, [^{3}H]-thymidine uptake, and brine shrimp assays. However, no significant difference was found between the E1 and E2 results using the MTS assay, this may have been due to a wide variation among the E3 results. The results of brine shrimp assay showed that E1 is more toxic to the nauplii than E2.

Table 19 Comparison of the results of *Euphorbia peplus L* extracts against CEM cells obtained using sulforhodamine B, ^{3}H-thymidine uptake, MTS, and brine shrimp assays.

Extract	NCI's Sulphorhodamine B assay GI50 μg/ml	[^{3}H]-thymidine uptake assay GI_{50} μg/ml	MTS assay GI_{50} μg/ml	Brine shrimp assay LC_{50} μg/ml
E1	0.54	15.5±1.9	0.74±0.22	13.5±3.1
E2	13.8	5.3±0.2	0.73±0.21	42.3±1.2
E3	ND	15.2±3.7	23.2±6.3	163±92
E4	ND	39.2±11.4	>125	>1000
E5	ND	955±259	>125	>1000
E6	ND	795±152	>125	>1000
EB	ND	471±129	>125	>1000

Values are the mean±SD of at least three replicate determinations, ND= not done

The sulforhodamine B and MTS assay results for E1 (GI50=0.54 and 0.74 μg/ml respectively) were approximately 100-fold lower than those (GI50=15.5 and 13.5 μg/ml respectively) obtained by using [^{3}H]-thymidine uptake and brine shrimp assays. The differences in E1 results obtained by [^{3}H]-thymidine uptake, MTS and brine shrimp assays were not significant (P=0.059, 0.743 and 0.160; >0.05).

For E2, GI50 results using the MTS assay (0.73 μg/ml) were approximately 10-fold lower than those (5.3 μg/ml) of the [^{3}H]-thymidine uptake assay whilst significant differences among the results of [^{3}H]-thymidine uptake, MTS and brine shrimp assays (P<0.05) were observed. The sulforhodamine B and brine shrimp assays gave mean LC50 values of 13.8 and 42.3 μg/ml, respectively, for E2, which were greater than those obtained using the [^{3}H]-thymidine uptake and MTS assays. No significant differences (P>0.05) were found among the GI50 results for E3.

The MTS and brine shrimp assays gave relatively lower (P<0.05) inhibitory potential (>125 μg/ml) for E4 compared with the [^{3}H]-thymidine uptake assay results (39.2 μg/ml). The sulforhodamine B assay results for E3, E4, and the aqueous extracts against

CEM cell line were not available from the NCI. The HCl (E5), NH_4OH (E6), and boiling water (EB) extracts gave very low inhibitory potentials (>125 μg/ml).

On the basis of the GI50 results for E2, it may be inferred that the sensitivity of the assays decreases in the order, MTS> [^{3}H]-thymidine>sulforhodamine B when CEM cells were used as the test cell line, whist the brine shrimp assay showed the least sensitivity.

3.4 Discussion

Suffness and Douros (1982) have reported previously that *in vitro* anticancer results have been interpreted, as an indication of cytotoxicity, although the toxic effect on cancer cells compared with normal cells has not been assessed. Subsequent NCI *in vivo* screening results have been interpreted as an indication of antitumour activity. A major limitation of *in vitro* screening systems is considered to be the inability of cells *in vitro* to respond to drug precursors or prodrugs. Hence lack of toxicity effect *in vitro* according to Masters *et al* (1992) does not preclude activity *in vivo* since some drugs require metabolism for activation. Although a strategy of using antibodies as vectors for enzymes capable of activating non-toxic precursors to potent cytotoxic moiety has been suggested (Melton and Sherwood, 1996), finding an activator from the same plant would avoid potential adverse toxicity resulting from such antibody directed prodrug therapy.

Euller (1994) isolated cucurbitacins from the plant, *Gonystylus keithii* using the pattern recognition from the mean graphs obtained from the NCI's 60-cell panel screen as a tool for dereplication. Since the *Euphorbia peplus* extracts, E1, E2 and E3 showed a selective cytotoxicity towards leukaemia and breast cancer cells lines, this may allow dereplication of extraction methods to isolate the active compounds. However, these extracts did not generate sufficient response in their TGI and LC50 mean graphs to enable computer-assisted pattern recognition using NCI's COMPARE program.

Cuello *et al* (2001) used the MTS assay to assess the toxicity of chemotherapeutic drugs using ovarian cancer cells. Although the present MTS results did not show a clear-cut results due to inconsistency, these plant extracts could be retested at relatively low concentrations to determine the cytotoxicity of the extracts.

Although the potent cytotoxic anticancer drug, paclitaxel (taxol®) had indicated an ED50 value of 4.1×10^{-5} μg/ml on KB cells as indicated from NCI's earlier screening

programme (Suffness, 1995), which indicated a 10^4-fold cytotoxicity compared to that shown by the E1, E2 and E3 extracts, the lowest dose of the naturally occurring cancer chemopreventive agent, protocatechuic acid, was 500 μg/ml according to earlier studies by Tanaka (1994).

The direct solvent extracts of *Euphorbia peplus*, when subjected to the NCI's previous screening programme against *in vitro* KB cells and *in vivo* mouse LE, PS, LL, SA and rat WA tumours, did not show sufficient growth inhibition to warrant further investigation. The highest *in vitro* growth inhibitory potentials shown by latex, aqueous/EtOH, EtOH and $CHCl_3$ extracts of *Euphorbia peplus* against KB cells were 21-36 μg/ml. However, when sequential soxhlet extracts, E1, E2 and E3 of *Euphorbia peplus* were used against the HOP-92 cell line of the human non-small cell lung cancer, the growth inhibitory potentials were further increased to 0.42, 0.45 and 0.15 μg/ml, respectively, which are much closer to the NCI's acceptance criterion of <0.1 μg/ml.

Although the results of the [^{3}H]-thymidine uptake assay and the MTS assay may not necessarily be consistent, the MTS results give more information about the cytotoxicity or cytostatic effect of the extract tested. The growth inhibitory potential given by the [^{3}H]-thymidine uptake assay was primarily due to the cytotoxic effect of the *Euphorbia peplus* extracts as shown by the MTS results.

Comparison of test results using different assays showed that the MTS method is more sensitive but the results were less precise. Although the results of the sulpharhodamine B, [^{3}H]-thymidine uptake, and MTS assays are somewhat consistent for the organic sequential extracts against CEM cell line, the brine shrimp assay results, except for E1 extract were not consistent with the other assays. Although very close IC_{50} values were obtained for E1 extract from, both the [^{3}H]-thymidine uptake assay and the brine shrimp assay, the difference between them was not significant ($P=0.743>0.05$).

A recurring problem in cancer chemotherapy drug discovery is thought to be due to limited solubility (Cragg and Newman, 1999) of natural product extracts and pure isolates in aqueous solvents. This may be avoided by using boiling water extracts that often contain saponins, which would assist dissolution of usually insoluble organic compounds (Farnsworth, 1994)

According to McLaughlin *et al* (1993), the mean graphs generated through the NCI cell panel with 48 hour cell-drug exposures for the clinically effective paclitaxel (Taxol®), etoposide, cis-platinum and several other agents have not been distinctive at all. Due to the complex nature of crude plant extracts, it is difficult to obtain a unique selective cytotoxicity pattern. McLaughlin *et al* (1993) suggested that crude extracts might only produce a pattern of general cytotoxicity that could well be screened in the P388 system. However, results in this project for the sequential crude extracts of *Euphorbia peplus*, E1 and E2 against the *in vitro* human 60-cell line panel indicated a weak but some selective cytotoxicity pattern towards the cell lines of leukaemia, breast and non-small cell lung cancer sub panels. In addition the E3 extract showed a weak but selective growth inhibitory activity against leukaemia and breast cancer sub-panels.

The NCI *in vitro* screening system is specifically directed at identifying anticancer agents having cell-type selective toxicity (McLaughlin *et al*, 1993). However, according to Boyd and Paull (1995) the NCI's 60 human cell-line screening programme, in addition to the information on "net cell killing" (LC50) or cytotoxic effects may also provides information on the growth-inhibitory (GI50) and "total growth inhibition" (TGI) or cytostatic effects of the test agent. Computerised COMPARE pattern-recognition algorithms which correlate similar patterns of differential cytotoxicity produced by extracts, fractions and pure compounds tested in NCI's 60-cell-line-panel are used for preliminary biological dereplication (Cardellina *et al*, 1993). Although the aim of the NCI's screening programme is directed towards discovering novel compounds with structures and functions different from the existing compounds in the NCI database, it may be used to detect sub panel selectivity and differential anticancer activity of plant extracts as shown by our results.

Although the suggested anticancer activity of the *Euphorbia peplus L* described in this chapter did not include a control normal cell line of each of the 60 cancer cell lines used in the NCI screen, such a control cell line would be essential in order to draw a scientific conclusion on the putative anticancer properties of some of our extracts. In addition, *in vivo* studies need to be carried out to confirm the antitumour and anticancer activity of the *in vitro* active extracts.

CHAPTER 4 INVESTIGATION OF ANTICANCER ACTIVITY OF *Viola odorata* L.

ABSTRACT

Viola odorata L, sweet violet, is used in traditional medical systems to treat uterine, throat, tongue, intestine, breast and stomach cancers. However, the extracts of *Viola odorata*, failed to show any significant *in vitro* anticancer activity using *in vitro* KB cells as the test system and against *in vivo* mouse tumours tested in the screening programme of the US National Cancer Institute (NCI). The lowest 50% growth inhibition value for KB cells was 21 μg/ml. Consequently, *Viola odorata*, has not been tested using a more extensive disease-oriented 60 cell-line human panel. Hence in the experiments described in this chapter, *Viola odorata* was investigated using an exhaustive sequential extraction procedure. The *in vitro* anticancer activity was tested against the human CEM leukaemia and LOVO colon cancer cells using both [^{3}H]-thymidine uptake and MTS assays. The NCI's *in vitro* 60 cell-line human panel in combination with the sulforhodamine B assay was also used as a test system.

The results showed that the highest 50% cell growth inhibition potential was 3 μg/ml given by the [^{3}H]-thymidine uptake CEM cell assay for the sequential $CHCl_3$-MeOH extract, V3. This result was also reflected by the MTS assay results. The corresponding result for RPMI-8266 leukaemia cells using NCI's sulphorhodamine B assay was 28 μg/ml. The panel averages for 50% growth inhibition as determined by the sulfarhodamine B assay for the V3 extract, its MeOH-soluble fraction and the 70% EtOH extract of the roots, VRT4 were 49, 7, and 263 μg/ml, respectively. Although these extracts showed a weak growth inhibition and cytotoxicity against the human cancers, they did not show any sub-panel selective growth inhibition. The [^{3}H]-thymidine assay results for the V3F5 fraction of V3, were 2±1 μg/ml and 67±28 μg/ml using the CEM and the LOVO cell lines, respectively.

The [^{3}H]-thymidine CEM cell assay indicated a 10-fold higher *in vitro* anticancer activity when compared with the sulforhodamine B assay against the RPMI-8226 cells and previous results obtained from testing direct solvent extracts and protein synthesis assay against the KB cells.

4.1 Introduction

Viola odorata L, sweet violet is a perennial plant belongs to the family, *Violaceae*, which bears heart-shaped leaves, purple flowers and rhizomes. According to Ewart (1939), *V. odorata* was recorded as growing in Victoria in 1893. The plant, *Viola odorata* (Sanskrit, Banaphasha) was described in the Ayurvedic (Kapoor, 1990) and Unani (Sajjad and Zaidi, 1984). These medical treatises describe plants as having emollient, demulcent, diuretic aperient, antipyretic and febrifuge properties. The flowers of *Viola odorata* have been used as a diaphoretic and have been given as an infusion or syrup to treat bilious affection, epilepsy, nervous disorders, prolapse of the rectum and uterus, coughs, sore throat, kidney diseases, liver disorders and inflammatory swelling (Kapoor, 1990).

Kroutil and Kroutilova (1968) reported that the *in vivo* anti-inflammatory activity of *Viola odorata* extract on rat paw oedema was comparable to extracts obtained from of *Radix ipecacuanhae* and emetine. Khattak *et al* (1985) reported the *in vivo* oral antipyretic activity in rabbits exerted by the hexane, chloroform and aqueous extracts of *Viola odorata*. The potencies of the antipyretic activity of these extracts were found to be comparable with aspirin (Khattak *et al*, 1985). The extracts were not toxic up to a dose of 1.6 g/kg. The reported antipyretic activity was more concentrated in the hexane extract of the plant; however the chemical compositions of the extracts have not been reported to date. In addition, Sajjad and Zaidi (1984) have previously described the antibiotic activity of an aqueous extract of *Viola odorata* against *Escherichia coli*, *Staphylococcus aureus*, *Streptomyces viridans*, *Bacillus proteus* and *Streptomyces hemolyticus*.

Viola odorata was reported to be efficient in the treatment of cancer according to Neil (1889). Preparations of various parts of this plant have been used to treat cancers of the uterine, throat, tongue, intestine, breast, stomach and skin (Hartwell, 1982; Table 10). Although various extracts of *Viola odorata* have been subjected to *in vitro* screening against mouse KB cells and various *in vivo* mouse tumour systems, its anticancer activity has not been fully investigated. As discussed in section 1.7.2.2 of Chapter1, the extracts of *Viola odorata*, have failed to show any significant *in vitro* growth inhibition against KB cells and mouse LE, PS, LL, SA and rat WA tumours *in vivo* in the screening programme of the US National Cancer Institute (Cragg, 1998, Screening Data Summary).

4. 2 Results and Discussion

4.2.1 *In vitro* screening of *Viola odorata* against CEM cell line

4.2.1.1 Screening of sequential extracts (aerial parts) using [^{3}H]-thymidine uptake assay

Results showing the anticancer activity of the extracts of the aerial parts of the plant *Viola odorata*, screened using the [^{3}H]-thymidine uptake assay described in Section 2.5.2.5 of Chapter 2, against the human leukaemia cell line (CEM) are illustrated in Figure 20. According to Figure 20, the dose-response curve for V3 extract increases gradually with increasing concentration of the extract. Hence it did not show the typical sigmoid dose-response relationship against the CEM cells. However, the next extract in the sequence of increasing polarity, V4, showed a modified sigmoid curve whereas the extracts, V2, V1 and VB showed a typical sigmoid dose-response relationship (Figure 20). Such a difference in the shape of the dose-response curve of V3 may be due to the slow response of the CEM cells to the crude extract rather than the differences in the cell cycle. The highest concentration of DMSO used was 100 μg/ml; which sets the upper limit of the dose-response curve for DMSO.

The organic solvent extracts V1, V2, V3, and V4 showed 50% growth inhibition potential (IC_{50}) values of 13±1, 9±3, 2±1 and 14±3 μg/ml respectively. The IC_{50} value for the aqueous extracts, VB was 99 ± μg/ml whereas V5 and V6 showed IC_{50} values > 1250 μg/ml. Hence the V3 extract showed (Figure 20) the highest 50% growth inhibitory potential of 2±1 μg/ml whereas V4 extract gave an IC50 value of 14±3 μg/ml which is approximately 10-fold higher ($p>0.05$) indicating a 10-fold lower inhibitory potential than that of V3.

The highest CEM cell growth inhibition as indicated by the V1, V2, V3 and V4 extracts were between 95-99%. In addition, the V3 extract showed a significantly higher ($p<0.05$) percent inhibition (51-83%) of *in vitro* CEM cell growth at a concentration of 1.2-78 μg/ml compared with V1, V2, V4, V5, V6 and VB extracts. Among the aqueous extracts of *Viola odorata,* VB showed the highest growth inhibition (70%) compared with the control at 1250 μg/ml whereas V5 and V6 showed lower growth inhibition of 32% and 45% respectively, even at 1250 μg/ml.

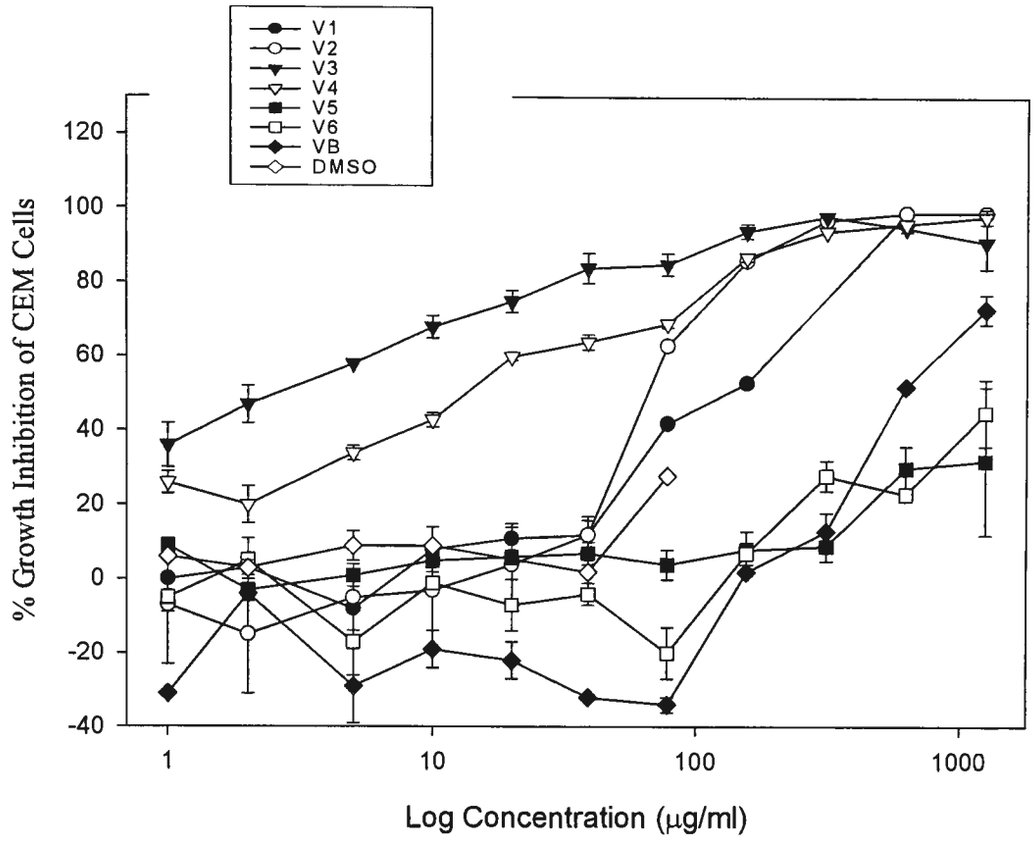


Figure 20 Percent growth inhibition of CEM leukaemia cells at different concentrations of *Viola odorata* extracts, V1-V6 and VB compared with a DMSO control as measured by the [^{3}H]-thymidine uptake assay. Each data point is represented by the mean ± S.E of four replicate determinations.

The pattern of higher CEM cell growth inhibition as shown by the organic extracts of *Euphorbia peplus* compared with the aqueous extracts, as illustrated in Figure 17 is also reflected by the extracts of *Viola odorata*. However, the highest inhibitory potential was shown by the $CHCl_3$ extract, E2 of *Euphorbia peplus*, whereas the $CHCl_3$-MeOH (1:1) extract (V3) of *Viola odorata* showed the highest inhibition potential.

4. 2.1.2 Screening of sequential extracts using MTS assay

The results obtained for the sequential extracts (aerial parts), V1, V2, V3 and V4 of *Viola odorata* using the MTS assay and the CEM leukaemia cell line are illustrated in Figure 21.

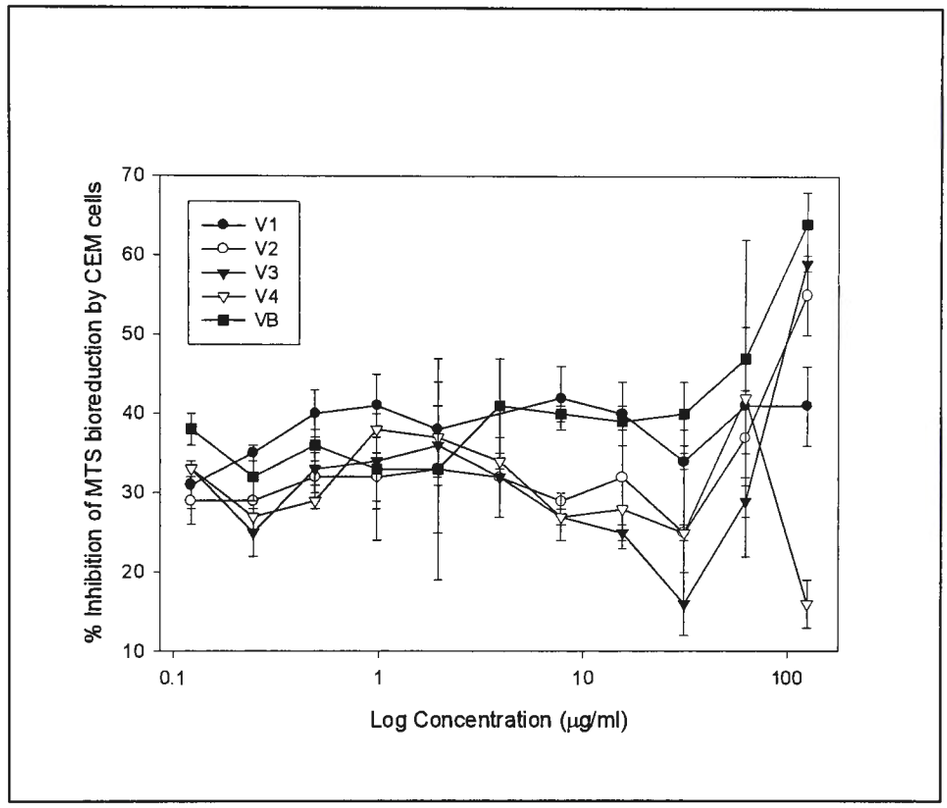


Figure 21 Percent growth inhibition of CEM leukaemia cells at different concentrations of *Viola odorata* extracts, V1, V2, V3, V4, and VB as measured by MTS assay. Each data point is represented by the mean ± S.E of two independent replicate determinations.

The extracts, V5 and V6 were not tested in this assay. Since the MTS assay measures the inhibition of cellular metabolism, the 50% inhibitory potentials of the extracts are given as IC50 values (the concentration of the extract that inhibits metabolism of 50% of the cell population), which correspond to the GI50 values described in Chapter 3. According to Figure 21, the sigmoid dose-response relationship started appearing towards the high concentration point of the curves. A concentration of 100 µg/ml indicated a low activity of the extracts on the metabolism of CEM cells as measured by the MTS assay.

The extracts V1, V2, V3, V4, V5, and VB gave IC50 values of 114±38, 99±15, 107±1, >74, and 82±16 μg/ml respectively. According to data shown in Figure 21, the significant differences among most of the extracts as determined by the [^{3}H]-thymidine assay (Figure 20) were not reflected in the MTS results.

Table 20 Comparison of CEM cell growth inhibitory potentials of *Viola odorata* extracts using [^{3}H]-thymidine uptake and MTS assays

Extract	[^{3}H]-thymidine uptake assay GI50 μg/ml	MTS assay IC_{50} μg/ml
V1	120±27	114±38
V2	112±50	99±15
V3	3±1	107±1
V4	14±3	74±0
V5	>1250	N/D
V6	>1250	N/D
VB	729±128	82±16

N/D= not done

The aqueous extract VB showed a lower IC50 value than those of the organic extracts, V1, V2, and V3. The extract, V4 gave an IC50 value close to that of VB. However, the difference between IC50 values of V4 and VB extracts was not statistically significant ($P>0.05$). The maximum inhibition of MTS reduction of 64±4% was shown by the VB extract at its tested highest concentration of 125 μg/ml. None of the tested extracts showed complete inhibition of MTS reduction at or below the highest concentration of 125 μg/ml. This may be partly due to the MTS reagent being unstable over several hours of the test.

The growth inhibitory potentials of the extracts of *Viola odorata* obtained from the [^{3}H]-thymidine uptake assay in comparison with the IC50 values obtained by using the MTS assay are given in Table 20.

According to data shown in Table 20, the highest inhibitory potential among the extracts given by V3 ($P<0.005$) using the [^{3}H]-thymidine uptake assay is not reflected in the results

of the MTS assay. Although the MTS results of V4 and VB extracts appear to have the highest inhibitory potential between 66 and 98 μg/ml, the differences between MTS results were not statistically significant (P>0.05).

4.2.2 *In vitro* screening of *Viola odorata* root extracts against CEM leukaemia system using MTS assay

In order to find out whether there is a higher anticancer activity in the roots of *Viola odorata*, sequential extracts of the roots were also tested. The results of the organic sequential extracts VRT1, VRT2, VRT3 and VRT4 and aqueous extracts, VRT5 and VRT6 of dried, ground roots of *Viola odorata* screened using the MTS assay and the CEM leukaemia cell line are given in Figure 22.

As shown in Figure 22, VRT4 extract made from the roots of *Viola odorata*, showed a typical sigmoid dose-response curve at high concentrations of the extract. Among the extracts tested, VRT4 showed the highest inhibition (P<0.05) at a 79±2% reduction of the MTS control and gave the highest (P<0.005) MTS inhibitory potential of 78±10 μg/ml. A similar dose-response relationship was observed for VRT2 but the effect was less marked compared to VRT4. However, VRT3, VRT4, and V3 showed an increase in the inhibition of MTS bioreduction as their concentrations increased beyond 62.5 μg/ml (Figure 22). There was no significant difference (P<0.05) between the MTS results of the V4 extract (IC50= 74 μg/ml) made from the *Viola odorata* aerial parts and that made from the roots, VRT4 (IC50=78 μg/ml).

The other root extracts did not show a considerable inhibition of MTS reagent in the assay. The extracts VRT1 and VRT2 did not show a dose-dependent inhibition of MTS reduction by CEM cells whereas VRT3 and VRT4 showed a dose-dependent inhibition within the concentration range used in the experiment. It appears that most of the activity of VRT1 and VRT2 results from a cytostatic effect whereas the inhibitory activities of VRT3 and VRT4 are mostly due to cytotoxicity.

The root extracts of *Viola odorata,* which did not contain dark coloured plant pigments observed in the aerial extracts, did not show unexpectedly low responses at their high concentrations as in the cases of E4 whole plant extract (illustrated in Figure 18) and V4

extract shown in Figure 21. It appears therefore, that the plant pigments at high concentrations may interfere with the OD measurement of MTS assay.

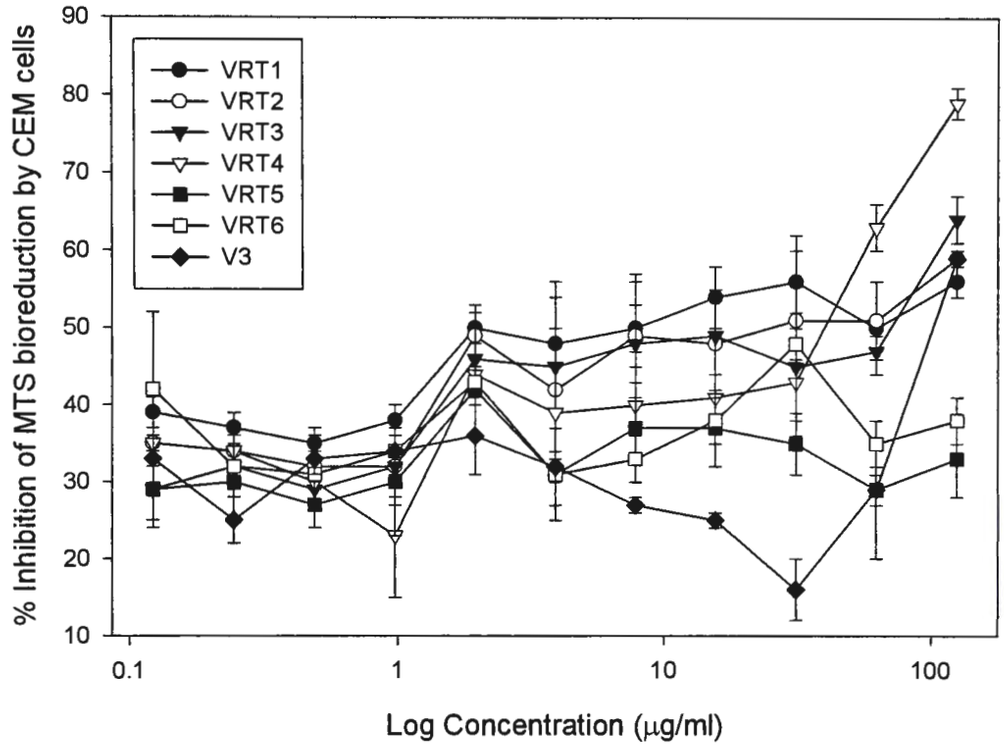


Figure 22 Percent growth inhibition of CEM leukaemia cells at different concentrations of *Viola odorata* root extracts, VRT1, VRT2, VRT3, VRT4, VRT5, and VRT6 compared with V3 as measured by MTS assay. Each data point is represented by the mean ± S.E of three independent replicate determinations.

4.2.3 *In vitro* screening of *Viola odorata* against NCI's human cancer panel

On the basis of the results obtained from the [^{3}H]-thymidine uptake assay and MTS assay, only the extracts that showed a high growth inhibition were selected for NCI's human panel screening due to limitations in accessing the screening facility. Hence the extracts V3, its MeOH soluble fraction and the sequential 70% EtOH root extract, VRT4 of *Viola odorata* were used in the NCI's *in vitro* screening program against the human panel. Dose response curves and mean graphs obtained for the three extract V3, its MeOH soluble fraction, and VRT4 extract, are given in Figures 23 and 24, 25 and 26, and 27 and 28,

respectively. The panel averages of 50% growth inhibition concentration for GI50 of V3, its MeOH soluble fraction and VRT4 extract were 49, 7 and 26 μg/ml, respectively (Figures 23, 26 and 28). The growth inhibitory potential of V3 extract was found to be approximately half that of the VRT4 extract. The MeOH soluble fraction of V3 showed the highest average GI50 (50% growth inhibition potential).

According to data shown in Figure 24, there was a weak growth inhibition for the extract V3 (IG50= 28 μg/ml) against the RPMI-8226 leukaemia cell line and TK-10 renal cancer cell line (IG50= 42 μg/ml). None of the other cell lines showed a GI50 (50% growth inhibition) below the highest tested concentration for the V3 extract at a dose of 50 μg/ml. Hence V3 was rated as "inactive" in the NCI's human panel.

Although the V3 extract (Figures 23 and 24) did not show considerable growth inhibition using the *in vitro* 60 human cell panel, the root extract of VRT4 did show (Figure 27 and 28) growth inhibition of 100% against a number of cell lines such as, the RPMI-8226 (TGI=37 μg/ml) leukaemia cells, EKVX (47 μg/ml), HOP-62 (26 μg/ml), NCI-H460 (30 μg/ml) cells of non-small cell lung cancer, U251 cell line (20 μg/ml) of CNS cancer, MALME-3M (30 μg/ml), UACC-257 (33 μg/ml) of melanoma subpanel, TK-10 (10 μg/ml) renal cancer cell line and DU-145 (28 μg/ml) prostate cancer cell line. The TGI mean graph of VRT4 (Figure 28) showed crossbars extending to the left from the vertical mean line for the above 9 cell lines. However, none of the adherent solid colon tumour cell lines showed 50% growth inhibition (GI50) to VRT4 within the concentration range, 0.01-50 μg/ml.

The lowest GI50 value of 28 μg/ml for the V3 extract was given by RPMI-8226 leukaemia cell line whereas TK-10 renal cancer cell line gave a GI50 of 42 μg/ml (Figure 24). However, TK-10 cells were very sensitive to both the MeOH soluble fraction (TGI=4 μg/ml) of the V3 extract and the VRT4 (TGI= 10 μg/ml) extract (Figures 26 and 28).

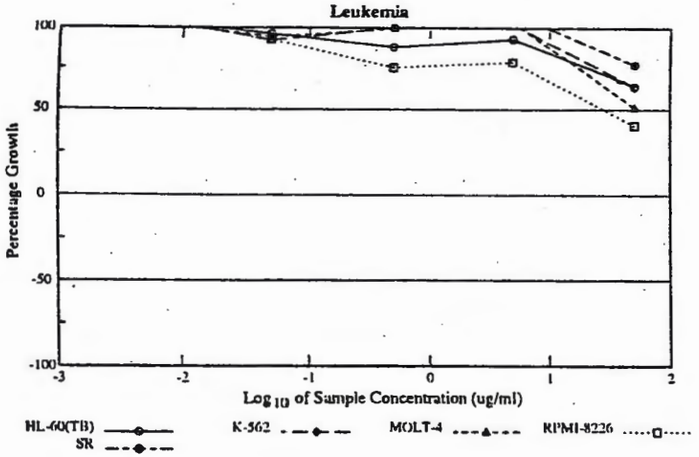
Although the LC50 mean graph for V3 extract did not show any horizontal bars projecting to the left of the mean (Figure 24), the LC50 mean graphs for the MeOH soluble fraction, the V3 extract and the VRT4 extract (Figure 26 and 28) indicated bars projecting to the left of the average showing a cell kill effect for some cell lines.

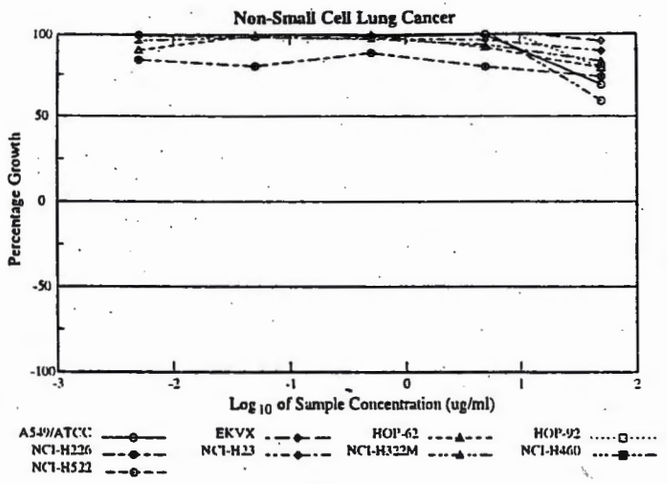
The GI50 mean graph of VRT4 (Figure 28) obtained using the TK-10 (GI50=2 μg/ml) cell line showed a bar projecting 1 unit to the left. This indicates that the GI50 for the TK-10 cell line occurred at a concentration of 10 times less than the average concentration required for all cell lines used in the panel. The LC50 panel averages of 34 and 50μg/ml for MeOH fraction and the VRT4 extract respectively indicate that these extracts have a weak cell killing or cytotoxic potency (Cragg and Newman, 1999). The 50% growth inhibition for the VRT4 extract using TK-10 cells occurred at a concentration of 2 μg/ml while the 50% cell killing (LC50) effect was exerted at 42.7 μg/ml. However, since the concentrations were relatively higher than the established NCI criterion (GI50=0.1 μg/ml), the VRT4 extract was rated as having a weak but non-subpanel selective growth inhibition in the NCI *in vitro* screen and was not forwarded for further investigation (Cragg and Newman, 1999).

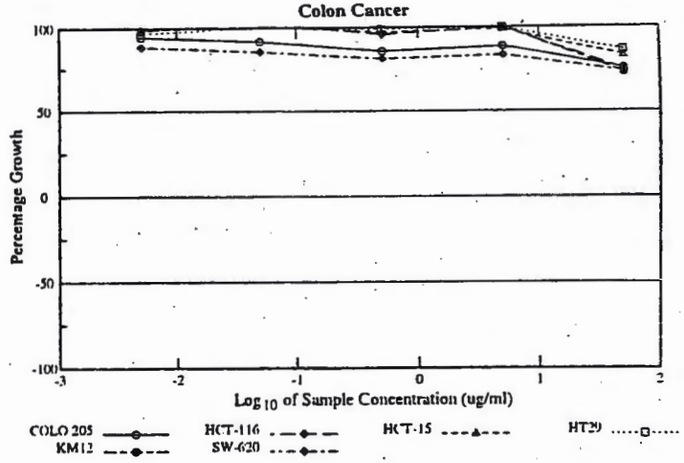
Although the V3 extract did not show a strong growth inhibition, the MeOH-soluble fraction obtained by simple solvent partitioning gave complete growth inhibition, with the TGI value occurring within the concentration range used in the test of 0.005-50 μg/ml. The MeOH-soluble fraction exerted a TGI value of 7 and 6 μg/ml respectively against TK-10 and RPMI-8226 cell lines. Hence it appears that the growth inhibitory activity of V3 is higher in the MeOH-insoluble fraction. No results of the effect of the V3 extract against the CEM cell line were available from NCI because of limitations of the service. When the NCI's sulfarhodamine B assay was used as the test method, the V3 extract did not show inhibitory activity against any of the cell lines of the human tumour panel.

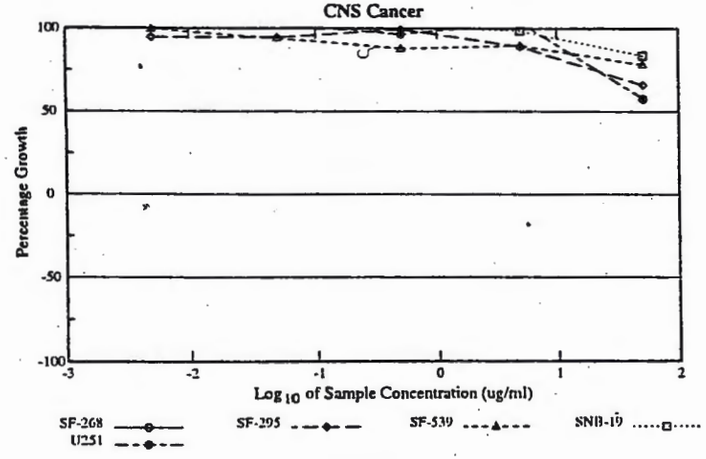
The GI50 values against the different leukaemia cell lines were >50 μg/ml whereas the RPMI-8226 and TK-10 cell lines gave values of 28 and 42 μg/ml, respectively. However, the V3 extract, when assayed using the [^{3}H]-thymidine assay using the CEM leukaemia cell line, showed a 50% growth inhibition at a concentration of 3±1 μg/ml. Hence the [^{3}H]-thymidine assay, using *in vitro* CEM leukaemia cell line, showed a sensitivity, which was 10-fold higher ($p<0.05$) than that found by the NCI's sulforhodamine B assay using the RPMI-8226 human leukaemia cell line and the TK-10 renal cancer cell line for the V3 extract.

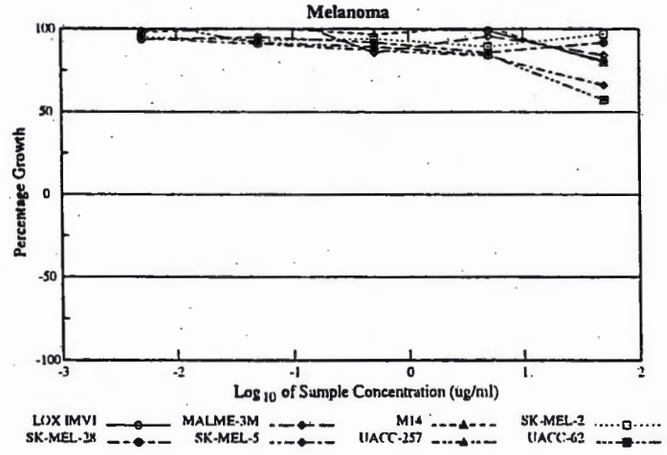
Figure 23 Dose response curves of V3 extract of *Viola odorata* against the human cancer panel using sulphorhodamine B assay. These data were provided by the Development Therapeutics Programme of the NCI (USA).

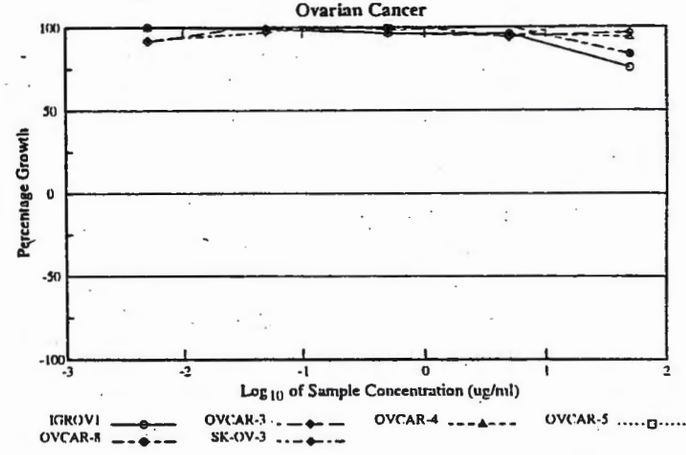

Leukemia
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
HL-60(TB)
K-562
MOLT-4
RPMI-8226
SR

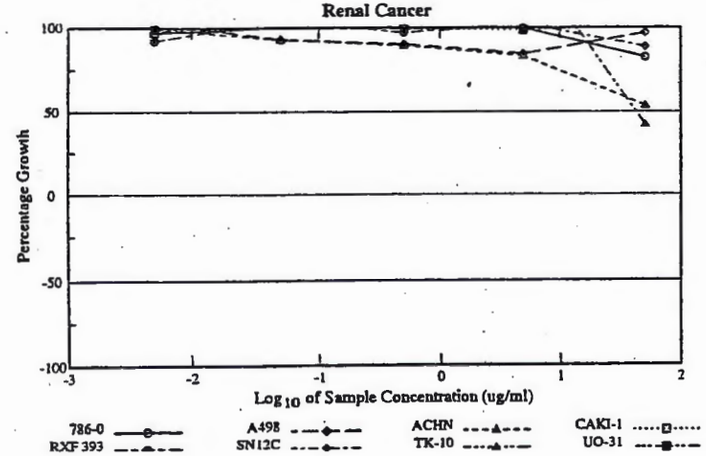

Non-Small Cell Lung Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
A549/ATCC
EKVX
HOP-62
HOP-92
NCI-H226
NCI-H23
NCI-H322M
NCI-H460
NCI-H522

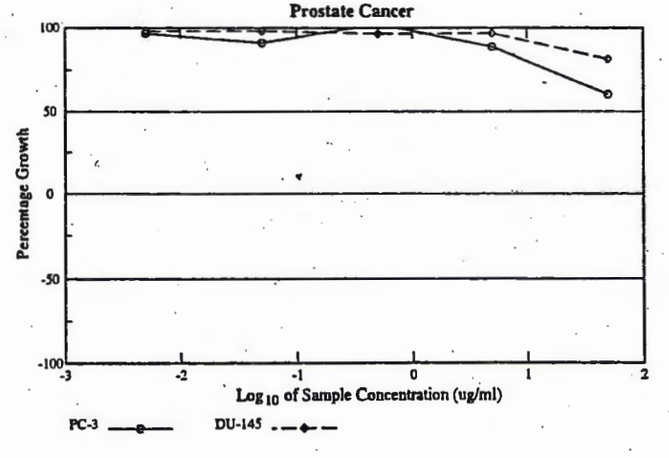

Colon Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
COLO 205
HCC-116
HCT-15
HT29
KM12
SW-620


CNS Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
SF-268
SF-295
SF-539
SNB-19
U251


Melanoma
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
LOX IMVI
MALME-3M
M14
SK-MEL-2
SK-MEL-28
SK-MEL-5
UACC-257
UACC-62


Ovarian Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
IGROV1
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
SK-OV-3


Renal Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
786-0
A498
ACHN
CAKI-1
RXF 393
SN12C
TK-10
UO-31


Prostate Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
PC-3
DU-145

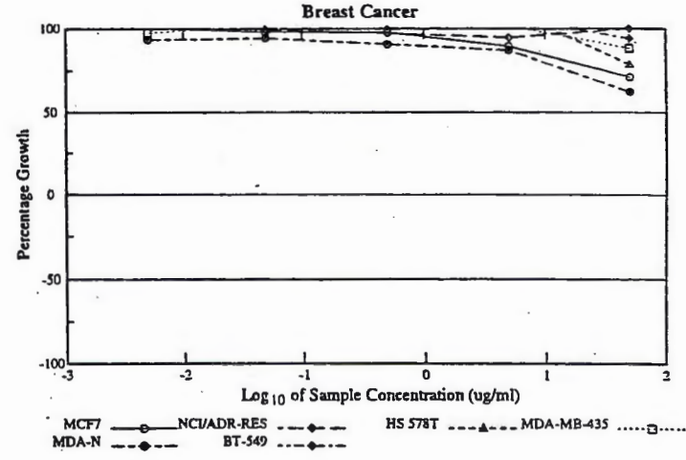
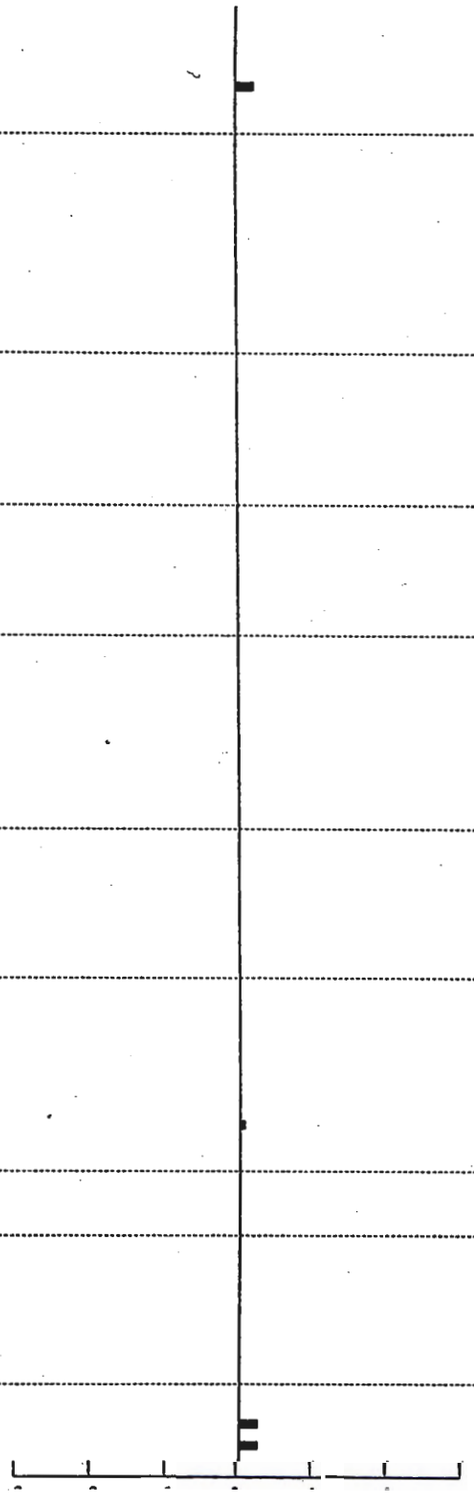
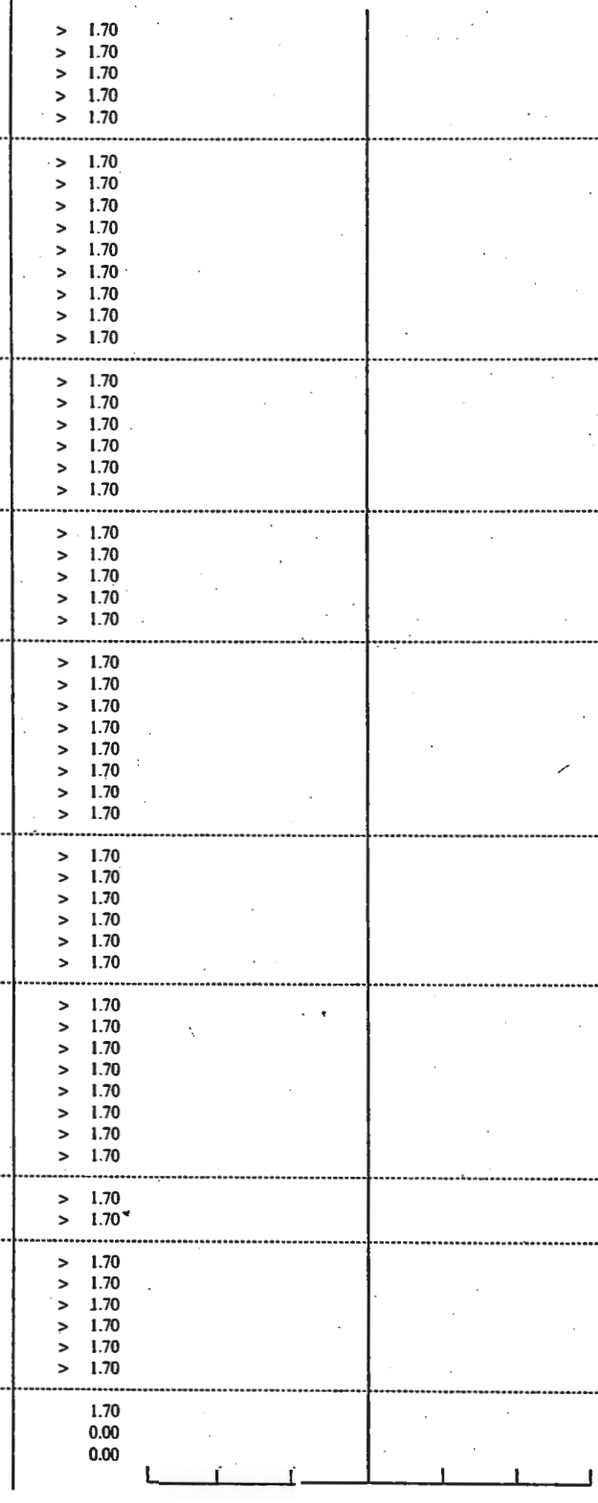

Breast Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
MCF7
NCI/ADR-RES
HS 578T
MDA-MB-435
MDA-N
BT-549

Figure 24 GI50, TGI, and LC50 Mean graphs of V3 extract of *Viola odorata* derived from the dose-response data of Figure 22. The $\log_{10}$ GI50, $\log_{10}$ TGI and $\log_{10}$ LC_{50} values respectively are given for each cell lines. These data were provided by the Development Therapeutics Programme of the NCI (USA).

Viola odorata

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
HL-60(TB)	> 1.70	> 1.70	> 1.70
K-562	> 1.70	> 1.70	> 1.70
MOLT-4	> 1.70	> 1.70	> 1.70
RPMI-8226	1.45	> 1.70	> 1.70
SR	> 1.70	> 1.70	> 1.70
Non-Small Cell Lung Cancer			
A549/ATCC	> 1.70	> 1.70	> 1.70
EKVX	> 1.70	> 1.70	> 1.70
HOP-62	> 1.70	> 1.70	> 1.70
HOP-92	> 1.70	> 1.70	> 1.70
NCI-H226	> 1.70	> 1.70	> 1.70
NCI-H23	> 1.70	> 1.70	> 1.70
NCI-H322M	> 1.70	> 1.70	> 1.70
NCI-H460	> 1.70	> 1.70	> 1.70
NCI-H522	> 1.70	> 1.70	> 1.70
Colon Cancer			
COLO 205	> 1.70	> 1.70	> 1.70
HCT-116	> 1.70	> 1.70	> 1.70
HCT-15	> 1.70	> 1.70	> 1.70
HT29	> 1.70	> 1.70	> 1.70
KM12	> 1.70	> 1.70	> 1.70
SW-620	> 1.70	> 1.70	> 1.70
CNS Cancer			
SF-268	> 1.70	> 1.70	> 1.70
SF-295	> 1.70	> 1.70	> 1.70
SF-539	> 1.70	> 1.70	> 1.70
SNB-19	> 1.70	> 1.70	> 1.70
U251	> 1.70	> 1.70	> 1.70
Melanoma			
LOX IMVI	> 1.70	> 1.70	> 1.70
MALME-3M	> 1.70	> 1.70	> 1.70
M14	> 1.70	> 1.70	> 1.70
SK-MEL-2	> 1.70	> 1.70	> 1.70
SK-MEL-28	> 1.70	> 1.70	> 1.70
SK-MEL-5	> 1.70	> 1.70	> 1.70
UACC-257	> 1.70	> 1.70	> 1.70
UACC-62	> 1.70	> 1.70	> 1.70
Ovarian Cancer			
IGROV1	> 1.70	> 1.70	> 1.70
OVCAR-3	> 1.70	> 1.70	> 1.70
OVCAR-4	> 1.70	> 1.70	> 1.70
OVCAR-5	> 1.70	> 1.70	> 1.70
OVCAR-8	> 1.70	> 1.70	> 1.70
SK-OV-3	> 1.70	> 1.70	> 1.70
Renal Cancer			
786-0	> 1.70	> 1.70	> 1.70
A498	> 1.70	> 1.70	> 1.70
ACHN	> 1.70	> 1.70	> 1.70
CAKI-1	> 1.70	> 1.70	> 1.70
RXF 393	> 1.70	> 1.70	> 1.70
SN12C	> 1.70	> 1.70	> 1.70
TK-10	1.62	> 1.70	> 1.70
UO-31	> 1.70	> 1.70	> 1.70
Prostate Cancer			
PC-3	> 1.70	> 1.70	> 1.70
DU-145	> 1.70	> 1.70	> 1.70
Breast Cancer			
MCF7	> 1.70	> 1.70	> 1.70
NCI/ADR-RES	> 1.70	> 1.70	> 1.70
HS 578T	> 1.70	> 1.70	> 1.70
MDA-MB-435	> 1.70	> 1.70	> 1.70
MDA-N	> 1.70	> 1.70	> 1.70
BT-549	> 1.70	> 1.70	> 1.70
MG_MID	1.69	1.70	1.70
Delta	0.25	0.00	0.00
Range	0.25	0.00	0.00





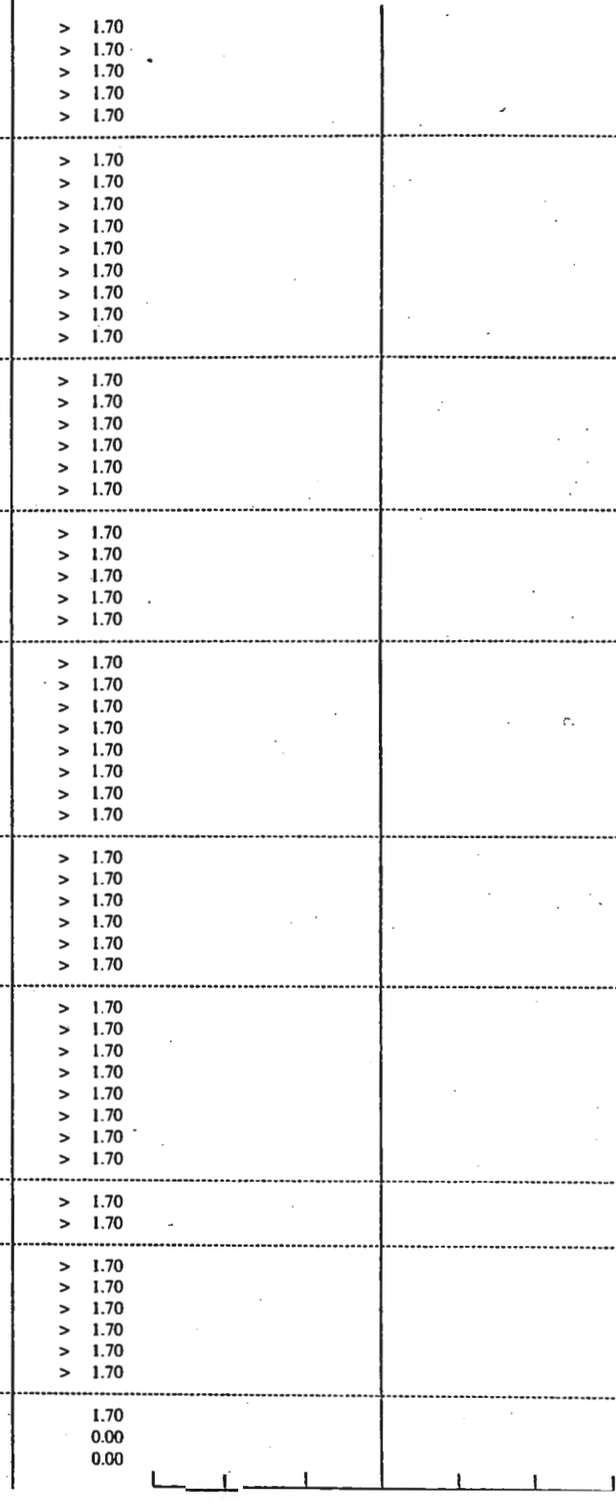
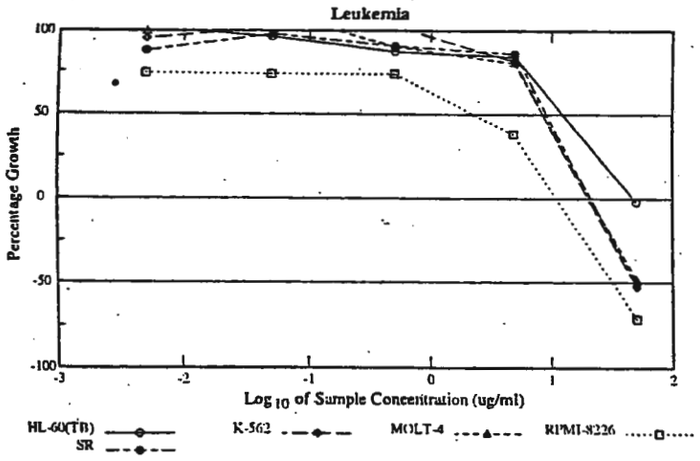
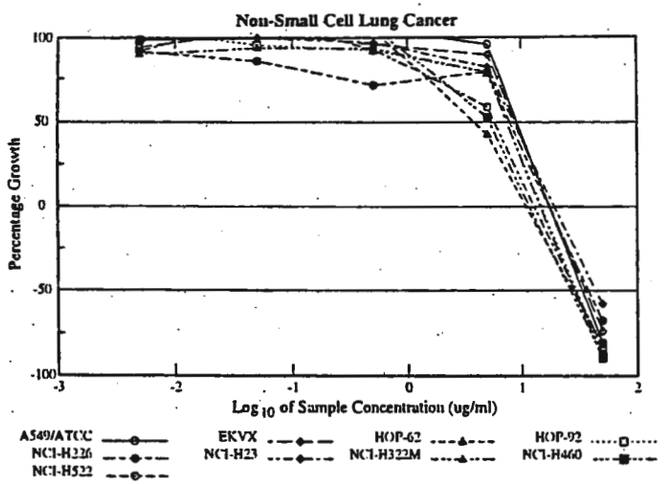
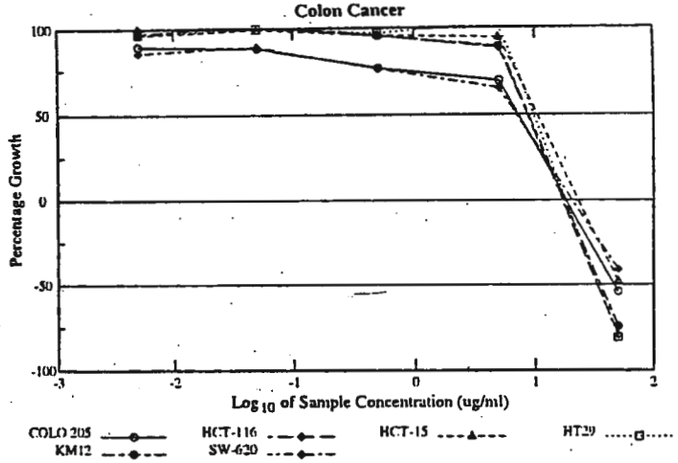
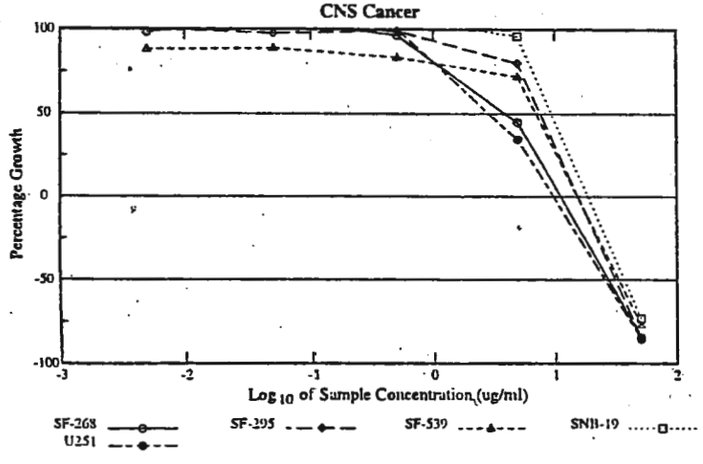


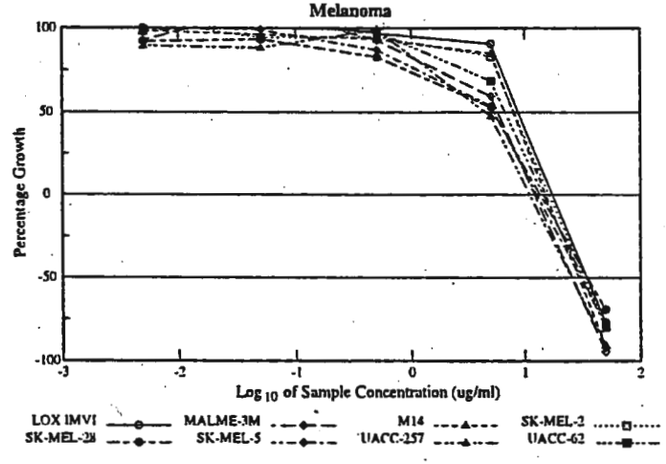
Figure 25 Dose response curves of MeOH-soluble fraction of V3 extract of *Viola odorata* against the human cancer panel using sulphorhodamine B assay. These data were provided by the Development Therapeutics Programme of the NCI (USA).

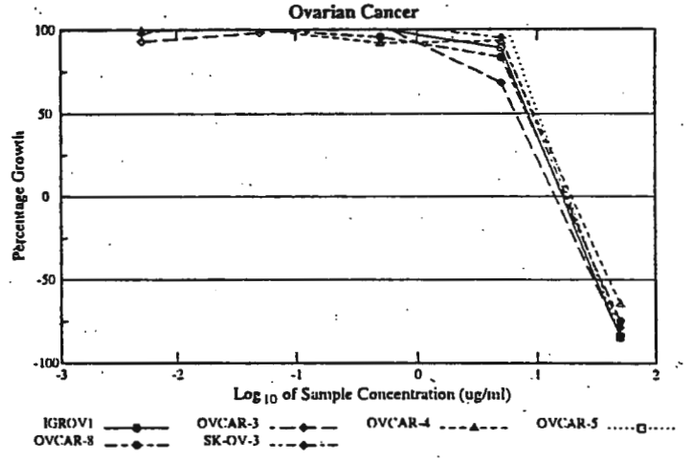

Leukemia
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
HL-60(TB)
K-562
MOLT-4
RPMI-8226
SR

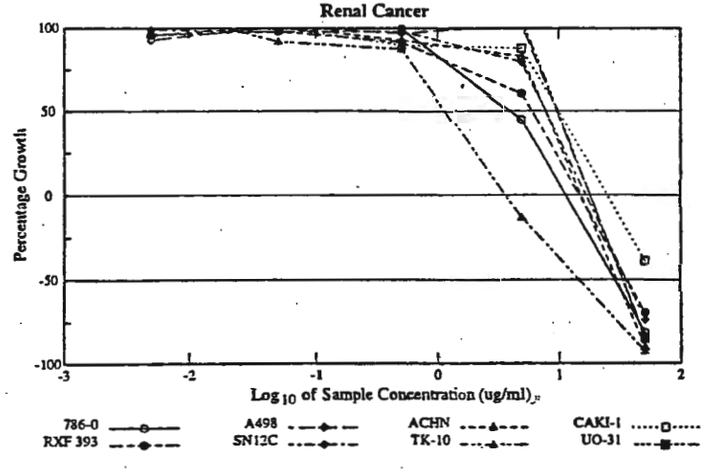

Non-Small Cell Lung Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
A549/ATCC
EKVX
HOP-62
HOP-92
NCI-H226
NCI-H23
NCI-H322M
NCI-H460
NCI-H522

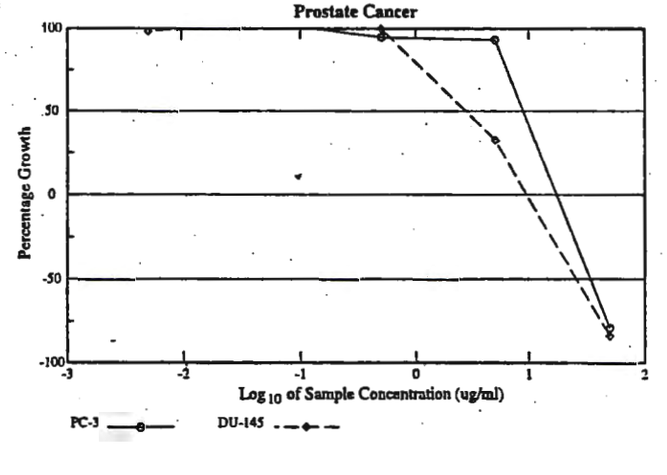

Colon Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
COLO 205
HCC-116
HCT-15
HT29
KM12
SW-620


CNS Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
SF-268
SF-295
SF-539
SNB-19
U251


Melanoma
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
LOX IMVI
MALME-3M
M14
SK-MEL-2
SK-MEL-28
SK-MEL-5
UACC-257
UACC-62


Ovarian Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
IGROV1
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
SK-OV-3


Renal Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
786-0
A498
ACHN
CAKI-1
RXF 393
SN12C
TK-10
UO-31


Prostate Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
PC-3
DU-145

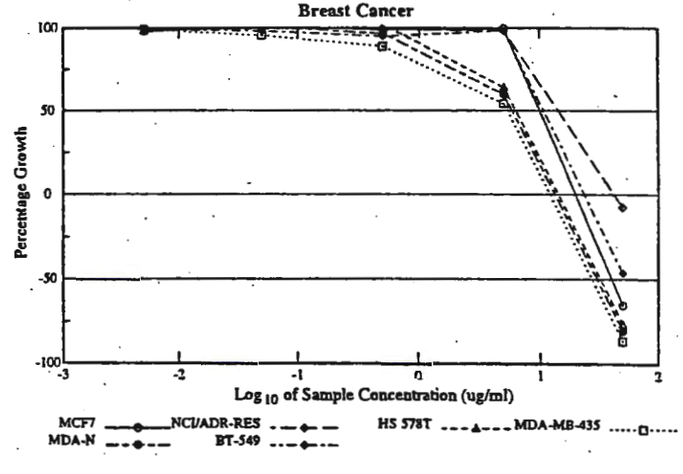

Breast Cancer
Percentage Growth
Log 10 of Sample Concentration (ug/ml)
MCF7
NCI/ADR-RES
HS 578T
MDA-MB-435
MDA-N
BT-549

Figure 26 GI50, TGI, and LC50 Mean graphs of MeOH-soluble fraction of V3 extract of *Viola odorata* derived from the dose-response data of Figure 24. The $\log_{10}$ GI50, $\log_{10}$ TGI and $\log_{10}$ LC_{50} values respectively are given for each cell lines. These data were provided by the Development Therapeutics Programme of the NCI (USA).

Mean Graphs

[illegible]
0H600007-T
Viola odorata

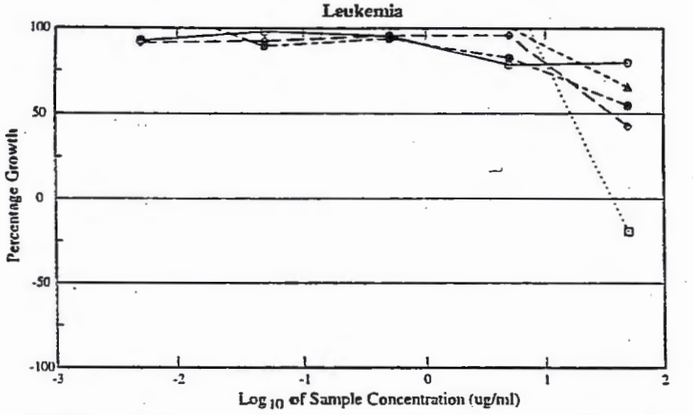
Report Date: October 28, 1999 | **Test Date:** October 11, 1999

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
HL-60(TB)	1.09	1.68	> 1.70
K-562	0.93	1.30	1.68
MOLT-4	0.93	1.32	> 1.70
RPMI-8226	0.36	1.05	1.50
SR	0.96	1.33	1.69
Non-Small Cell Lung Cancer			
A549/ATCC	0.96	1.24	1.53
EKVX	0.94	1.25	1.55
HOP-62	0.58	1.04	1.44
HOP-92	0.87	1.17	1.47
NCI-H226	0.90	1.24	1.58
NCI-H23	0.93	1.29	1.64
NCI-H322M	0.87	1.16	1.46
NCI-H460	0.72	1.07	1.42
NCI-H522	0.76	1.11	1.46
Colon Cancer			
COLO 205	0.85	1.26	1.66
HCT-116	0.93	1.23	1.52
HCT-15	1.01	1.36	> 1.70
HT29	0.98	1.26	1.53
KM12	0.94	1.25	1.55
SW-620	0.84	1.31	> 1.70
CNS Cancer			
SF-268	0.60	1.05	1.43
SF-295	0.88	1.18	1.49
SF-539	0.85	1.18	1.52
SNB-19	0.97	1.27	1.56
U251	0.46	0.99	1.40
Melanoma			
LOX IMVI	0.94	1.24	1.54
MALME-3M	0.76	1.09	1.41
M14	0.90	1.18	1.47
SK-MEL-2	0.90	1.21	1.52
SK-MEL-28	0.72	1.13	1.54
SK-MEL-5	0.73	1.10	1.48
UACC-257	0.65	1.04	1.40
UACC-62	0.82	1.16	1.50
Ovarian Cancer			
IGROV1	0.92	1.21	1.50
OVCAR-3	0.82	1.15	1.48
OVCAR-4	0.97	1.29	1.61
OVCAR-5	1.01	1.27	1.52
OVCAR-8	0.91	1.23	1.54
SK-OV-3	0.96	1.25	1.53
Renal Cancer			
786-0	0.60	1.05	1.45
A498	0.98	1.25	1.52
ACHN	0.89	1.18	1.47
CAKI-1	1.00	1.39	> 1.70
RXF 393	0.78	1.16	1.55
SN12C	0.89	1.22	1.54
TK-10	0.07	0.57	1.16
UO-31	0.99	1.25	1.51
Prostate Cancer			
PC-3	0.95	1.24	1.53
DU-145	0.44	0.98	1.41
Breast Cancer			
MCF7	1.00	1.30	1.61
NCI/ADR-RES	1.16	1.63	> 1.70
HS 578T	0.80	1.16	1.51
MDA-MB-435	0.73	1.08	1.44
MDA-N	0.77	1.13	1.48
BT-549	1.03	1.38	> 1.70
MG_MID	0.84	1.20	1.53
Delta	0.77	0.63	0.37
Range	1.09	1.11	0.54

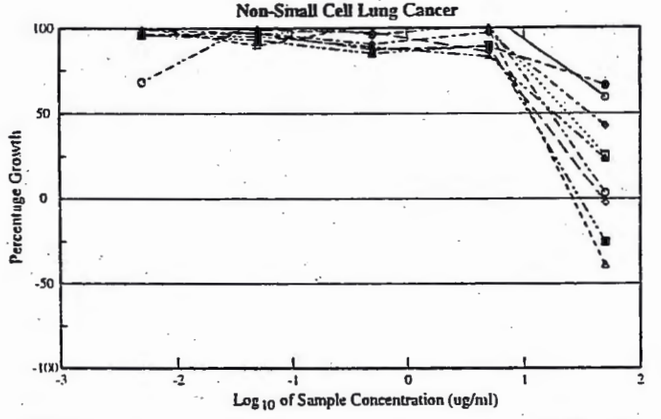
GI50, TGI, LC50 bar-graph axes: +3 +2 +1 0 -1 -2 -3

Figure 27 Dose response curves of VRT4 extract of *Viola odorata* against the human cancer panel using sulphorhodamine B assay. These data were provided by the Development Therapeutics Programme of the NCI (USA).

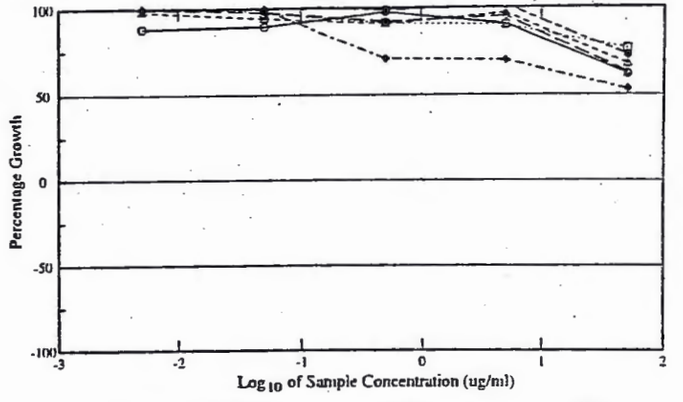
Leukemia



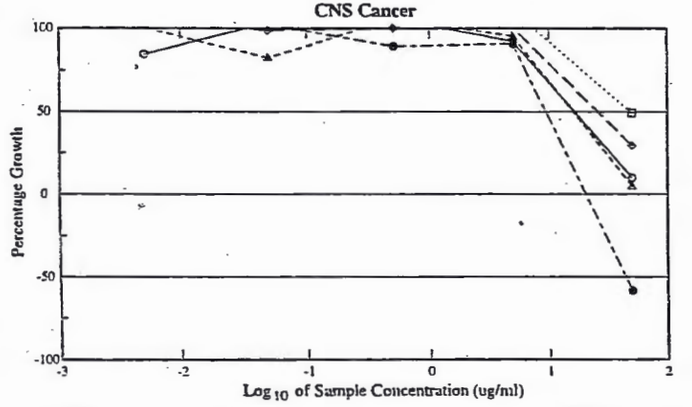
Non-Small Cell Lung Cancer



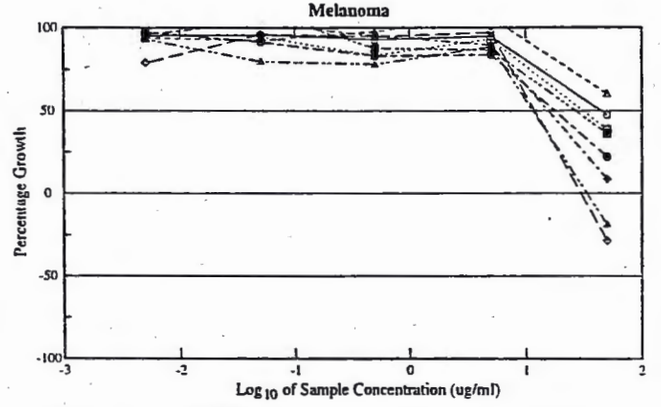
Colon Cancer



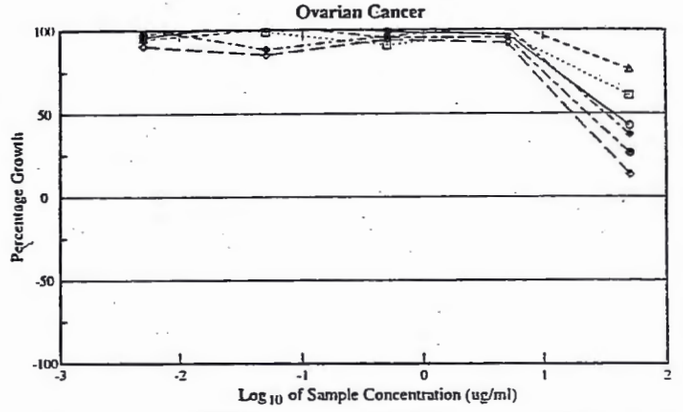
CNS Cancer



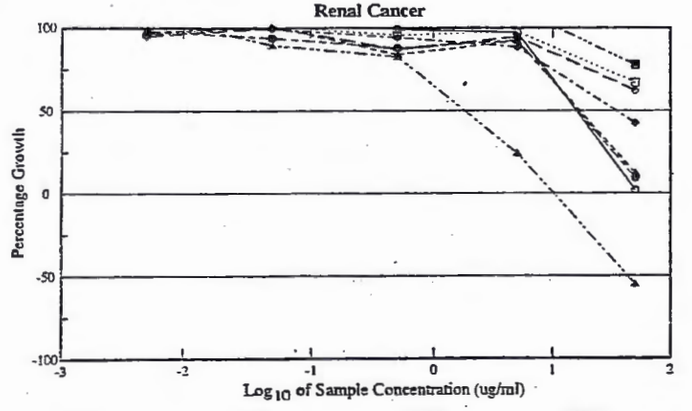
Melanoma



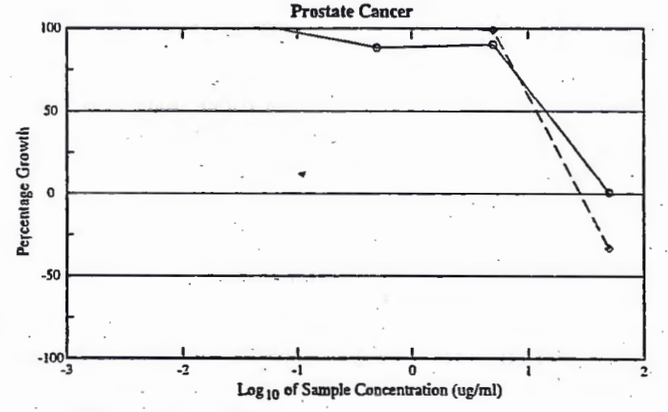
Ovarian Cancer



Renal Cancer



Prostate Cancer



Breast Cancer

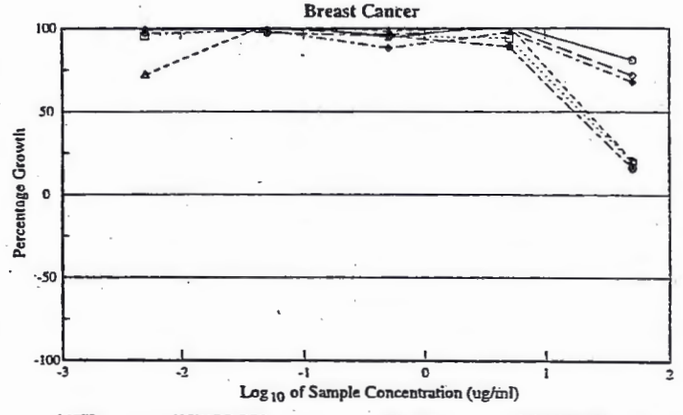


Figure 28 GI50, TGI, and LC50 Mean graphs of VRT4 extract of *Viola odorata* derived from the dose-response data of Figure 26. The $\log_{10}$ GI50, $\log_{10}$ TGI and $\log_{10}$ LC_{50} values respectively are given for each cell lines. These data were provided by the Development Therapeutics Programme of the NCI (USA).

Viola odorata

Panel/Cell Line	Log_{10} G150	Log_{10} TGI	Log_{10} LC50
Leukemia			
HL-60(TB)	> 1.70	> 1.70	> 1.70
K-562	1.56	> 1.70	> 1.70
MOLT-4	> 1.70	> 1.70	> 1.70
RPMI-8226	1.21	1.56	> 1.70
SR	> 1.70	> 1.70	> 1.70
Non-Small Cell Lung Cancer			
A549/ATCC	> 1.70	> 1.70	> 1.70
EKVX	1.11	1.67	> 1.70
HOP-62	1.06	1.42	> 1.70
HOP-92	1.37	> 1.70	> 1.70
NCI-H226	> 1.70	> 1.70	> 1.70
NCI-H23	1.56	> 1.70	> 1.70
NCI-H322M	1.26	> 1.70	> 1.70
NCI-H460	1.05	1.48	> 1.70
NCI-H522	1.23	> 1.70	> 1.70
Colon Cancer			
COLO 205	> 1.70	> 1.70	> 1.70
HCT-116	> 1.70	> 1.70	> 1.70
HCT-15	> 1.70	> 1.70	> 1.70
HT29	> 1.70	> 1.70	> 1.70
KM12	> 1.70	> 1.70	> 1.70
SW-620	> 1.70	> 1.70	> 1.70
CNS Cancer			
SF-268	1.21	> 1.70	> 1.70
SF-295	1.41	> 1.70	> 1.70
SF-539	1.20	> 1.70	> 1.70
SNB-19	1.69	> 1.70	> 1.70
U251	0.97	1.31	1.64
Melanoma			
LOX IMVI	1.65	> 1.70	> 1.70
MALME-3M	1.08	1.47	> 1.70
M14	> 1.70	> 1.70	> 1.70
SK-MEL-2	1.49	> 1.70	> 1.70
SK-MEL-28	1.25	> 1.70	> 1.70
SK-MEL-5	1.19	> 1.70	> 1.70
UACC-257	1.06	1.52	> 1.70
UACC-62	1.43	> 1.70	> 1.70
Ovarian Cancer			
IGROV1	1.57	> 1.70	> 1.70
OVCAR-3	1.23	> 1.70	> 1.70
OVCAR-4	> 1.70	> 1.70	> 1.70
OVCAR-5	> 1.70	> 1.70	> 1.70
OVCAR-8	1.35	> 1.70	> 1.70
SK-OV-3	1.50	> 1.70	> 1.70
Renal Cancer			
786-0	1.20	> 1.70	> 1.70
A498	> 1.70	> 1.70	> 1.70
ACHN	1.22	> 1.70	> 1.70
CAKI-1	> 1.70	> 1.70	> 1.70
RXF 393	1.21	> 1.70	> 1.70
SN12C	1.53	> 1.70	> 1.70
TK-10	0.26	1.00	1.63
UO-31	> 1.70	> 1.70	> 1.70
Prostate Cancer			
PC-3	1.15	> 1.70	> 1.70
DU-145	1.07	1.45	> 1.70
Breast Cancer			
MCF7	> 1.70	> 1.70	> 1.70
NCI/ADR-RES	> 1.70	> 1.70	> 1.70
HS 578T	1.33	> 1.70	> 1.70
MDA-MB-435	1.29	> 1.70	> 1.70
MDA-N	1.23	> 1.70	> 1.70
BT-549	> 1.70	> 1.70	> 1.70
MG_MID	1.42	1.66	1.70
Delta	1.17	0.65	0.06
Range	1.44	0.69	0.07

G150 / TGI / LC50 scale: +3 +2 +1 0 -1 -2 -3

Although the bioassay-guided fractionation and dereplication approach led to the discovery of cucurbitacins from the plant *Gonystylus keithii* (Fuller *et al*, 1994), the cytotoxicities shown by the V3 extracts in this study were not high enough to warrant further investigation.

4.2.4 *In vitro* screening of the HPLC fractions of the V3 extracts

The results of the NCI's screen showed that even though the sequential crude extract, V3 did not show growth inhibition or cell killing properties, the MeOH soluble fraction did indicate a 10-fold increase in growth inhibition.

In order to provide the first step in the isolation of the active substance, the HPLC fractions of the V3 extract, were screened for *in vitro* CEM cell growth inhibitory activity, using the [^{3}H]-thymidine uptake and MTS assays. In addition, adherent solid LOVO colon cancer cells were also used in the experiment.

The results showing UV traces of the V3 extract using and a Photodiode Array Detector as described in section 2.4.3 of Chapter 2, are illustrated in Figure 29.

4.2.4.1 *In vitro* screening of the HPLC fractions against CEM cells

Due to limitations of the NCI's human panel screening, the HPLC fractions of V3 obtained according to the method described in Section 2.9 of Chapter 2, were investigated for anticancer activity using both the [^{3}H]-thymidine uptake and the MTS assays.

4.2.4.1.1 ^{3}H-thymidine uptake assay

The results obtained for the HPLC fractionation of the V3 extract of *Viola odorata* against CEM leukaemia cell line using the [^{3}H]-thymidine assay are illustrated in Figure 30.

According to the data shown in Figure 30, the HPLC fractions V3F1, V3F2, V3F3, V3F4, V3F5, V3F6, V3F7 and V3 F8 gave IC50 values of 57±15, 38±5, 90±32, 48±1, 2±1, 2±1, 19±4 and 24±1 μg/ml, respectively. Fractions, V3F5 and V3F6 showed the highest ($P<0.05$) growth inhibitory potential against the CEM cells. There was no

Figure 29 High-performance liquid chromatogram of the V3 extract of *Viola odorata* indicating the HPLC fractions, V3F1, V3F2, V3F3, F3F4, V3F5, V3F6, V3F7 and V3F8. Vydac, C_{18} column (250 x 46 mm i.d.). Eluent: 0.1% trifluoroacetic acid in dearated Milli Q water and acetonitrile; gradient 100-0% in 60 min at a flow rate of 0.5 ml/min. The UV spectrum is at 254 nm.

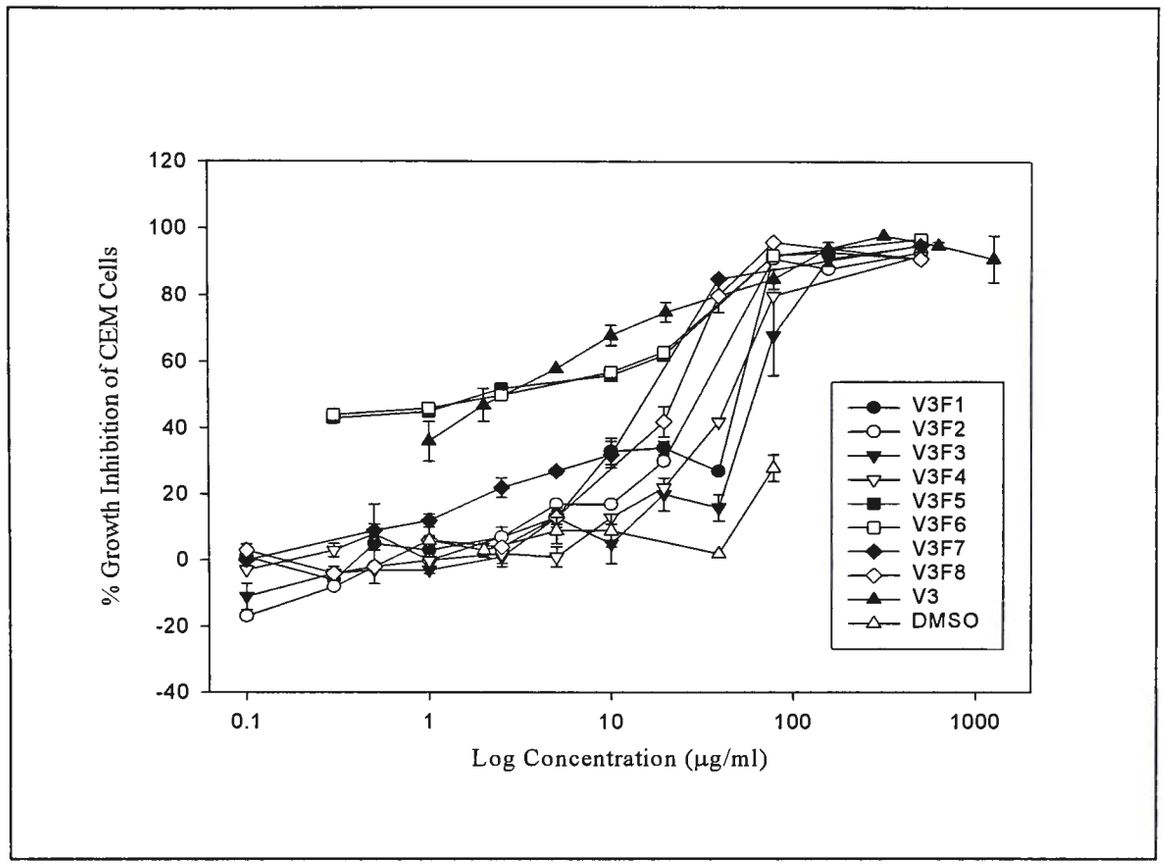


Figure 30 Percent growth inhibition of CEM leukaemia cells by the HPLC fractions of V3 extract of *Viola odorata* compared with V3 and DMSO as measured using the [^{3}H]-thymidine uptake assay. Each data point is represented by the mean ± S.E of three independent replicate determinations.

significant difference (P>0.05) between the IC50 values of the V3F5 (1.8±0.6 µg/ml) and V3F6 (1.8±0.7 µg/ml) fractions. All the other fractions of V3 extract, except for V3F5 and V3F6, showed inhibitory potentials lower than that of V3 (3±1 µg/ml). The V3 extract, as well as all of the other fractions, showed almost complete growth inhibition at the highest concentration of these fractions (Figure 30).

Although it was difficult to determine the exact quantity of the original extract in the fractions used for the experiment, the IC50 values of both fractions were significantly

higher than that of V3 extract (IC50=3±1) showing an efficient fractionation of the V3 extract using the semi-preparative Vydac (218TP54) C_{18} HPLC column.

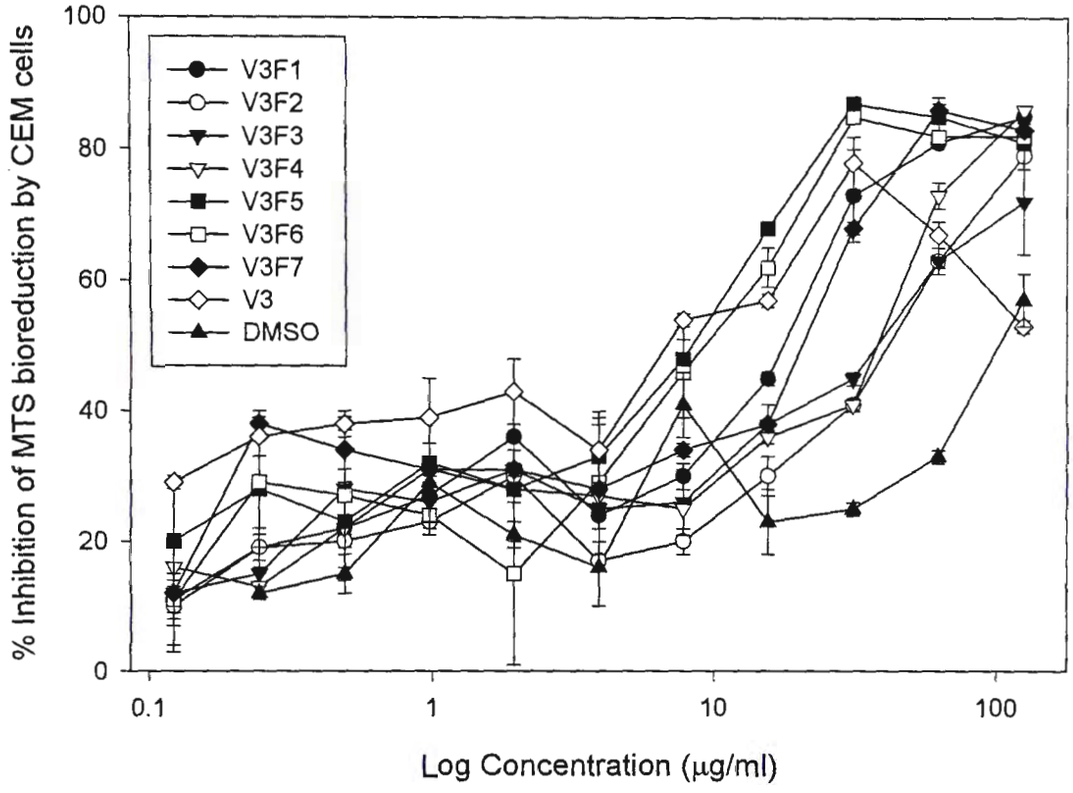


Figure 31 Percent growth inhibition of CEM leukaemia cells by the HPLC fractions of V3 extracts of *Viola odorata* compared with V3 and DMSO as measured using the MTS assay. Each data point is represented by the mean ± S.E of three independent replicate determinations.

The inhibition of MTS bioreduction, for each of the above fractions, was assayed against CEM cells using MTS assay. These the results were compared with those obtained using [^{3}H]-thymidine uptake assay.

4.2.4.1.2.1 MTS assay

The results of growth inhibition of the CEM leukaemia cell line, obtained for the HPLC fractions V3F1, V3F2, V3F3, V3F4, V3F5, V3F6, and V3F7 of the V3 extract of *Viola odorata* using the MTS assay are illustrated in Figure 31.

The V3F1, V3F2, V3F3, V3F4, V3F5, V3F6, and V3F7 fractions gave IC50 values of 19±2, 44±6, 42±7, 40±2, 10±2, 11±2, and 22±1 μg/ml, respectively. It would seem, on the basis of these results, that the V3F5 and V3F6 fractions had the highest ($P<0.05$) CEM cell growth inhibitory potentials (10±2, 11.5±2 μg/ml, respectively) using the MTS reduction assay. There was no significant difference ($P>0.05$) between the IC50 values of the fractions V3F5 and V3F6. However, the IC50 values of these two fractions were lower ($P<0.05$) than that of V3 extract (107±1 μg/ml).

According to the data shown in Figure 31, a dose dependent relationship was shown using the MTS assay for the HPLC fractions of the V3 extract. The MTS results also provided the highest activity ($P<0.05$) for the V3F5 and V3F6 fractions as indicated by the [^{3}H]-thymidine uptake assay. However, at higher concentrations the V3 extract did not show a dose-dependent relationship (Figure 31). This may have been due to interference of plant pigments with OD measurement of MTS assay.

As shown in Figure 31, the inhibitory potential of DMSO (LC50>100 μg/ml) was lower ($P<0.05$) than that of all the fractions tested. These results indicate that DMSO does not appear to affect the inhibitory activity of the fractions below a concentration of 100 μg/ml.

The MTS results of the active extracts V3, VRT4 and HPLC fractions were also compared with the results obtained using the [^{3}H]-thymidine uptake assay against the LOVO colon cancer cell line. These results are discussed below.

The CEM leukaemia cell line has been used as a soft form of cancer whereas colon cancer, such as LOVO has been used as the adherent form of solid or hard cancers. The LOVO cells are usually resistant to many anticancer drugs. Therefore, in this investigation, the LOVO cell growth inhibitory activity of the HPLC fractions of V3 extract of *Viola odorata* was tested using the [^{3}H]-thymidine uptake assay.

4.2.5 *In vitro* screening of HPLC fractions against human LOVO tumour system using [^{3}H]-thymidine uptake assay

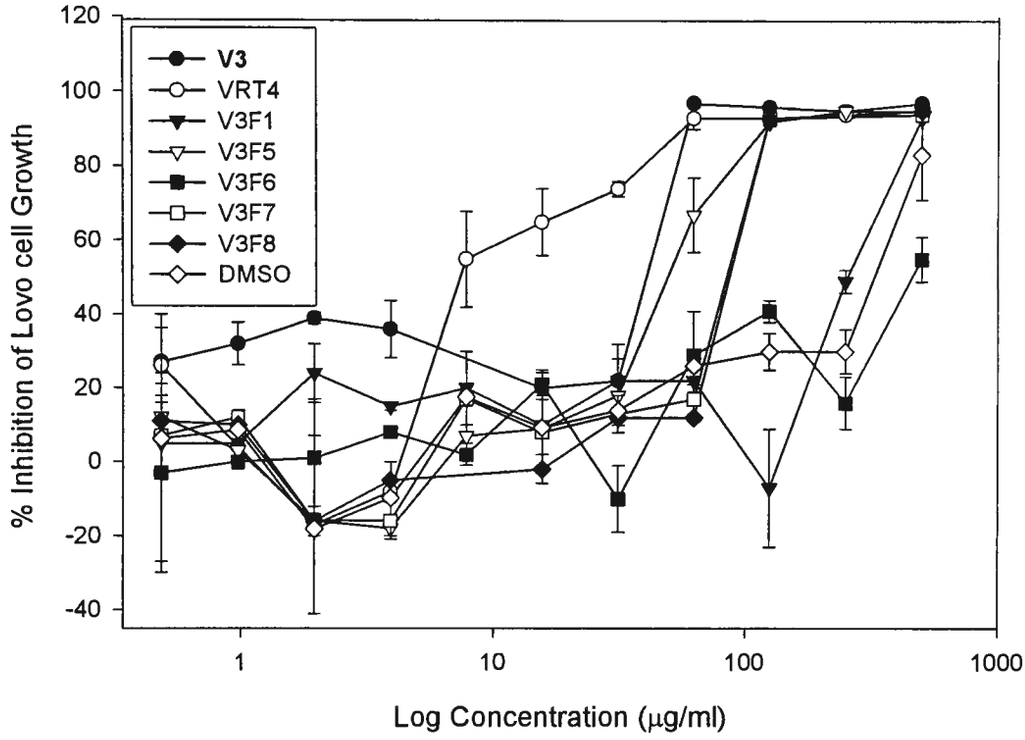


Figure 32 Percent LOVO cell growth inhibition of *Viola odorata* extracts, V3 from aerial parts, VRT4 from roots and some fractions of V3 at different concentrations using the [^{3}H]-thymidine uptake assay.

The results of the anticancer activity of the HPLC fractions of V3 extract of *Viola odorata* against the LOVO colon cancer cell line compared with those of VRT4 and V3 extracts are given in Figure 32. The IC50 values of the extracts, V3, VRT4 and the fractions V3F1, V3F5, V3F6, V3F7, V3F8 and the solvent DMSO were 52±19, 10±2, 262±7, 67±28, 229±76, 126±58, 164±97 and >313 µg/ml, respectively. The fractions, V3F2, V3F3 and V3F4 were not tested for the LOVO cell growth inhibitory activity.

As illustrated in Figure 32, the root extract, VRT4 (IC50=10±2 μg/ml) showed the highest (P>0.05) inhibition of solid LOVO tumour cell growth, which was higher (P>0.05) compared with the activity shown by the aerial part extract, V3 (IC50=52±19 μg/ml). Among the fractions tested, V3F5 showed the highest LOVO cell growth inhibitory activity (IC50=67±28 μg/ml) compared with the other fractions, V3F1, V3F6, V3F7, V3F8 and the solvent control DMSO.

The DMSO control showed a much lower inhibitory potential (LC50 of >313 μg/ml) compared with other extracts and fractions tested using LOVO cells as the test system. The inhibitory effect of DMSO was below 30% at the concentration of 250 μg/ml. Hence DMSO does not appear to have contributed to the inhibitory activity found for some of the samples tested.

In summary, the sequential root extract, VRT4 showed the highest (P<0.05) CEM cell growth inhibitory activity using both MTS assay (Figure 31) and the [^{3}H]-thymidine uptake assay using LOVO cells (Figure 32) whereas the V3 extract exerted the highest CEM cell growth inhibition (Figure 20 & 30) and inhibition of the LOVO cells (Figure 31) according to [^{3}H]-thymidine uptake assay results. The HPLC fraction, V3F5 showed the highest growth inhibitory activity for both cell lines using the MTS and [^{3}H]-thymidine uptake assays, compared with the different HPLC fractions of the V3 extract.

Interestingly, although there was no significant difference (P>0.05) between the CEM growth inhibitory potentials of V3F5 and V3F6 (2±1 and 1.8±0.7 μg/ml respectively), a significant (P<0.05) difference of the LOVO growth inhibitory potential was seen between the [^{3}H]-thymidine uptake assay results of V3F5 and V3F6 (67±28 and 229±76 μg/ml, respectively).

The "net cell killing" or cytotoxic effects as discussed previously by Boyd and Paull (1995) is prominent as indicated by the horizontal bars extended to the left of the LC50 mean graph (Figure 26) of the NCI's 60 human cell-line screening profile for the V3-MeOH fraction of *Viola odorata*.

4.3 Discussion

The investigation of the plant *Viola odorata* for anticancer activity has revealed the following;

In the NCI's *in vitro* screening programme, an EtOH/$CHCl_3$ extract of *Viola odorata* showed a weak activity against human epidermoid carcinoma, KB, giving the highest 50% growth inhibitory potential of 21 μg/ml. The EtOH/$CHCl_3$ extracts extended the survival time (T/C%=137) of P388 leukaemia (PS) implanted mice beyond that of the control animals (T/C%=125%), and showed growth inhibition against sarcoma 180 (SA) and human Friendvirus leukaemia. However, these extracts failed in the NCI's screening programme since they did not meet the selection criteria (NCI's Screening Data Summary, 1964-1977).

However, in this study the sequential $CHCl_3$-MeOH extract V3, when assayed using the [^{3}H]-thymidine uptake method, gave the highest CEM cell growth inhibitory potential of 3±1 μg/ml among the sequential *Viola odorata* extracts tested, which is approximately 10-fold higher than the previous NCI's result of 21 μg/ml. In addition the V3 extract gave an IC50 value of 52±19 μg/ml when using the LOVO colon cancer cells whereas the root extract, VRT4 gave a higher ($P<0.05$) inhibitory potential of 10±2 μg/ml.

The V3, V3-MeOH and VRT4 extracts when screened against the NCI's human panel using sulforhodamine B method gave GI50 panel averages of 49, 7 and 26 μg/ml, respectively. According to the NCI's selection criteria, the V3 extract was inactive whereas V3-MeOH fraction showed some toxicity and the VRT4 extract indicated a weak but non-selective growth inhibition on the cell lines of the NCI's human panel. However in this study a much higher growth inhibitory anticancer potential of 2 μg/ml was shown by the sequential 70% EtOH root extract, VRT4 against TK-10 renal cancer cell line of the human panel when measured using sulphorhodamine B assay.

Hence the NCI's current screen comprising a panel of 60 human cancers may enable *in vitro* detection of selective as well as differential growth inhibitory anticancer activity of the appropriately made extracts of putative medicinal plants, which may be more effective

than the previous single cell prescreen as suggested by Schwartsmann and Workman (1993) or proposed pre-screen using three sensitive cell lines (NCI's Website, 2000)

The HPLC fraction, V3F5 of the V3 extract gave a higher growth inhibitory potential of 2 μg/ml for the CEM cells and 67±28 μg/ml for the LOVO cell line using the [^{3}H]-thymidine uptake assay. The same V3F5 fraction gave an IC50 value of 10 μg/ml for the CEM cells using MTS assay. Hence the V3F5 fraction of the V3 extract showed a significantly higher ($P<0.05$) growth inhibitory potential than that of V3 crude extract using the HPLC technique for fractionation of the V3 extract. A similar procedure was used previously by De Guilio *et al* (1992) to isolate cytotoxic metabolites from the plant, *Chelodonium majus*.

The MTS assay did not yield results comparable with the [^{3}H]-thymidine uptake assay against the same cell line for the dark-coloured plant extracts E3 and V3 at a relatively high concentration of >100. μg/ml. However, MTS results for fractions and the colourless extracts were similar to those obtained with the [^{3}H]-thymidine assay results.

The active extracts V3 and its fraction V3F5, gave CEM growth inhibitory potentials of 3, and 2 μg/ml, respectively, compared with the standard anticancer drugs doxorubicin and idorubicin, (0.44-0.51 and 0.02-0.05 μg/ml, respectively) when assayed using the [^{3}H]-thymidine uptake assay. Our results for the standard agent, doxorubicin, lie within the range of results (0.003-0.92 μg/ml) obtained for doxorubicin by NCI against the human cancer panel (NCI's Website, 2001) and close to the results (0.13-0.76 μg/ml) obtained by Fridborg *et al* (1995).

Although the crude V3 extract did not show a cytotoxic effect, the MeOH fraction of V3 did. It would seem from this result that further fractionation of an inactive or weakly active plant extract may sometimes lead to an unmasking of cytotoxic activity. Investigation of a non-cytotoxic extract like V3 for other indirect anticancer activities may also be useful for detecting other anticancer compounds. As a result of this study, which identified a number of different bioactive fractions that showed different anticancer activities, it may be possible to design a broad-spectrum treatment model using a combination of these different anticancer agents.

In this regard it is of interest that Clark (1996) previously indicated that, bryostatins, from marine dinoflagellates, could be used as a basis for a broad-based anticancer treatment. As further functional roles of oncogenes and tumour suppressor genes were discovered, Schwartsmann and Workman (1993) suggested that the inclusion of drug targets into a broad anticancer activity treatment should be used to treat cancer.

Because of additive or synergistic efficacy of compounds with less toxic effects, a combination therapy is considered to be an attractive option (Steele *et al*, 1994) since it offers the possibility of combining agents with different primary mechanisms of action such as, antiproliferative, antiestrogen, immunomodulatory, and antioxidant for broad spectrum alleviative effect

It is suggested that the modified extraction method when used in combination with a sensitive cell line and an appropriate method of detection may provide a procedure for determining whether or not the plant possesses any useful *in vitro* anticancer activity. In addition the NCI's selection criteria for complex extracts such as plant extracts may need to be modified as the method of detection is not sensitive enough to determine *in vitro* anticancer activity from crude plant extracts.

Chapter 5 Determination of antioxidant activity of *Euphorbia peplus* L. and *Viola odorata* L.

ABSTRACT

Low antioxidant state or enhanced oxidant state has been observed in cancer patients. Induction of cancer cell death (apoptosis), prevention of cancer progression and possible immunological involvement due to antioxidants, have also been suggested. Oxy radicals formed during lipid peroxidation and other pathways are also suggested to cause destruction of DNA structure and inactivation of the tumour suppression gene, p53. Antioxidants may play an important role in preventing cancer. Other studies have shown that putative anticancer plants used in traditional medicine have been found inactive in the *in vitro* anticancer screening assays based on cytotoxicity. It was considered to be important to investigate the antioxidant activity of the anticancer plants because of the possible role played by antioxidants in preventing as well as curing cancer.

An appropriate antioxidant measurement should reflect the putative therapeutic antioxidant effect of the plant extract on the human body. Hence, application of a suitable analytical method for the determination of antioxidant potential of plant extracts is imperative. In this part of the study, we compared the inhibition of lipid peroxidation in a linoleic acid model system using modified TBARS and FTC measurements, DPPH free radical scavenging activity and ferric reducing antioxidant potential (FRAP) using a Fe(III)-TPTZ method, of the *Euphorbia peplus* and *Viola odorata* plant extracts with the standards, α-tocopherol and trolox. The E4 and V3 extracts were found to effect linoleic peroxidation inhibitory, DPPH free radical scavenging and TPTZ-ferric reducing antioxidant activities. According to the present results, the modified TPTZ method yielded results comparable with those of DPPH while the FTC and TBARS results for inhibition of linoleic peroxidation by plant extracts E4 and V3 were significantly higher ($P>0.001$, $n=5$) than those given in the DPPH and FRAP assays. The modified TPTZ method gave a better precision (within-run CV, 5%, $n=25$) and reproducibility (between-runs CV, 6%, $n=5$) than the other methods investigated.

5.1 Introduction

As discussed in Chapter 1, because of the possible causative role played by antioxidants in generating cancerous cell growth, it was considered important to investigate putative anticancer plants such as *Euphorbia peplus* and *Viola odorata* for antioxidant activity. A number of different methods have been used to determine the antioxidant activity of samples of different origin. In order to select the appropriate antioxidant assay with a reasonable reproducibility and precision, it is important to define antioxidant activity.

Traditionally, food antioxidants are defined as the chain-breaking antioxidant inhibitors of lipid peroxidation. Indeed lipid peroxidation may be responsible for cellular damage to all oxidisable substances found in living cells including proteins, DNA, and carbohydrates as well as the lipids by *in vivo*-generated free radicals. Halliwell and Gutteridge (1989 and 1995) defined an antioxidant as ":any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate". However, according to Benzie (1996) an antioxidant can be "any substance that inhibits an oxidative reaction or favours a reducing reaction in a redox equilibrium" whereas Bors *et al* (1992) defined antioxidants as "scavengers of oxygen radicals formed under oxidative stress conditions".

As discussed in Chapter 1, section 1.6.3.1, when reactive oxygen species are generated in living systems, the body's self-defensive endogenous antioxidant systems comprising a wide variety of antioxidants are activated against the generated reactive oxygen species (ROS). According to Halliwell (1995), the relative efficacy of these antioxidants as protective agents depends upon which ROS is generated, how it is generated, where it is generated and what target of damage is measured. Halliwell (1995) noted different results, when a different target of oxidative damage is measured while oxidative stress is kept the same, thus indicating the importance of the source of stress and the target or the oxidisable substrate.

Although the broader definition of an antioxidant includes any substance that inhibits oxidative damage to a target, on the basis of assays that have little biological relevance, every chemical would be classified as an antioxidant or a prooxidant (Halliwell, 1995),

such assay models may be utilized, as tools, to determine biologically useful antioxidant activity of a plant extract. According to Prior and Cao (1999), it is the existence of various harmful pro-oxidants or reactive free radical species *in vivo* that makes anti-oxidants essential for the maintenance of a healthy life. However, when the redox equilibrium favours the oxidant state, there is the potential to generate oxy-free radicals (ROS). Hence, a two-pronged approach could be proposed to circumvent the disease state by two general pathways, one would be to reinstate the redox balance by administering an antioxidant(s) to restore the deficient antioxidant power or capacity and the other would be to neutralise the free radical species formed. Whether all biological antioxidants possess the dual functions of restoring the disturbed redox state by increasing the antioxidant capacity of the body while scavenging the free radicals or whether the different functional antioxidant have different targets and mechanisms of action, are yet to be established.

Given the potential of antioxidants to act as therapeutic agents in certain diseases, it is therefore imperative to develop an appropriate analytical method or methods for assessing antioxidant activity. The method of choice in many recent publications for determining the antioxidant activity for medicinal purposes has been the free radical scavenging activity (FRSA) method, which provides a measure of free radical species. Mantle *et al* (1998) used $ABTS^{\cdot+}$ [2,2'-azino-bis(3-ethyl benzthiazoline-6-sulphonic acid], hydroxyl and superoxide radicals to estimate the FRSA of plant oils. The antioxidant activity was presented as mM equivalent of trolox. According to Mantle *et al* (1998) the apparent FRSA of plant extracts depends entirely on the assay method utilised and the free radical species used. Bors and Saran (1987) used a stable azide radical scavenger as a measurement for the antioxidant activity of flavonoids. Lee *et al* (1996), Kirby and Schmidt (1997) and Jin and Chen (1998) used the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radicals for the determination of antioxidant activity of plant extracts.

Although there are many different analytical methods currently being used for the determination of antioxidant activity, a reliable rapid method that measures the total antioxidant potential with reasonable precision and reproducibility is lacking (Packer, 2001). The TBARS method that measures the oxidative products formed during lipid peroxidation shows a wide variation in the results (Esterbauer, 1996).

Although the radical-scavenging and metal-chelating capabilities are thought to account for the antioxidant activity of plant constituents such as flavonoids (Bors and Saran, 1987), the relationship between the FRSA and the total antioxidant potential of a plant extract corresponding to the redox status of the body has not yet been established.

5.2 Aim of the investigation

The aim of this part of the study was to develop different assay methods for measuring the *in vitro* inhibition of lipid peroxidation (using the TBARS and FTC methods), free radical scavenging (using the DPPH method) and ferric reducing using the TPTZ-FRAP assay) antioxidant activities and to utilize these assays for determining the *in vitro* antioxidant activity of the putative anticancer plants, *Euphorbia peplus* and *Viola odorata.*

5.3 Results and discussion

The extraction methods described in Section 2.4.1 and 2.4.2 of Chapter 2 were used to prepare the extracts used in the antioxidant assays, results of which are discussed below.

5.3.1 Selection of extracts

The rapid TBARS method described in Section 2.6.1, was used for testing of the *Euphorbia peplus* extracts and the TPTZ method (Section 2.6.2) were used for testing extracts from both plants.

The results obtained for the sequential extracts of *Euphorbia peplus*, according to the rapid TBARS method of Sroka *et al* (1994) described in section 2.8.1 of chapter 2, are illustrated in Figure 33. According to the results shown in Figure 33, the E4 extract showed significantly lower ($P<0.05$) absorbance values indicating higher inhibition of lipid peroxidation compared with the sequential extracts and the boiling water extract of *Euphorbia peplus* tested. The standard α-tocopherol indicated the highest antioxidant activity using the rapid TBARS assay.

In addition, the results obtained for the sequential extracts of *Euphorbia peplus* as well as *Viola odorata,* for ferric reducing antioxidant activity measured using the rapid Fe (III)-TPTZ method of Benzie (1996) described in section 2.8.2 of Chapter 2, are shown in Figure 34. According to the results illustrated in Figure 34, E4 (70% EtOH-aqueous) extract of *Euphorbia peplus* and V3 ($CHCl_3$-MeOH) extract of *Viola odorata* gave

significantly higher ($P<0.05$) absorbance values indicating higher antioxidant activity compared with the other extracts. Hence, the E4 and V3 extracts of the two plants were used in experiments aiming at developing an appropriate method for antioxidant activity.

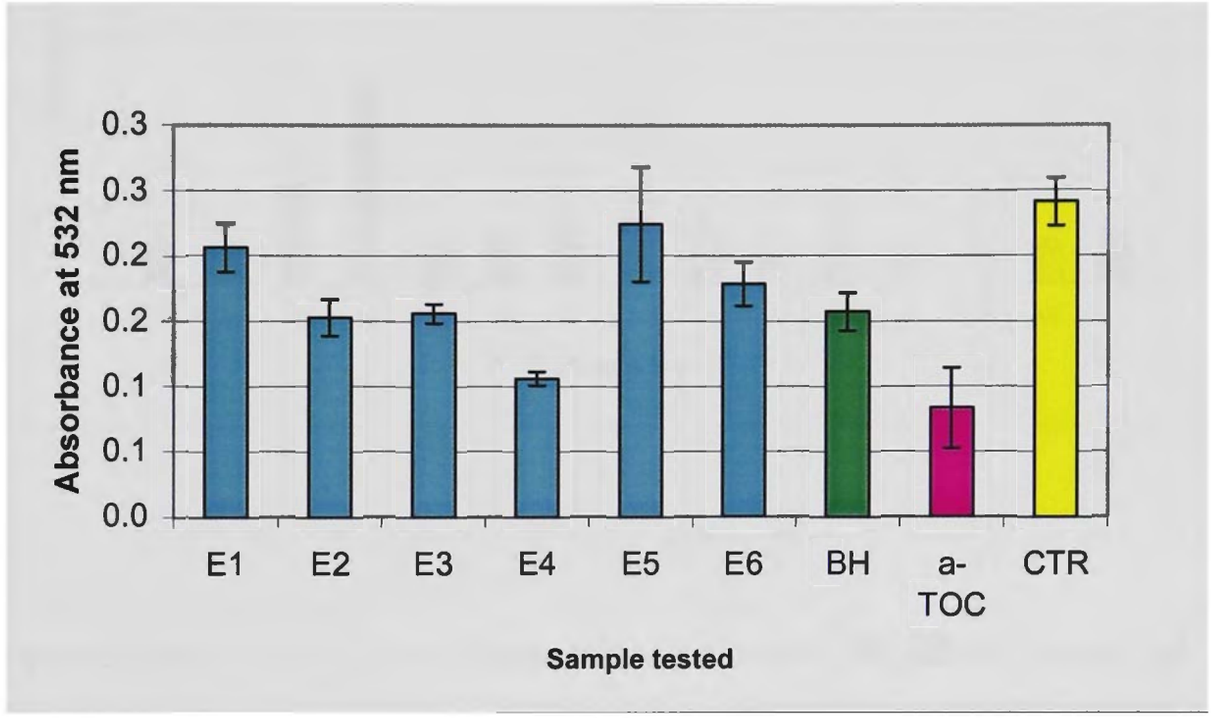


Figure 33 Absorbance values of the sequential extracts E1, E2, E3, E4, E5, and E6 and the boiling water extract EB of *Euphorbia peplus* compared with the standard, α-tocopherol and the control, measured using modified TBARS method of Sroka *et al* (1994). Each data point is the average of 3 replicate measurements ± SD. The absorbance value of E4 was significantly different from those of the other samples ($P<0.05$).

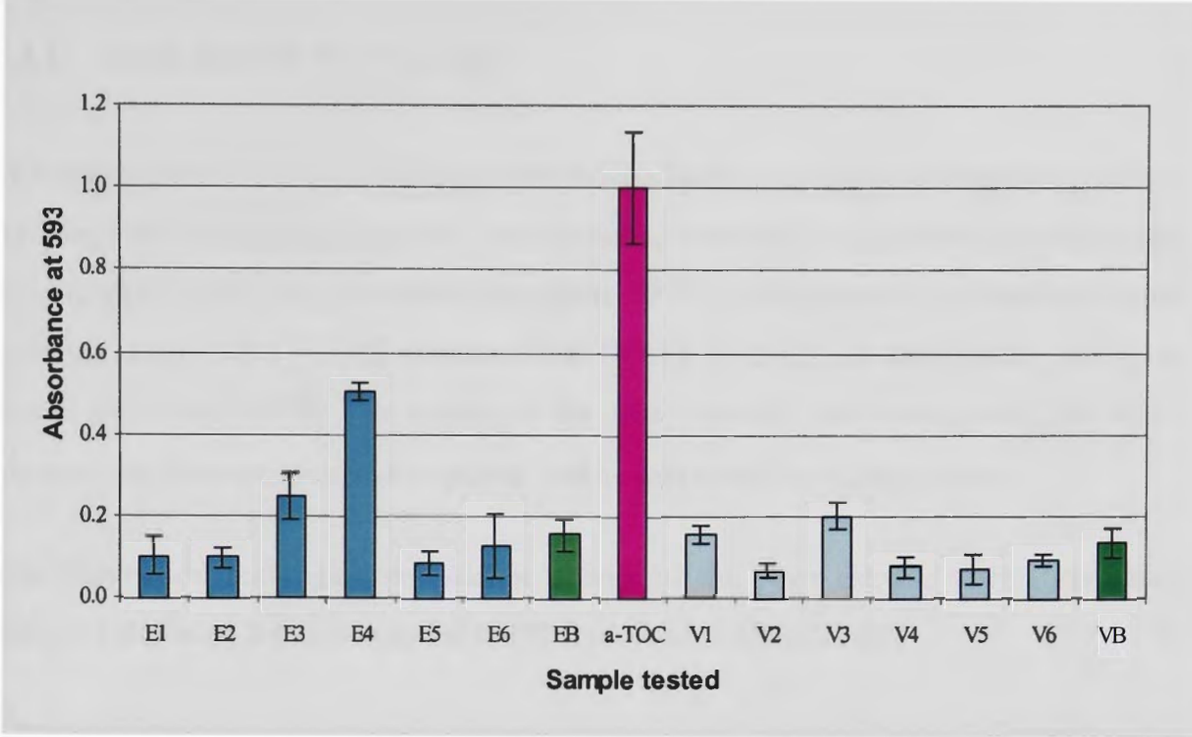


Figure 34 Absorbance values of the sequential extracts E1, E2, E3, E4, E5, and E6 and the boiling water extract EB of *Euphorbia peplus* and the sequential extracts, V1, V2, V3, V4, V5, and V6 and the boiling water extract VB of *Viola odorata* compared with the standard, α-tocopherol and the control, measured using TPTZ method of Benzie (1996). Each sample concentration was 200 μg/ml. Each data point is the average of 3 replicate measurements ± SD. The results for E3, and E4 & V1 and V3 were significantly different from each other ($P<0.05$).

According to the results given in Figure 34, the moderately polar sequential $CHCl_3$-MeOH extract, E3, and the polar 70% EtOH extract, E4, gave the highest ($P<0.05$) absorbance values indicating higher ferric reducing antioxidant activity among the extracts of *Euphorbia peplus*. As for *Viola odorata,* the non-polar, petroleum spirit (40-60^{0}C) extract, V1 and the moderately polar, $CHCl_3$-MeOH extract, V3, gave the higher absorbance values, indicating higher ferric reducing antioxidant activity among the extracts of *Viola odorata.* Interestingly, both EB and VB, the crude, boiling water extracts of *Euphorbia peplus* and *Viola odorata*, respectively, also showed relatively low antioxidant activities. However, the

highest ferric reducing antioxidant activity was shown by the standard, α-tocopherol as indicated (Figure 34) by its highest ($P<0.001$) absorbance value.

5.3.1 Assay method development

The antioxidant effect of the plant extracts on the lipid peroxidation (autoxidation) of the aqueous-EtOH-linoleic acid system, was measured according to the method of Kikuzaki and Nataniki (1993), using the ferric thiocyanate (FTC) method and the thiobarbituric acid reducing substances (TBARS) method. The TBARS method was modified according to Buege and Aust (1978). The results of the plant extracts were compared with those obtained for the standards, α-tocopherol and its water-soluble analogue, trolox.

The free-radical scavenging antioxidant activity of the plant extracts were determined using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method of Blois (1958).

The modifications made to each of these methods are described below followed by the discussion of the results obtained using each method.

5.3.2.1 Inhibition of lipid peroxidation

5.3.2.1.1 Substrate selection

The investigation of plant antioxidants was originally directed towards finding an effective antioxidant for the prevention of autoxidation of lipid peroxidation. In a typical model experimental system, the use of a more simple form of auto-oxidation was preferred to the induced oxidation since the oxidation reaction can ideally be controlled over the incubation period. Therefore, we utilized linoleic acid [(*Z, Z*)-9,12-octadecadienoic acid], (Figure 35) as the substrate, to investigate the possible inhibitors of lipid peroxidation.

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Figure 35. Structure of linoleic acid

Because of its higher molecular weight as well as the two allylic double bonds in the linoleic molecule (Figure 35), linoleic acid generates sufficient amounts of thiobarbituric acid reacting substances (TBARS), for it to be used routinely.

When a peroxidation initiator was used in the test system the incubation period before the TBARS measurement was reduced to 90 minutes comparing to 14 days of incubation in a typical TBARS procedure (Yamamoto *et al*, 1982). This was done to eliminate the effect of plant extracts on the initiator. Thus avoiding the use of an initiator.

Osawa and Namiki (1981) used a linoleic acid peroxidation test system without any free radical initiator and a 40^0 C incubation temperature. The atmospheric oxygen trapped above the surface of the incubation mixture allowed auto-oxidation of linoleic acid in this system, generating degradative products of linoleic acid during the incubation period of up to 40 days. The inhibitory activity of the plant extracts on the auto-oxidation of the linoleic acid system has been determined as the antioxidant activity of the plant extracts when the FTC, TBARS total carbonyl value and weighing methods were used. According to the results presented by Osawa and Namiki (1981), a distinguishable difference was seen between the antioxidant activities of the plant extracts as well as the standards and the control, during a 10-day incubation period.

The purpose of using long (e.g., 40 day) incubation periods in lipid peroxide systems is to determine how long the test antioxidant could be used to keep the lipid from peroxidation. Also a long incubation period would enable a slow active antioxidant sufficient time to initiate its inhibitory activity. The purpose of the investigation carried out by Osawa and Namiki (1981) was to identify an effective natural antioxidant that would prolong the oxidation of food. Thus a long incubation period was unavoidable when these antioxidant assays were used.

5.3.2.1.1.1 FTC Method

The visible spectra obtained from the ferric thiocyanate (FTC) method as described in Section 2.6.3.1 of Chapter 2, for both plant extracts and the standards, when used at relatively low concentrations, are given in Figure 36. According to Figure 36, the absorbance maxima obtained for all the samples tested was approximately at 500 nm. Although the absorbance maxima obtained at low absorbance values were in a broad range as in the cases of plant extracts, E4, EB VB and V3, the absorbance maxima,

obtained from the control, α-tocopherol and trolox, at higher absorbance values gave relatively sharper maxima.

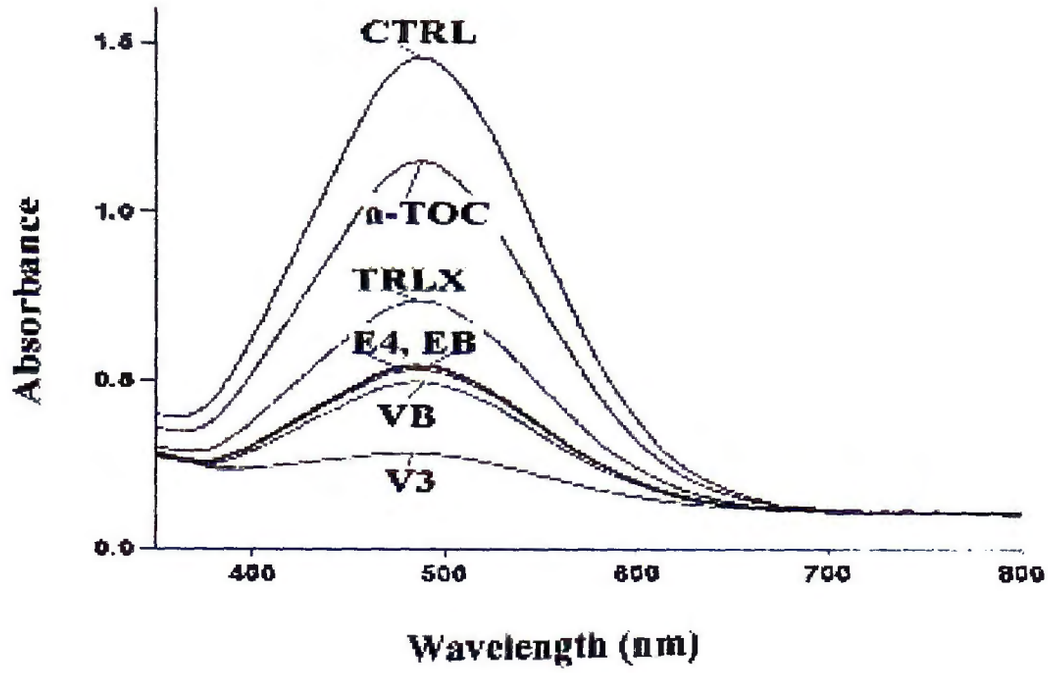

Figure 36. Visible spectra showing absorbances of the E4 and EB extracts of *Euphorbia peplus* and V3 and VB extracts of *Viola odorata* compared with the control (CTRL) and the standards, α-tocopherol and trolox, using the ferric thiocyanate (FTC) method.

The changes of absorbance measured at 500 nm during the incubation period of 14 days for the standards, α-tocopherol and its water soluble analogue, trolox, the E4 and EB extracts of *Euphorbia peplus* and V3 and VB extracts of *Viola odorata* are illustrated in Figure 37.

As illustrated in Figure 37, the control sample, which contained only the linoleic acid and the alcoholic buffer and the air space, gave the highest absorbance values until day 9,

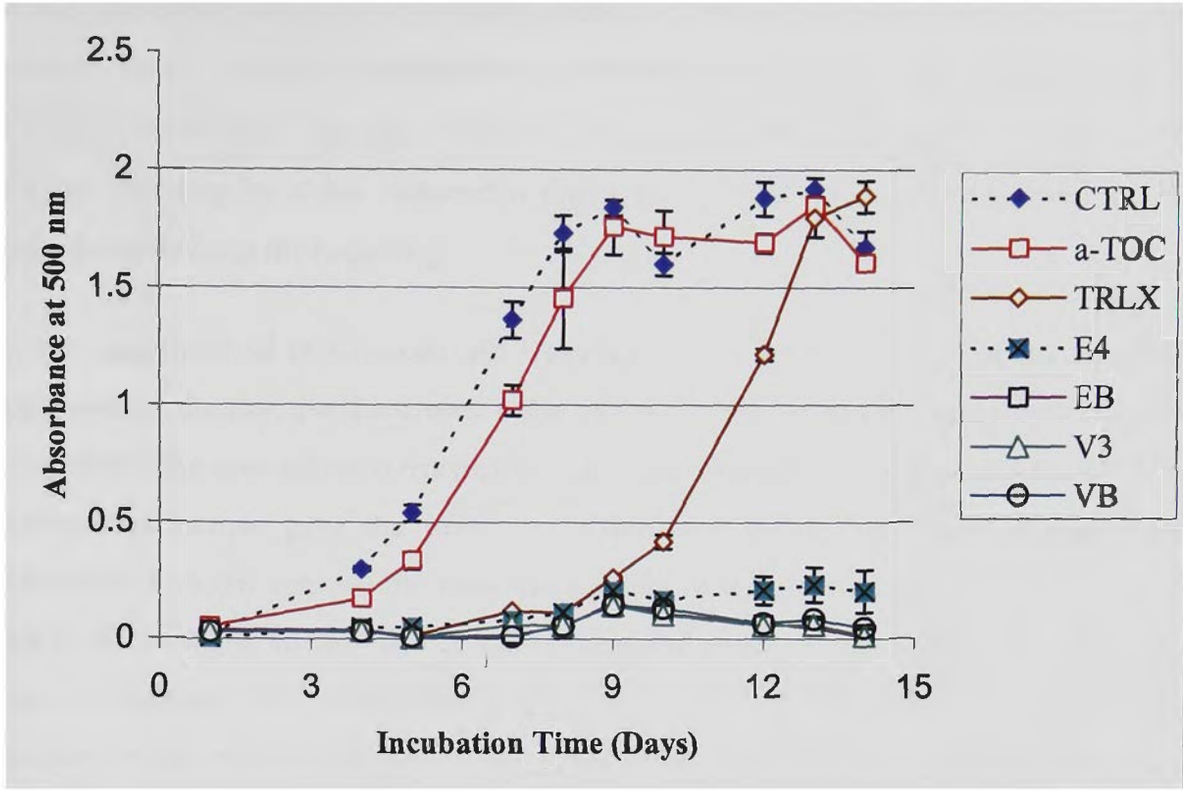

Figure 37. Absorbance values at 500 nm vs. incubation time of the E4 and EB extracts of *Euphorbia peplus* and V3 and VB extracts of *Viola odorata* compared with the control (CTRL) and the standards, α-tocopherol and trolox, using ferric thiocyanate (FTC) method. Each sample concentration was 200 μg/ml. Each data point is the average of 5 replicate measurements ± SD.

indicating the maximum quantity of peroxidation products that could be derived from linoleic acid auto-oxidation. According to the data shown in Figure 37, the absorbance values of the incubate, containing α-tocopherol, although higher than the extracts and trolox, were significantly lower ($P<0.05$) than those of the control on day 4, 5, 7 and 8. However, on day 9 there was no significant difference ($P>0.05$) between the absorbance value of α-tocopherol and that of the control showing the loss of inhibitory activity of α-tocopherol against the lipid peroxidation on day 9.

Although there was no significant difference between ($P>0.05$) the absorbance values of the plant extracts, E4, EB, V3 and VB, and trolox until day 9, from day 9 until day 14, trolox gave significantly higher ($P<0.05$) absorbance values than those given by the plant extracts. Hence the linoleic peroxidation inhibitory activity of the plant extracts E4, EB, V3 and VB remained low up to the 14^{th} day of incubation whereas the reduction of inhibitory activity by trolox occurred at day 9. In contrast, α-tocopherol showed a very low inhibition from the beginning.

In the assay method of Kikuzaki and Nakatani (1993), the FTC results of the samples, measured on the day, the absorbance value of the control reached maximum was selected to compare the antioxidant activity of the samples. Although day 9 appeared to be when the control sample gave the maximum absorbance value, there was no significant difference ($P<0.05$) between the absorbance values of the control measured on day 8 and day 9. Accordingly, the absorbance values measured either on day 8 or on day 9 may be used to compare the results of the samples. In order to compare the effect of different samples on the inhibition of linoleic acid peroxidation, the FTC measurements were made on day 7, 8 and 9 and the results are summarised in Table 21.

Although there was no significant difference ($P>0.05$) between the results of the control measured on day 8 and day 9, there was a significant difference ($P<0.05$) between the results of each sample measured on day 8 and 9, as given in Table 21. Hence the results obtained either on day 8 or day 9 could be selected for comparison purposes. There was no significant difference ($P>0.05$) between the results of trolox, E4 and V3 measured on days 7 and 8.

Although Kikuzaki and Nataniki (1993) reported a significantly higher antioxidant activity of α-tocopherol compared with the control using the FTC method, the above results, obtained using a similar assay, showed very high absorbance values (Figure 37) for α-tocopherol, which were much closer to the absorbance values of the control, indicating very low antioxidant activity for α-tocopherol.

The FTC method of Mitsuda *et al* (1966), was adopted by Osawa and Namiki (1982), to isolate and quantify β-diketones from *Eucalyptus globulus* L. leaf wax. The method involved measurement of absorbance at 500 nm after reaction with $FeCl_2$ and ammonium thiocyanate at intervals during the incubation period.

Table 21. Percent inhibition of linoleic peroxidation using the FTC method

Sample	DAY 7		DAY 8		DAY 9	
	% Inhibition	SD	% Inhibition	SD	% Inhibition	SD
CTRL	0	8	0	3	0	5
α-TOC	25	21	16	12	4	11
TRLX	91	1	94	1	86	3
E4	94	2	93	4	89	3
EB	99	0	97	2	92	1
V3	96	5	97	1	92	2
VB	99	1	97	2	92	2

The control (CTRL) containing no added samples or a standard represents the 100% lipid peroxidation or the 0% inhibition of antioxidant activity. The % inhibition values are the averages of 5 replicate determinations. SD, standard deviation.

Osawa and Namiki (1981 and 1985) identified and isolated the antioxidant, *n*-tritriacontan-16,8-dione from the leaf wax of *Eucalyptus globulus* using the FTC method to monitor antioxidant activity.

5.3.2.1.1.2 TBARS Method

The visible spectra obtained using the thiobarbituric acid reacting substances (TBARS) method described in Section 2.6.3.2 of Chapter 2, for the plant extracts and the standards are given in Figure 38.

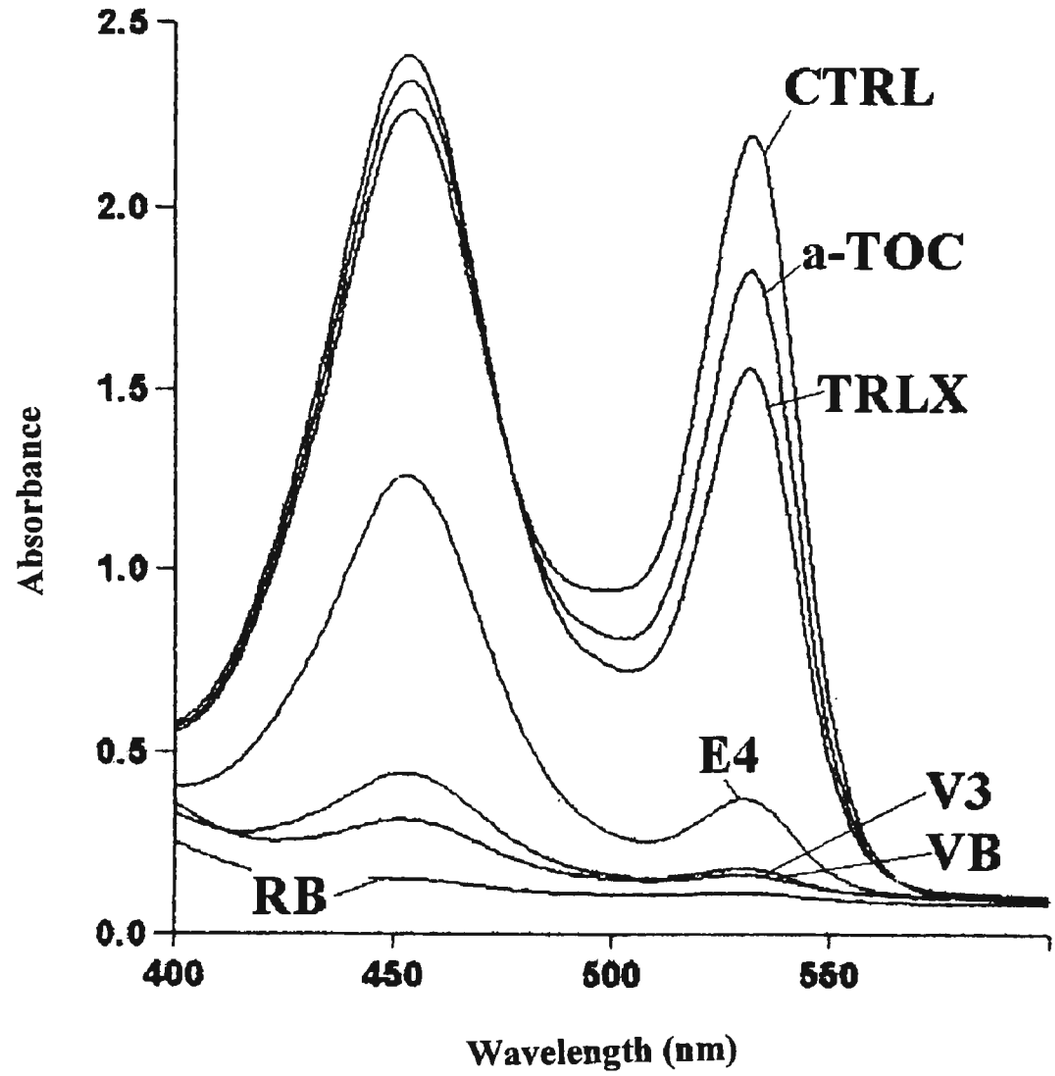

Figure 38. Visible spectra showing absorbance of the E4 and EB extracts of *Euphorbia peplus* and V3 and VB extracts of *Viola odorata* compared with the control (CTRL) and the standards, α-tocopherol and trolox, using TBARS method.

According to the data shown in Figure 38, the absorbance maxima obtained for all the samples tested was 450 nm. However the second peak at 532 nm in the same spectrum for all the samples tested was not only proportional to the peak height of the absorbance maxima at 450 but also relatively sharper than the absorbance maxima at 450 nm. The absorbance spectrum of malandialdehyde (MDA) suggested that the absorbance at 532 was due to the presence of MDA formed during the lipid peroxidation. Thus 532 nm was

selected as the optimum wavelength at which the TBARS measurements were made for the standards as well as the plant extracts.

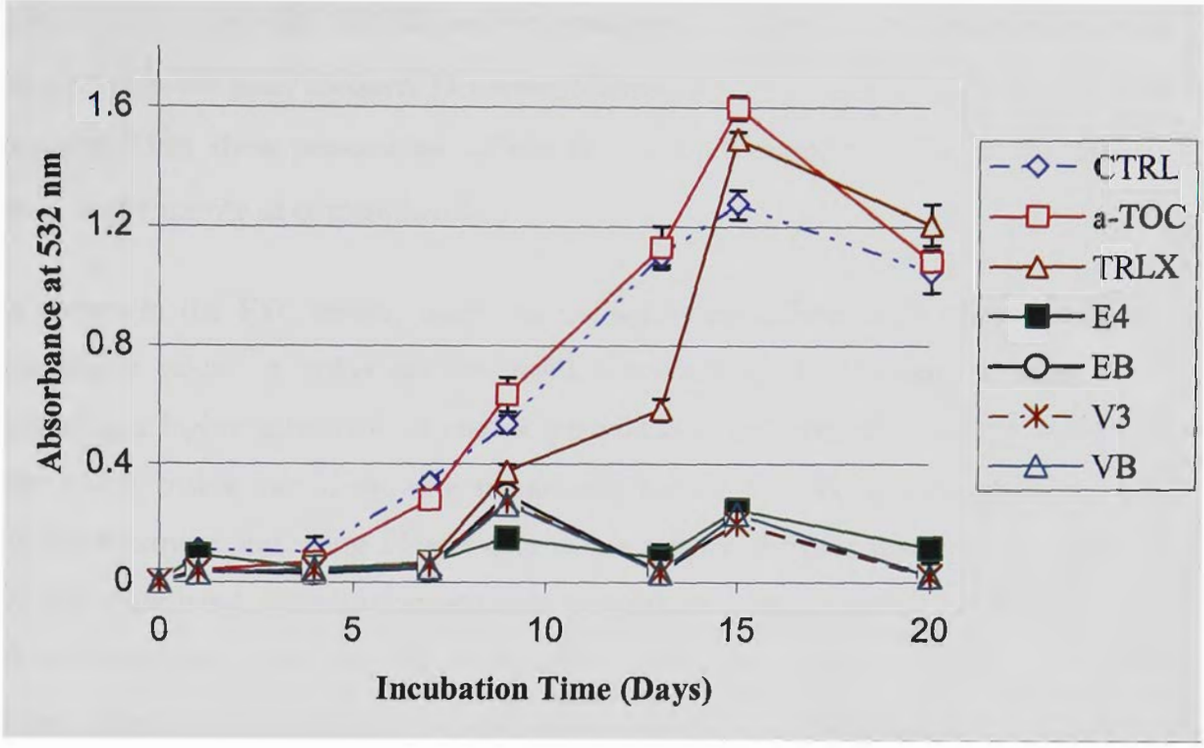

Figure 39. Absorbance values at 532 nm vs. incubation time of the E4 and EB extracts of *Euphorbia peplus L* and V3 and VB extracts of *Viola odorata L* compared with the control and the standards α-tocopherol and trolox, using the modified TBARS method according to Kikuzaki and Natarniki (1993). Each sample concentration was 200 μg/ml. Each data point is the average of 5 replicate measurements ± STDEV.

The change of absorbance, measured at 532 nm, during the incubation period of up to 20 days for the standards, α-tocopherol and trolox, the E4 and EB extracts of *Euphorbia peplus* and V3 and VB extracts of *Viola odorata* using TBARS method, are illustrated in Figure 39.

As shown in Figure 39, the control sample, had the highest absorbance values up to day 9, but after this time had lower absorbance values compared with α-tocopherol. The difference between the absorbance values ($P<0.05$) of the control and α-tocopherol was

significant ($P<0.05$) from day 1 to 9, indicating enhanced lipid peroxidation or prooxidant activity of α-tocopherol after 9 days of incubation. The pro-oxidant activity shown by α-tocopherol may be due to the use of aqueous-EtOH reaction medium. Kikuzaki and Nataniki (1993) used the same assay medium and found very low absorbance values for α-tocopherol using the TBARS method, indicating a higher antioxidant activity of α-tocopherol in the assay medium. However, Halliwell's (1995) observation showing that α-tocopherol can show pro-oxidant activity *in vitro*, lends support to our results showing pro-oxidant activity of α-tocopherol.

As shown in the FTC results, there was no significant difference ($P>0.05$) among the absorbance values of trolox and the plant extracts, E4, EB, V3 and VB until day 7, indicating a higher inhibition of linoleic peroxidation or antioxidant activity. However, after day 9, trolox started showing significantly higher ($P<0.05$) absorbance values than the plant extracts, indicating a lower antioxidant activity of trolox than the plant extracts. On the other hand, the plant extracts gave consistently lower absorbance values until day 20, indicating high lipid peroxidation inhibitory antioxidant activity than that of trolox and α-tocopherol.

The inhibitory activity of the samples tested for peroxidation of linoleic acid on day 9, 13 and 15 are summarised in Table 22. As illustrated in Figure 39, the day maximum absorbance shown by the control was day 15th. However, according to the assay method of Kikuzaki and Nataniki (1993), the TBARS measurements have been made on the same day the control gave the maximum absorbance using the FTC method. According to our results the TBARS measurements made on day 9, for the E4 and EB extracts of *Euphirbia peplus* gave 81 and 86% inhibition whereas V3 and VB extracts of *Viola odorata* gave 82 and 86 % inhibition of lipid peroxidation respectively, using TBARS method.

The control (CTRL) containing no added samples or standard represents 100% lipid peroxidation of 0% inhibition or antioxidant activity. The % inhibition values are the averages of 5 replicate determinations given with their standard deviations (SD).

The α-tocopherol and trolox standards resulted in a 17% and 80% inhibition of lipid peroxidation using the same method. Hence the plant extracts gave results from the TBARS method, which were equal to or higher than those given by the trolox standard as in the case of the FTC method.

Table 22. Percent inhibition of linoleic peroxidation using the TBARS method

	DAY 9		DAY 13		DAY 15	
Sample	%Inhibition	SD	%Inhibition	SD	%Inhibition	SD
CTRL	0	3	0	5	0	7
α-TOC	17	6	-3	4	-25	4
TRLX	80	2	47	2	-17	7
E4	81	1	93	3	81	3
EB	86	1	97	1	84	4
V3	82	1	97	2	84	0
VB	86	0	97	1	82	4

As shown in Table 22 both the standards, trolox and α-tocopherol gave significantly different results on day 9, 13 and 15 ($P<0.05$) whereas results of plant extracts did not show much variation. Indeed the results showing prooxidant activity for both α-tocopherol and trolox on day 15 contrast with the results obtained on day 9, which showed antioxidant activities of both the standards at this time.

Samples drawn from each replicate incubate using FTC and TBARS methods gave maximum absorbance values for the control, on the 8th or 9th day using the FTC method and on the 15th day using the TBARS method.

During the oxidation process of linoleic acid, peroxide is gradually decomposed into compounds with lower molecular weights. The degradation products formed were measured by the TBARS method and the amount of peroxide formed during the initial

stages of lipid peroxidation was measured by the FTC method (Kikuzaki and Nataniki, 1993). The highest level of peroxide formation in the control as given by the FTC results, was measured on the 8th or 9th day (Figure 37) whereas further degradation of those peroxide products into TBARS products, reached the maximum on the 15th day as shown by the TBARS results of the control Figure 39).

Although an initiator has been used extensively to induce peroxidation in many peroxidation assay systems, in this research the air gap above the incubation mixture in the screw-capped bottle induced the autoxidation of linoleic acid. In the presence of an initiator, lipid peroxidation occurs rapidly. Previous studies have shown that the initiator can affect the antioxidant activity of an extract resulting in inaccurate measurements, making it difficult to interpret the results of the antioxidant determination.

The results of both the FTC and TBARS methods indicate that the E4 and EB extracts of *Euphorbia peplus* and V3 and VB extracts of *Viola odorata* possess higher inhibitory activity than the standards, α-tocopherol and trolox, at concentrations of 200 μg/ml when the peroxidation of linoleic acid is undertaken in an ethanolic-aqueous medium *in vitro*. According to Wanasundara and Shadhidi (1994), the purpose of using the linoleic acid assay system was to prolong the induction period of autoxidation, in order to improve the oil stability. As evident from the above results, incorporation of all the extracts prolonged the incubation period of linoleic acid, as shown by the low rate of accumulation of oxidant products compared to the control and the standards used.

5.3.2.2 DPPH-Free radical scavenging activity method

As discussed in Section 1.6.3.1.2 of Chapter 1, oxygen radicals and lipid peroxidation play a crucial and perhaps causative role in the pathogenesis of a number of chronic and acute diseases, such as cancer (Esterbauer, 1996). Since formation of superoxide anion (O_2^-), which can transform into more reactive hydroxyl radical and other reactive oxygen species that could induce tissue damage, putative anticancer activity of the plant extracts could well be tested on the basis of their ability to scavenge these free radicals.

Since radical reactions of peroxyl radicals are kinetically complex, the reactions of hydroxyl radicals are extremely rapid, rather unspecific and they do not easily lend

themselves for investigation. In an earlier study, Bors and Saran (1987), selected azide radicals as the primary oxidising species.

In the present study, we used the stable, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical using the assay for 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity described in Section 2.6.4 of Chapter 2.

The DPPH assay is based on the incubation of methanolic solution of DPPH in acetate buffer to produce the DPPH radicals. The DPPH is a purple coloured, relatively stable free radical. It becomes reduced to yellow coloured diphenylpicrylhydrazine by radical scavenging antioxidants. The absorbance of DPPH in different incubation mixtures having different pH values has been measured at different absorbance maxima, i.e., pH 7 at 540 nm (Kirby and Schmidt, 1997), pH 5.5 at 517 nm (Sassa *et al*, 1990; Hong, 1994).

However, according to our results, the final DPPH concentration of 250 μM, specified in the method used by Kirby and Schmidt (1997) and the concentration of 200 μM, used by Duh and Yen (1997), did not allow the measurement of DPPH radical scavenging activity of slow reacting plant extracts due to the low sensitivity of the reaction mixture. In addition, the presence of DPPH radicals in an acidic media at a pH below 7 used by Sassa *et al* (1990) and a pH of 5.5 were not stable enough to complete an experiment involving a number of samples and replicate measurements within the specified time window of 30 minutes.

Increasing the pH of the medium increased the stability of the free radicals. The pH of the acetate buffer was made up to 8.8 in order to maintain stable DPPH radicals as it has been shown that an alkaline pH stabilizes the azo free radicals (Bors and Saran, 1987). Hence the DPPH assay methods adopted by Sassa *et al* (1990) and Lee *et al* (1996) was modified according to Bors and Saran (1987) and Hong (1994), the final DPPH concentration was changed to a concentration of DPPH of 100 μM and a pH of 7.8.

Before the measurements were made, the absorbance maxima of the DPPH incubation mixture was determined after obtaining the visible spectrum of the DPPH incubation mixture against a reagent blank, using a UV-VIS spectrophotometer.

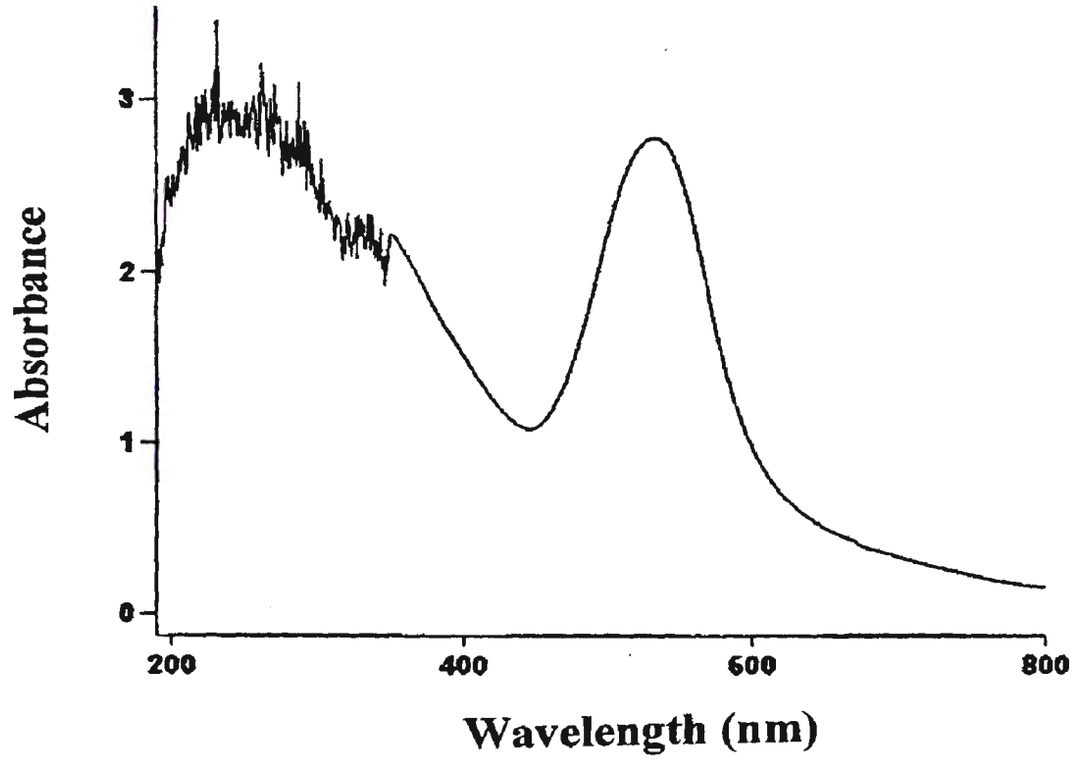

Figure 40. The UV-Visible spectrum showing absorbance of DPPH reagent in the control, using DPPH method.

The UV-VIS spectrum of the DPPH incubates obtained for the plant extracts and the standards are given in Figure 40. According to Figure 40, the absorbance maxima obtained for the DPPH incubation mixture was 530 nm. The visible spectra of the plant extracts, E4 and V3 at the same concentration used in the DPPH method, were obtained and examined for any interfering plant pigments at 530 nm. The E4 and V3 extracts did not contribute to any detectable interfering absorbance at 530 nm at the concentrations used in the experiment. Although Arnao (1999) suggested possible interference from wine extracts in DPPH measurements at 515 nm, our results indicated no interference from the matrices of E4 and V3 extracts in DPPH measurement at 530 nm.

The results of experiments designed to measure 1,1-diphenyl-2-picryl-hydrazyl free radical scavenging activity (FRSA) using the methods described in section 2.8.3 of Chapter 2 are illustrated in Figure 41.

According to the results shown in Figure 41, the *in vitro* DPPH scavenging activity in the methanol medium, given by α-tocopherol, trolox and the E4 extract of *Euphorbia peplus* and V3 extract of *Viola odorata* at 200 μg/ml were 78±3, 80±2, 31±2 and 13±3% respectively. Although there was no significant difference ($P>0.05$) between the higher % DPPH scavenging values of α-tocopherol and trolox, both plant extracts, E4 and V3 showed significantly ($P<0.05$) lower activity *in vitro* in terms of DPPH scavenging values. In addition, the concentrations that scavenge 50% of the DPPH radicals of the control (IC50) were 63±13, 65±16, 626±23 and ~1171 μg/ml for α-tocopherol, trolox, and for the extracts, E4 and V3 respectively.

The results showed that the E4 extract showed less than half the DPPH scavenging activity compared to the standards whereas the V3 extract gave a DPPH scavenging activity approximately 3 times lower than that of E4 extract.

Hence the sequential, 70% EtOH-aqueous (E4) extract of the putative anticancer plant, *Euphorbia peplus* and to a lesser extent the CHCl3-MeOH (1:1) extract, V3 of the putative anticancer plant, *Viola odorata* showed *in vitro* DPPH free radical scavenging activity within the time window employed in the experiment.

Both the radical scavenging and metal-chelating activities of plant flavonoids have been proposed for their antioxidant functions (Bors and Saran, 1987).

The presence of flavonoids in extracts of both the plants, *Euphorbia peplus* (Dunkow & Pohl, 1973) and *Viola odorata* (Rizk *et al*, 1980) has been reported. The flavonoid antioxidant effect is often considered only in terms of a direct single biochemical reaction with free radicals (DiSilvestro, 2001). Scavenging of free radicals is defined as the direct reaction of plant flavonoids with free radicals wherein the unpaired electron of a radical becomes paired without ultimately generating another free radical (Kehrer, 1993). Free radical scavenging mostly observed in plant extracts containing phenolic compounds might be interpreted according to Sherwin (1978) and Dziezak (1986) as the probable interception of the free-radical chain of oxidations by the antioxidants in the extracts.

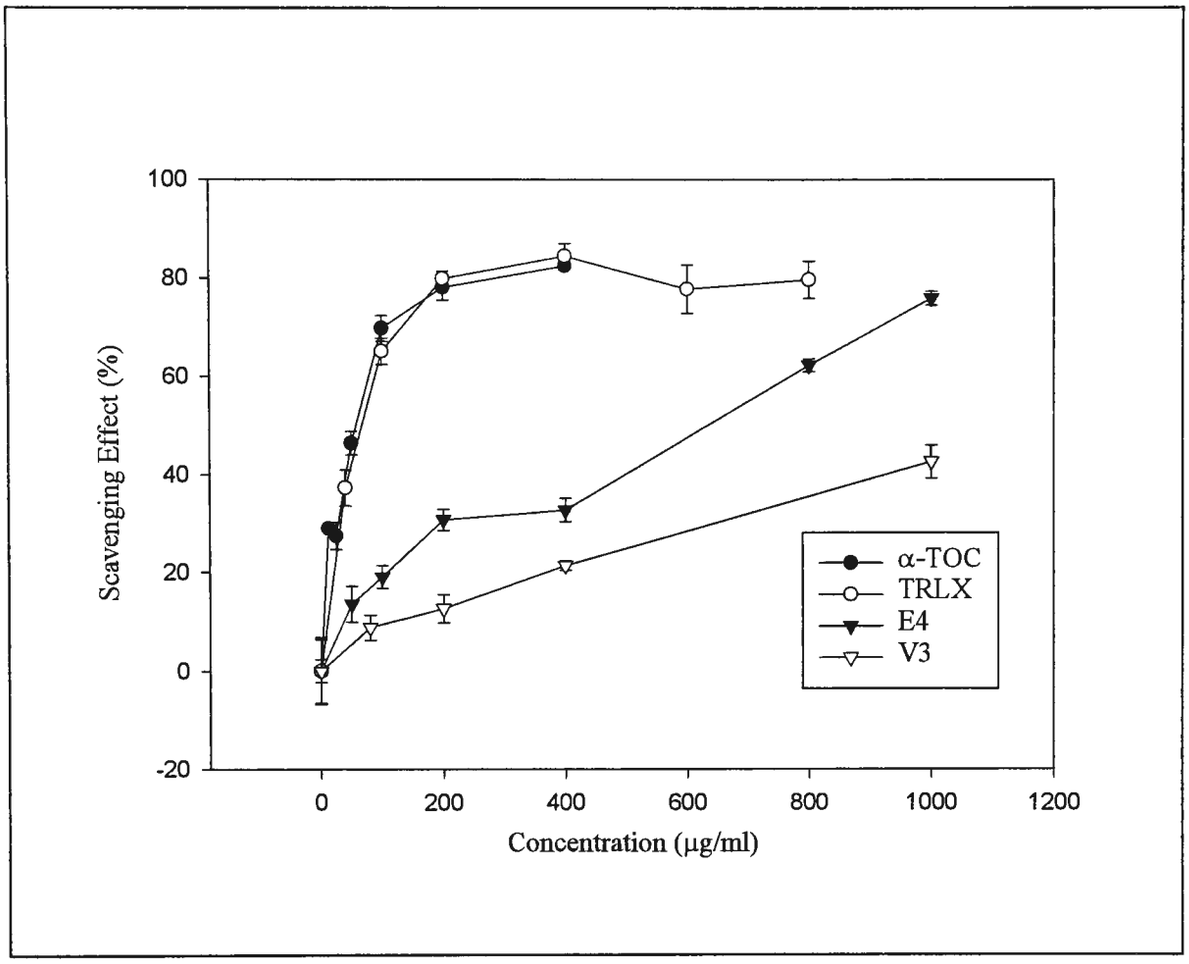

Figure 41. Percent DPPH free radical scavenging activity with increasing concentration of E4 extracts of *Euphorbia peplus* and V3 extracts of *Viola odorata* compared with the standards, α-tocopherol and trolox, using the DPPH method. Each data point is the average of 4 replicate absorbance measurements ± SD obtained at 530 nm and calculated on the assumption that the absorbance of the control was 0% of the scavenging activity.

The mechanism of action may involve the release of hydrogen from the phenolic hydroxyl group to form stable free radicals, which do not initiate or propagate further oxidation of lipids.

Given the dual function of many plant antioxidants to exert both the pro-oxidant activity and antioxidant activities, further research is required to understand whether the reported

prooxidant activity of some plant antioxidants is due to the reaction medium, use of isolated tissue *in vitro* models, the concentration of antioxidant used, the nature of the antioxidant or whether it is synthetic stereo isomer or the naturally occurring isomer.

The DPPH has been used as a free radical to evaluate antioxidant activity of some natural extracts (Shimada, 1992; Yen and Chen, 1995; Yen and Duh 1995).

In living systems, a wide variety of antioxidants play a protective role in scavenging reactive oxygen species. When the indigenous antioxidants are no longer capable of scavenging the ROSs in a disease state, administration of antioxidant therapy using natural antioxidants may be useful. However, the efficacy of a natural antioxidant may depend on which ROS is generated, how it is generated, and what target of damage is measured (Halliwell and Gutteridge, 1995). Hence, it is important to establish an *in vitro* assay, which will be predictive of the *in vivo* free radical scavenging antioxidant activity of a plant extract. The correlation of the DPPH results with the results using other ROSs need to be established. Although our results based on *in vitro* assays may not necessarily predict the same effect *in vivo*, these results can give an insight into some possible actions *in vivo*.

5.3.2.3 FRAP (TPTZ) method

The FRAP assay, that measures the Fe^{3+} reducing ability of an antioxidant, has been used to determine the ascorbic acid content in the plasma (Day *et al*, 1979; Lui, 1982; Benzie, 1996), serum iron (Ichida *et al*, 1968; O'Malley *et al.*, 1970) and urate (Morin *et al*, 1974) contents, to compare the antioxidant potential of propofol with α-tocopherol (Ansley, 1998) and to measure the ferric reducing antioxidant power of plasma (Benzie and Strain, 1996). The direct reaction between an antioxidant and the Fe^{3+}-TPTZ may qualify the application of the TPTZ method to plant extracts as a direct, convenient and relevant method to determine not only the antioxidant capacity of the plasma but also that of a plant extract.

In this study, we tested the possibility of using the FRAP assay for the determination of Ferric-TPTZ reducing antioxidant activity of plant extracts. In doing so, we carried out preliminary absorbance measurements using the sequential extracts of *Euphorbia peplus* and *Viola odorata,* the boiling water extracts EB and VB and the standards, α-tocopherol and

trolox. The extracts that showed higher TPTZ-absorbance values indicating higher ferric-TPTZ reducing antioxidant activity were selected for TPTZ method development.

In order to determine whether iron in the form of Fe^{2+}, interferes with the TPTZ determination, all samples were tested for the presence of Fe, using an atomic absorbance spectrophotometer. We found no detectable Fe present in the plant extracts.

The fixed–time FRAP results for the 200 μg/ml solutions of the extracts, E1 to E6 of *E. peplus*, (Figure 34) showed that the average absorbance value obtained for the E4 extract, was significantly higher at 37±1 (SD) ($P<0.05$) when compared with the other samples. Similar determinations for V1 to V6 of *V. odorata*, (Figure 34) showed a significantly ($P< 0.05$) higher result of 11±1 (SD) for V3 compared with the other samples. On the basis of these results, which showed a high sensitivity, for the E4 and V3 extracts were used for the TPTZ method development.

The absorbance spectra of the selected plant extracts, E4, V3 and the standard, α-tocopherol, after reacting with the Fe- (III)-TPTZ reagent, are shown in Figure 42. According to data shown in Figure 42, α-tocopherol as well as the plant extracts E4 and V3 gave absorbance maxima of 593 nm after reducing the Fe- (III)-TPTZ reagent to a blue colour Fe- (II)-TPTZ complex.

5.3.2.3.1 TPTZ reaction kinetics

In order to test the applicability of the TPTZ method for plant extracts, reaction kinetics of the TPTZ system with the plant extracts, E4, EB, V3 and V4 and the standard, α-tocopherol, were investigated for 12 minutes.

According to the data shown in Figure 43, an almost linear absorbance curve were obtained for the plant extracts as well as for α-tocopherol. On the basis of these data, the reaction follows first-order reaction kinetics over the 12 minutes reaction period. A rapid reaction was shown by α-tocopherol with TPTZ resulting in a higher absorbance soon after addition of the reagent whereas E4 showed a lower absorbance and a slower reaction compared with α-tocopherol (Figure 43).

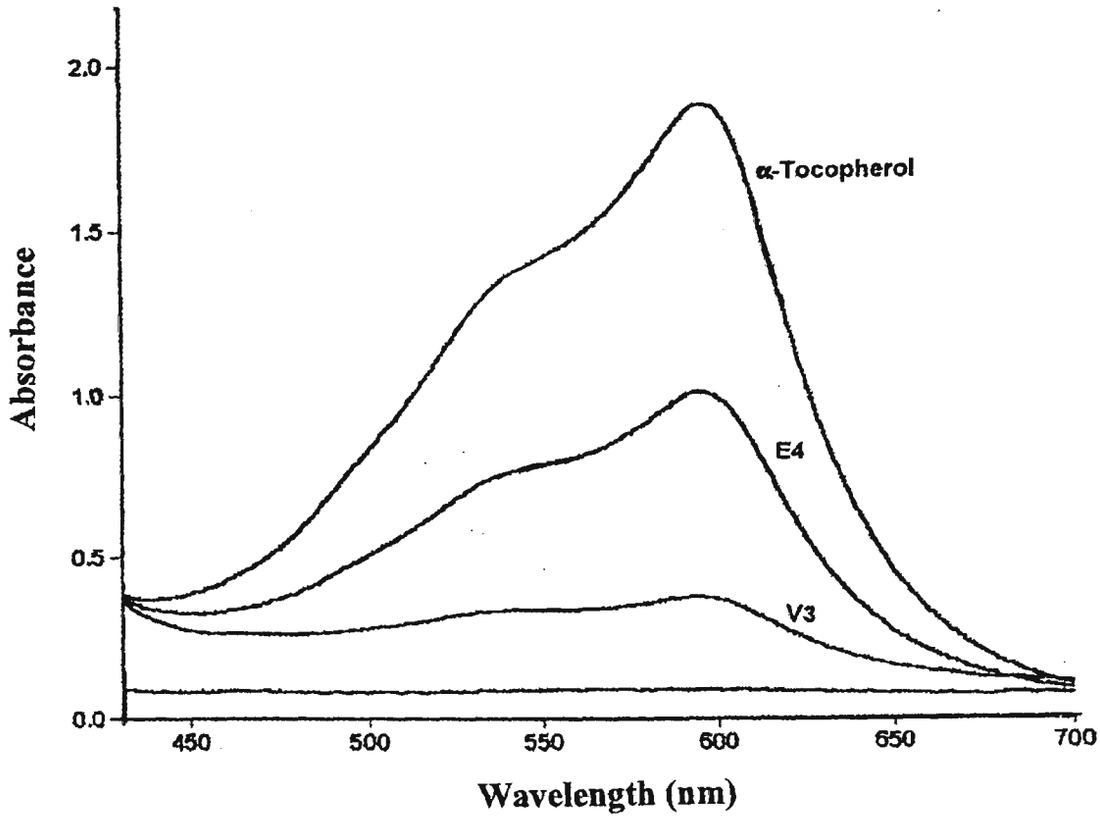

Figure 42. UV-Visible spectra showing absorbance of the Fe (II) TPTZ complex after reacting with, E4 extract of *Euphorbia peplus*, V3 extracts of *Viola odorata* and the standard, α-tocopherol compared with the control (shown as a horizontal line), using the TPTZ method.

The absorbance reading for the sample, V3 was lower than that of E4 sample indicating that the reaction of E4 and V3 with TPTZ were slower than that of α-tocopherol. Although the automated Cobas Fara analyzer allowed the measurement of initial absorbance values for α-tocopherol and other standards as reported by Benzie and Strain (1996), the delay between the addition of reagent and activation of the cell transport assembly of the semi automated multi-cell chamber used in our experiments did not allow the measurement of initial data points.

Hence the initial data points were actually measured around 40 seconds after addition of the reagent to all the samples in the multi-cell chamber (Figure 43).

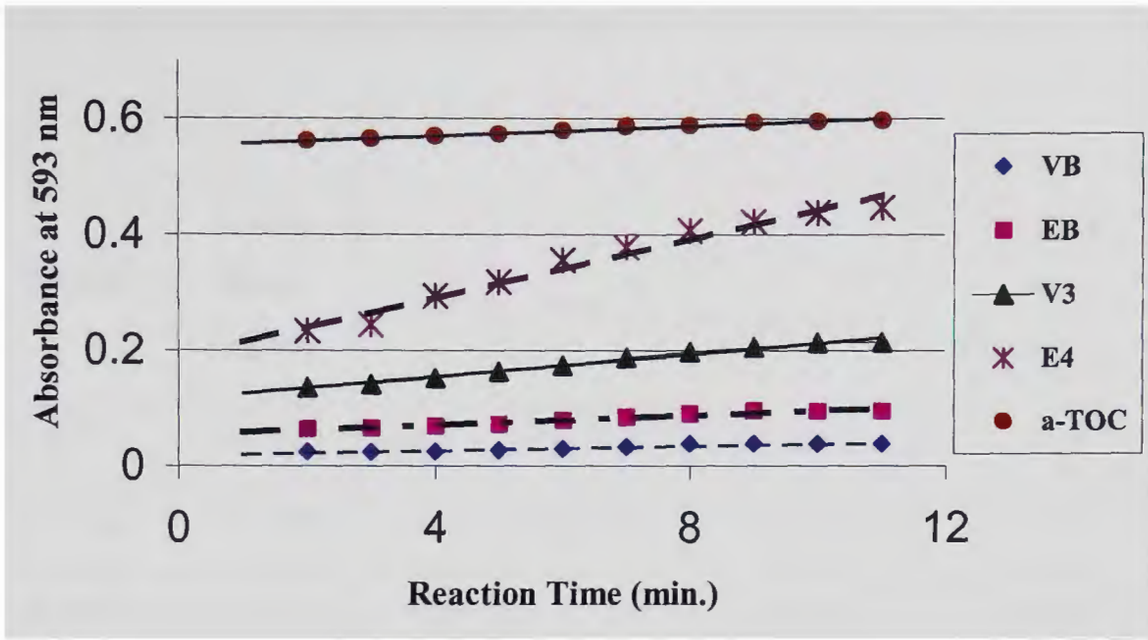

Figure 43. Reaction kinetics of TPTZ with plant extracts, E4 and EB of *Euphorbia peplus* and V3 and VB of *Viola odorata* compared with the standard, α-tocopherol.

A 5 min. reaction time was chosen as an arbitrarily point to obtain a comparable measurements among samples. This reaction time was used to optimise the other parameters including the dose-response curve discussed below.

According to data shown in Figure 43, the absorbance values for the samples tested continued to increase beyond the 5-minutes monitoring window. However, the selection of 5 minutes reaction time point did allow comparison of the ferric-TPTZ reducing rate, thus the ferric-TPTZ reducing power of the antioxidant sample.

5.3.2.3.2 Linearity of the dose-response curves

The dose-response results obtained for the two plant extracts, E4 and V3 and for the standards, Fe^{+2}, α-tocopherol, trolox, and quartering, are given in Table 23.

Table 23. Summary of results obtained for plant extracts and the standards using TPTZ method. Showing Ferrous standards (Fe^{2+}), DL-α-Tocopherol (α-Toc), Trolox and the extracts, E4 of *Euphorbia peplus* and V3 of *Viola odorata*.

Sample	Linear Range (µg/ml)	r^2 [a]	Slope [b]	%FRAP Value [c] (Slope based)	%FRAP Value [d] (Fixed Time)
Fe^{2+}	20 – 400	0.979	5.90×10^{-3}	100±6	100±5
α-Toco	50 – 400	0.985	5.60×10^{-3}	95±7	83±10
Trolox	10 – 200	0.998	11.5×10^{-3}	195±6	164±10
E4	50 – 1000	0.991	2.20×10^{-3}	37±3	36±2
V3	50 – 1000	0.958	6.00×10^{-4}	10±4	14±4

[a] The correlation coefficient of the dose-response line
[b] The slope of the linear dose response trend line given as the absorbance units per unit sample concentration.
[c] The relative ferric reducing antioxidant potential (FRAP) values were derived by dividing the sample slope value by the slope value of the ferrous standards, which was shown as a percentage (n=11±SD).
[d] The fixed time, FRAP values obtained for 200 µg/ml solution of the samples (n=11±SD). FRAP values were calculated taking FRAP of ferrous standard as 100%.

According to the results shown in Table 23, the extracts, E4 and V3 showed a linear response within a wider concentration range (50-1000 µg/ml; with r^2=0.991 and 0.958 respectively) compared to the standards. The FRAP results were calculated, by dividing the slope of the linear dose-response curve of each sample by the slope of that of Fe^{+2} standards, taking as unity. These results, which are shown in Table 23, support the observation made by Benzie *et al* (1999) that the antioxidant efficiencies of the standards and the samples tested are not dependent on their concentrations within their linear ranges.

The Fe^{+2}-based, FRAP values indicate the stoichiometry of the antioxidant to the TPTZ reactant for ferrous standard, α-tocopherol and trolox, these values were 100±6, 95±7,

195±6 % (close to 1, 1, and 2) respectively. The corresponding values obtained by Benzie and Strain (1996) for FRAP of both trolox and α-tocopherol, using TPTZ method in an automated Cobas Fara analyser, were 1. The reduced values obtained for α-tocopherol (Table 23) could have been due to prooxidant activity in the aqueous medium.

As for the plant extracts, E4 and V3, the slope-based FRAP values (37±3 and 10±4) were significantly ($P<0.05$) lower than that of Fe^{+2} (100±6) and the standards. However, the E4 and V3 extracts gave low FRAP values whereas the antioxidant standards, trolox and α-tocopherol gave higher FRAP values of 195±6 and 95±7 respectively over a narrow (10-200 and 50-400 μg/ml) linear range.

5.3.2.3.3 Precision of the TPTZ method

The automated measurement of ascorbic acid in plasma using TPTZ method according to Benzie (1996) gave an average within-run (W/R) CV of 5% (n=5). The average W/R CV was <6% (n=25) for the standards at 200 μg/ml concentration using TPTZ method. The comparable W/R CV for the TPTZ method obtained for the plant extracts was 4.5% (n=25) showing a good precision for both the standards and the plant extracts.

5.3.2.3.4 Reproducibility of the TPTZ method

Although higher between-runs (B/R) CVs were observed for lower absorbance measurements in all samples, a reasonably good reproducibility (B/R CV = 4.5%; n=11) was shown for the standards using TPTZ method. Liu *et al* (1982) reported a B/R CV of 3% for the TPTZ method using ascorbic acid-supplemented serum. Our results with plant extracts gave a B/R CV of =6.8% showing a good reproducibility of the method for crude plant extracts such as E4 and V3.

5.3.2.3.5 Interference with measurements

The visible spectra of the Fe^{2+}-TPTZ chromogens of E4, V3 and α-tocopherol were shown earlier, in Figure 42. As shown in Figure 42, the absorbance maxima of both E4 and V3 are similar to that of α-tocopherol and very close to 593 nm. The concentration of the acetate buffer and the pH of the reaction mixture were shown to be important in obtaining accurate measurements at 593 nm.

The absorbance spectra obtained for 1000 μg/ml solutions of the plant extracts in the reagent blank, without the reagent, added showed no absorbance around 593 nm. This may be due to the extraction of pigments into petroleum spirit and $CHCl_3$ during the early stages of the sequential solvent extraction of the plants. On the other hand, interfering plant pigments would have led to an overestimation of the TPTZ measurements.

5.3.2.3.6 Recovery of added trolox

The matrix effect of a plant extract on the accuracy of method was studied by adding known amounts of trolox to the E4 plant extract. Trolox was used for these recovery studies since it is a stable analogue of α-tocopherol. Our results indicated that 97-105% (n=5, CV=5%) of the trolox was recovered from the E4 extract over a concentration range of 25–200 μg/ml showing a good recovery.

The expected recovery was calculated based on the absorbance values obtained for the same concentrations of trolox solution without any E4 added. On the basis of our results the plant extract matrix did not significantly ($P>0.05$) inhibit or synergistically enhance the reducing reaction of trolox with the TPTZ reagent.

5.3.2.3.7 FRAP values

The fixed-time FRAP values given in Table 23, were the % absorbance values of the samples at a concentration of 200 μg/ml against the TPTZ-reagent blank. For all of the samples tested, except for trolox, there was no significant difference between the slope-based and fixed-time FRAP values. The significant difference shown between slope-based and fixed-time FRAP values of trolox was due to the measurement taken at the upper limit of the linear dose-response curve for trolox. Although the slope-based FRAP measurements were made using different concentrations of the sample, absorbance readings at each concentration, were made at a fixed time. Hence the slope-based FRAP values that depict the TPTZ-reaction kinetics of the test sample also can be expressed as a fixed-time FRAP values, provided the absorbance measurement are taken within the linear range of the dose-response curve.

According to our results, there was no significant difference ($P> 0.05$) between the fixed time absorbance values and the values calculated on the basis of the slopes of the dose-response curves for E4 and V3 (37±3, 36±2, $p>0.05$ and 10±4, 14±4; $P>0.01$,

respectively). However, trolox and α-tocopherol showed much lower fixed time absorbance values of 164±10 and 83±10 compared to the slope-based values of 195±6 and 95±7 respectively. The low fixed time absorbance value compared with the slope-based value obtained for trolox may be due to the relatively high trolox concentration of 200 μg/ml, which was slightly higher than the linear range of the assay. Had the assay been repeated at a concentration of 150 μg/ml for each sample, more comparable slope-based and fixed time FRAP values might have been obtained. This disparity of results can be overcome by using slope values obtained from the linear dose-response line.

Overall, the TPTZ method, based on the fixed time absorbance measurement, is rapid, required only one concentration, and if used within the linear range would provide results which are very close to those obtained from the slope of the dose response data. During this study we did find that antioxidant standards with different structures and functional groups, because of steric hindrance, reacted very slowly with the ferric-TPTZ solution, indicating a limitation of the fixed time FRAP assay from being a one-off measurement of total antioxidant activity.

5.3.2.4 Comparison of Methods

In order to enable a comparative evaluation, the antioxidant activities, for several samples at a concentration of 200 μg/ml, were determined for ferric reducing antioxidant activity (TPTZ), DPPH-free radical scavenging activity, and linoleic peroxidation inhibition by thiobarbituric acid reacting substances (TBARS) and the ferric thiocyanate (FTC) methods. These results are shown in Figure 44.

Figure 44 shows the quantitative results of the percent antioxidant activity values obtained for the plant extracts, E4 and V3 and for the antioxidant standards, trolox and α-tocopherol at the concentration of 200 μg/ml using TPTZ, FRSA, TBARS methods. The antioxidant activity values obtained using these assays were adjusted on the basis of the activity given by 200 μg/ml of trolox in each assay as 100%. Since ferrous standard was not used in the assays of DPPH and linoleic peroxidation using FTC and TBARS, all values were calculated based on a trolox activity value of 100%.

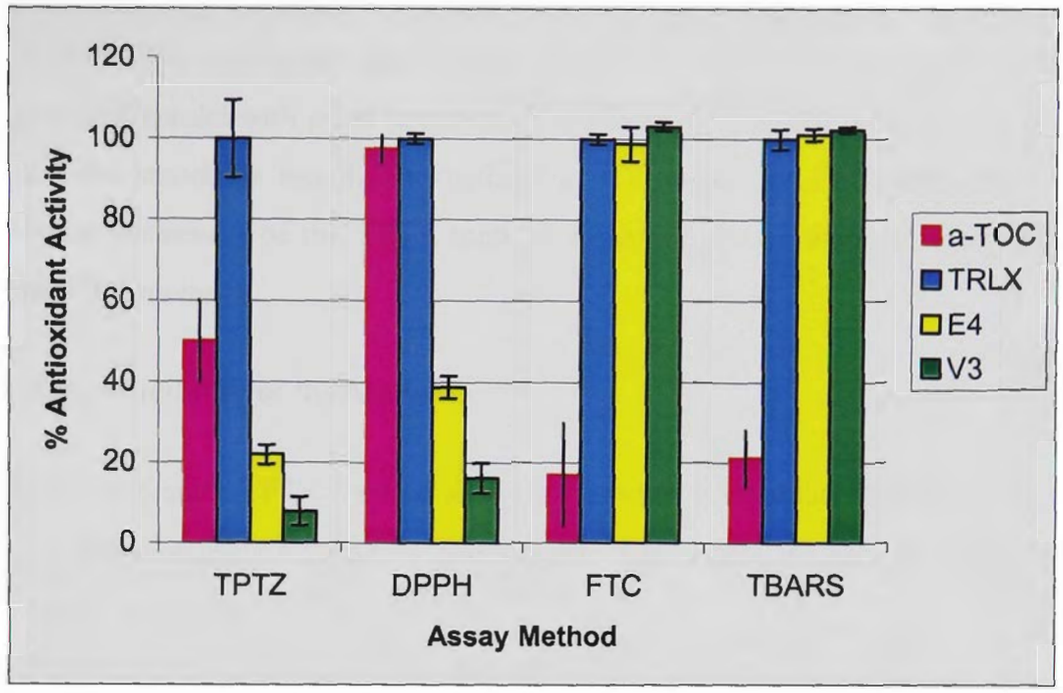

Figure 44. Comparison of antioxidant activity using TPTZ, DPPH, TBARS and FTC methods. Results of 200 μg/ml solutions of standards, α-tocopherol (a-TOC), trolox (TRLX) and plant extracts, *Euphorbia peplus* (E4), and *Viola odorata* (V3). Results are illustrated as the percent mean ± SD of 5 measurements.

The comparative FTC and TBARS were based on the day 8 FTC results (Figure 37) and the day 9 of TBARS results (Figure 39). According to data shown in Figure 44, the plant extracts, E4 and V3 gave a linoleic peroxidation inhibitory antioxidant activity of 99±4, 101±1 and 103±1 and 102±1% for both the FTC and TBARS methods, respectively. These results were significantly higher ($P<0.001$) compared with the α-tocopherol standard, which gave values of 17±13 and 21±7, respectively.

The quantitative values obtained using TPTZ and DPPH-FRSA methods for the extracts, E4 and V3 (22±2, 39±3 and 8±4, 16±4 respectively) were significantly lower ($P<0.001$) than those of the standards, α-tocopherol and trolox (50±10, 98±3 and 100±10, 100±1 respectively). There was no significant difference ($P>0.001$) between the results obtained by TPTZ and FRSA methods for trolox.

5.3.2.4.1 Precision of the Methods:

Both the TBARS and FTC methods showed large variation in within-run CV values for the standards (5-15%) and for the plant extracts (8-25% W/R CV). In contrast the TPTZ method provided results with good precision (<5% W/R-CV, n=25) for both the plant extracts and the standards tested. The within-run CV values for FRSA method were <9.4%. Hence the results of the TPTZ method are more precise than those of FRSA, TBARS and FTC methods.

5.3.2.4.2 Reproducibility of the Methods:

According to our results, TPTZ method showed a good reproducibility for plant extracts, E4, and V3 (between-runs CV<6.6%) and for the standards, α-tocopherol and trolox (between-runs CV<7.6%).

However, a large variation was seen in FRSA results of E4 and V3 (B/R CV<27%) and of α-tocopherol and trolox (B/R CV<11%), which may be due to poor stability of the free radicals during the incubation period

The α-tocopherol and trolox results from the TBARS and FTC assays showed a poor reproducibility (36, 43 and 58, 50% B/R CV respectively). However the TBARS and FTC results of the plant extracts, E4 and V3 showed a very good reproducibility (8, 5 and 3, 3% B/R/ CV respectively).

According to Gray (1978), the FTC method showed an excellent reproducibility in comparison with iodometric and 2,6-dichlorophenolindophenol methods for peroxide levels following methyl linoleate lipid peroxidation. Although very high B/R CV values were observed for the standards, our FTC results for the plant extracts showed a very good reproducibility (<5% B/R CV) although precision was poor.

Despite poor reproducibility and possible interference by many substances the comparison of TBARS results with other methods for measuring lipid peroxidation such as diene formation, chemiluminescence, oxygen uptake, poly-unsaturated fatty acid loss and hydroperoxide has been satisfactory enough to warrant continued usage of the TBARS method (Slater, 1984). Both TBARS and FTC results show that the potency of the plant extracts to inhibit the peroxidation of linoleic acid are significantly ($P<0.05$)

higher than that of the standards. Mantle *et al* (1998) found wide variation in the results of FRSA for plant essential oils using three different free radical species and he stressed the importance of specifying the radical species employed in the experiment.

The high antioxidant activities observed in the results from the TBARS and FTC methods for both plant extracts may be due to a number of reasons including; a reduction of available oxygen or oxy free radicals at the initiation step; lipid free radicals at the propagation step or neutralization of the TBARS or FTC reacting peroxides in the incubation mixture. If the Inhibition of lipid peroxidation is due to scavenging of linoleic peroxide radical (LOO•), then both the TBARS and FTC results we obtained indicate that the E4 and V3 extracts exert stronger scavenging effect on LOO• radicals than on DPPH radicals.

According to our results the modified TPTZ method described in this study showed high precision and reproducibility for both the plant extracts and the standards compared to results obtained by the TBARS and FTC methods. The TPTZ method is labour efficient with 15 minutes per sample whereas incubation period for lipid peroxidation in the TBARS and FTC methods spans over 15 days.

The TPTZ method directly measures the ferric reducing antioxidant potential (FRAP), capacity or activity of an antioxidant whereas DPPH method directly measures the DPPH free radical reducing, scavenging or neutralising activity of an antioxidant. Both the TPTZ and DPPH measurements are made within a fixed time window, in order to rapidly compare the antioxidant activity of a substance at a certain concentration, with a known concentration of an antioxidant standard. Although these rapid fixed time measurements do not involve both the reaction time (TPTZ) or inhibition time (DPPH) and the degree of reaction or inhibition measurements into a ingle quantity, as in the case of area-under-curve technique used in the ORAC assay (Cao *et al*, 1997), they still may be used to determine whether a sample possesses a ferric reducing and DPPH scavenging antioxidant capacity within the fixed time window and the degree of reducing power or inhibition within the given time compared to a known standard antioxidant.

Ansley (1998) used TPTZ results of α-tocopherol and propofol to evaluate oxygen derived free radical scavenging activity of propofol. Our results also suggest that the rapid

TPTZ method may be a substitute for DPPH-FRSA method when determining antioxidant activity of the plant extracts such as E4 and V3.

Nollet (1996) have indicated the difficulties in using the Fe- (III)-TPTZ method in determining the antioxidant activity of food antioxidants such as BHA, BHT and NDGA, because of the fact that this method measures the absorbance, of the Fe- (II)-complex formed from the reaction of Fe- (III)-TPTZ reagent with the antioxidant. It was shown that the reaction depends only on the concentration of the antioxidant Nollet (1996). Low selectivity of the reagent, interference of any other reductant capable of reducing the reagent, necessity of a time control for slow reacting antioxidants like BHT and the poor stability of the reagent in the light have been cited as the drawbacks of the TPTZ method (Nollet, 1996).

Further testing of different polyphenolic antioxidant standards with the Fe- (III)-TPTZ reagent, indicated that compounds having large molecular structures reacted more slowly with the reagent than the ones with relatively smaller molecular structures. This slow reactivity may be due to the steric hindrance caused by the three pyridyl rings surrounding the central triazine ring of the Fe- (III)-TPTZ molecule partially shielding the molecule being reduced by the antioxidant.

The lower absorbance values given by the TBARS and FTC methods, for the E4 and V3 extracts, obtained after twelve days of incubation showed a strong inhibition of linoleic acid autoxidation for incubation times up to twelve days. Hence these extracts prolonged the induction period of linoleic autoxidation *in vitro*. Similarly, E4 and V3 extracts may inhibit peroxidation of membrane lipid under oxidative stress conditions in biological systems.

The results obtained for linoleic peroxidation inhibitory antioxidant activity, using the FTC and TBARS methods for α-tocopherol (16±12, 17±6%) were significantly ($P<0.05$) less than of those (83±6 and 164±12) obtained using TPTZ method indicating a higher sensitivity of TPTZ method for α-tocopherol and trolox. On the other hand the difference in results may also be due to the reaction medium used in the assays.

The sensitivity of the TPTZ and FRSA methods for both α-tocopherol and trolox were approximately double those of the TBARS and FTC methods (Figure 44). Although the

possibility of interference by plant pigments in the TBARS measurements at 532 nm was proposed previously by Hagege *et al* (1990), the use of the sequential extraction procedure, as evidenced by the lack of absorbance around 532 nm in the visible spectra of E4 and V3 extracts, may have eliminated most of such interfering pigments during the early stages of extraction. However, both the TPTZ and FRSA methods showed low sensitivity to the plant extracts, E4 and V3, compared to results obtained using the TBARS and FTC methods.

The E4 extract from *Euphorbia peplus* gave an average value of 37±1 % for relative ferric reducing antioxidant potential using the TPTZ method, 31±7% DPPH-free radical scavenging activity using the FRSA method and 81±13% inhibition of linoleic autoxidation, on the basis of the averaged TBARS and FTC results.

The *Viola odorata* extract, V3 showed an 11±1% relative ferric reducing antioxidant potential, 14±4% DPPH-free radical scavenging activity and 96±1% inhibition of linoleic autoxidation.

We found that the inhibitory potential of the plant extracts on the lipid peroxidation as measured by TBARS and FTC methods do not correspond directly with the DPPH free radical scavenging and ferric reducing potentials. Hence the plant extracts tested show differential antioxidant activities towards lipid peroxidation and free radical scavenging.

There was no significant difference ($P>0.05$) between the results of the TPTZ method and those obtained using the FRSA method for the plant extracts and α-tocopherol standard. Hence the rapid TPTZ method may be a substitute for DPPH free radical scavenging activity (FRSA) method for determining ferric reducing antioxidant activity of the plant extracts such as E4 and V3. Although the TPTZ assay does not measure the SH-group containing antioxidants, Prior and Cao (1999) found a weak but significant linear correlation between serum oxygen radical absorbance capacity (ORAC) and serum TPTZ (FRAP) results.

In summary, the TPTZ method, on the basis of the present results, can be used to determine the ferric reducing antioxidant activity of crude plant extracts such as E4 from *Euphorbia peplus* and V3 from *Viola odorata* with no apparent interference from the complex plant matrix.

Chapter 6 Determination of immunomodulatory activity of *Euphorbia peplus* L. and *Viola odorata* L.

ABSTRACT

Immunomodulatory activity is defined as a biological or pharmacological effect on humoral or cellular factors, which results in a specific, nonspecific or combined effect. An immunomodulatory effect suggested to be attributed to a plant extract with claimed anticancer activity that does not show a direct anticancer activity when applied in a small quantity. A number of immunomodulatory compounds isolated from natural sources have shown antitumour activity *in vivo*. Although anticancer, inflammatory, antipyretic and irritant activities of *Euphorbia peplus* have been investigated, its immunomodulatory activity and that *of Viola odorata* has not been reported to date. Hence an investigation was carried out to determine whether the putative anticancer effect of *Euphorbia peplus* and *Viola odorata* is due to their immunomodulatory activity.

Anti-inflammatory activities, including anti-platelet aggregation and cyclooxygenase-2 (COX-2) inhibition are considered to have immunomodulatory activities because of the mediation of the immune system. Chronic inflammatory conditions are known to be associated with lung, bladder, bowel, breast, skin and stomach cancers. Most platelet aggregation antagonists when used at high doses, are also known as potent cytotoxic anticancer agents and act as immunostimulators at low doses. Malignant cells as in the case of intestinal cancer reportedly generate the cyclooxygenase (COX-2) enzyme to accelerate their growth. Hence COX-2 inhibitors may possess both anti-inflammatory as well as anticancer activities.

Hence, in this study, sequential extracts of *Euphorbia peplus* were investigated for *in vitro* anticomplement activity via classical and alternative pathways using complement from pooled human sera. In addition, the anti-inflammatory activity was studied, using an assay that measured inhibition of platelet aggregation. COX-2 activity was determined using a TLC separation of the reaction mixture, followed by measurement of the radioactive arachidonic acid and/or its metabolites.

The results indicated that sequential $CHCl_3$-MeOH (E3) and 1M NH_4OH (E5) extracts and the boiling water extract (EB) of *Euphorbia peplus* inhibited complement activity via the

classical pathway giving IC50 values of 30±9, 94±14, and 22±16 μg/ml, respectively, compared to a value of 37±8 μg/ml for inulin. Inhibition of complement activity however, via the alternative pathway, by these extracts was not pronounced as in the case of classical pathway inhibition. The HPLC fractionation of the $CHCl_3$-MeOH extract yielded five fractions using a gradient of acetonitrile-water (with 0.5% trifluoric acid) on a semi-preparative C_{18} column. The fifth collected fraction (E3F5) gave a 15-fold increase (2 μg/ml) of the anticomplement activity via the classical pathway. The above results of *in vitro* study showed that the putative anticancer activity of *Euphorbia peplus* might be due to its immunomodulatory activity by potential activation of complement *in vivo*.

Results of the platelet aggregation assay indicated a 73±9% and 19±8% inhibition of platelet aggregation for the extracts, V3 and VB of *Viola odorata L* respectively. The extracts E4 and EB of *Euphorbia peplus* gave 47±6% and 6±3% antiplatelet activity respectively.

The cyclooxygenase-2 assay gave a very high inhibition of 90% by the E4 extract of *Euphorbia peplus* whereas the V3 extract showed 85% inhibition of the COX-2 activity, at the concentration of 200 μg/ml compared with 94% inhibition by the potent COX-2 inhibitor, indomethacin at a concentration of 40 μg/ml.

According to our results E4 and V3 extracts of both plants *Euphorbia peplus* and *Viola odorata* showed a considerable antiplatelet aggregation and COX2 inhibition activities *in vitro*. In addition, E3 extract of *Euphorbia peplus* indicated a considerable *in vitro* anticomplement activity. Hence, the putative anticancer effect of *Euphorbia peplus* and *Viola odorata L* may be due to their immunomodulatory activities, mediated possibly by the platelet activating factor, COX2 and complement in case of *Euphorbia peplus*.

6.1 Introduction

The immune system of the body functions basically to recognise and destroy the non-self or foreign organisms or substances that has entered into the system. This task of the immune system is achieved by cellular as well as molecular (humoral) immune factors exerting antigen-specific, as well as antigen-nonspecific immune responses.

Major known immuno factors that exert non-specific immune response are the complement system including C3a and C5a peptides; the phagocytes (polymorphonuclear neutrophilic leukocytes (PMNL), monocytes and macrophages); the reactive oxygen species (ROS) produced by the activated PMNL; monokines produced by other phagocytes that interact with the lymphocytes and the mediator cells (basophills, eosinophils, mast cells and platelets). These immunofactors release soluble mediators including, vasoactive amines, heparin, chemotactic factors, platelet-activating factor (PAF) and arachidonic acid metabolites (prostaglandins and leukotrienes) resulting an inflammatory reaction to the foreign organism or substance (Labadie, 1993). The major immuno factors that exert an antigen-specific immune response are T-lymphocytes including T-helper cells, T-suppresser cells, and cytotoxic T-cells), B-lymphocytes, natural killer cells (NK-cells); the immunoglobulins (IgM, IgG, IgA, IgD, IgE); and the lymphokines or interleukines (ILs) (Labadie, 1993).

The major known mediators that play an important role in an inflammatory condition are vasoactive amines (histamine), prostaglandins (PGD_2, PGE_2, PGF_2), thromboxane, leukotrienes (LTB_4, LTC_4, LTD_4, LTE_4), platelet-activating factor (PAF), peptides (substance P), adenosine and bradykinin (Labadie, 1993). Hence modulation of activities, of platelets and cyclooxygenase-2 (COX-2) (that metabolises prostaglandins), are also considered immunomodulatory activities.

As discussed in sections 1.6.3.2 to 1.6.3.3.2.2 of Chapter 1, it was suggested that the *in vitro* anticomplement activity might be exerting an immunomodulatory effect *in vivo*, resulting an anticancer effect. Anti-inflammatory activities, including anti-platelet aggregation and cyclooxygenase-2 (COX2) inhibition are also considered as immunomodulatory activities because of the mediation of immune factors. Chronic inflammatory conditions are known to be associated with lung, bladder, bowel, breast, skin and stomach cancers. Most antagonists of platelet aggregation, when used at high doses, are also known as potent cytotoxic agents in cancer treatment and act as immunostimulators at low doses.

Malignant cells, as in the case of intestine cancer, reportedly generate cyclooxygenase (COX-2) to accelerate their growth. Hence COX-2 inhibitors may possess both anti-inflammatory as well as anti-cancer activities.

A number of immunomodulatory compounds isolated from natural sources have shown antitumour activity *in vivo*. Although anticancer, paw-inflammatory, antipyretic and irritant activities of *Euphorbia peplus* has been investigated, its immunomodulatory activity and that *of Viola odorata* has not been reported to date. Hence an investigation was carried out to determine whether the putative anticancer effects of *Euphorbia peplus* and *Viola odorata* are due to their immunomodulatory activity.

6.2 Aim of the investigation

The aim of the investigation described in this chapter is to determine whether the putative anticancer effect of *Euphorbia peplus* and *Viola odorata* is due to their immunomodulatory activity. In order to achieve this goal the following investigations were carried out.

1. Determine *in vitro* anti-complement activity of *Euphorbia peplus* extracts and fractions and to investigate whether this occur via classical and alternative pathways.

2. Determine the *in vitro* anti-inflammatory activities, anti-platelet aggregation and inhibition of COX-2 activities of *Euphorbia peplus* and *Viola odorata* extracts.

6.3 Results and discussion

The sequential soxhlet extracts of *Euphorbia peplus* and *Viola odorata* used in the assays discussed in this chapter are summarised in Table 24. The assay method used are described in Section 2.7 of Chapter 2.

In addition to the sequential extracts given in Table 24, the boiling water extracts EB and VB of *Euphorbia peplus* and *Violoa odorata* were used in some of the assays described in this chapter. The method, used to prepare the extracts is given in Section 2.4.1 of Chapter 2.

Table 24 Sequential extracts used in the immunomodulatory assays

Sequence of Solvents	Extracts	
	Euphorbia peplus	*Viola odorata*
Petroleum spirit (40-60)	E1	N/D
$CHCl_3$	E2	N/D
$CHCl_3$-MeOH (1:1)	E3	V3
70% EtOH	E4	N/D
1N HCl	E5	N/D
10% NH_4OH	E6	N/D

N/D is not done

6.3.1 *In vitro* Screening for inhibition of human complement activity

The *in vitro* inhibition of human complement activation via the classical (CP) and alternative (AP) pathways was studied using sensitised sheep erythrocytes or normal rabbit erythrocytes, respectively, as target cells according to Kroes *et al* (1996). The assay method used was described in Chapter 2. The extracts of *Viola odorata* were not tested for the anticomplement activity, as the plant had not been collected at the time of testing. The results obtained using sequential extracts and HPLC fractions of *Euphorbia peplus* are discussed in the following sections.

6.3.1.1 *In vitro* Screening of *Euphorbia peplus* extracts for inhibition of human complement activity via the Classical Pathway (CP)

The results obtained using sequential extracts and HPLC fractions of *Euphorbia peplus* are discussed in the following sections.

The dose-response curves obtained for the extracts of *Euphorbia peplus* for the inhibition of complement activity via classical pathway (CP) are given in Figure 44.

According to Figure 44, a dose-dependent relationship was shown for the sequential extracts of *E. peplus*. The highest ($P<0.05$) *in vitro* human complement inhibitory potential via CP (IC50 <5 μg/ml) was shown by dextran sulphate whereas inulin, and the extracts, E3, EB, E5 gave IC50 values of 37±8, 30±9, 22±16, and 94±14 μg/ml, respectively. The extracts E4 and E6 gave IC50 values of 137±42 and 316±78 μg/ml, respectively, whereas

E1 and E2 gave IC50 values of > 1500 μg/ml. The boiling water extract, EB showed a significantly higher ($P<0.05$) inhibitory potential than the sequential extracts of *Euphorbia peplus*. Both EB and E3 showed significantly higher ($P<0.05$) *in vitro* complement inhibitory potential than inulin.

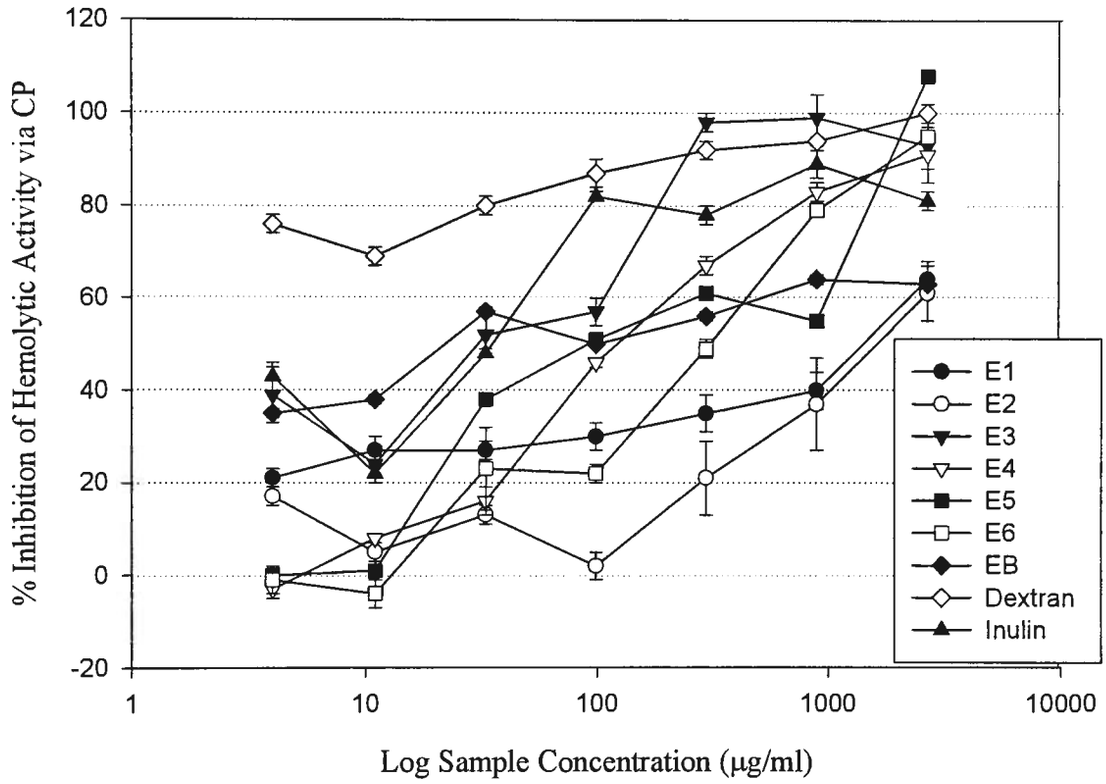

Figure 45 Dose-response curves for the percent inhibition of haemolytic complement activity via classical pathway (CP) against log sample concentration of the sequential extracts, E1, E2, E3, E4, E5 and E6 and the boiling water extract (EB) *of Euphorbia peplus* compared with dextran sulphate and inulin. Data points shown are the mean values with the error bars showing standard error of the mean (SEM) value of four replicate determinations.

Although EB showed a high ($P<0.05$) CP inhibitory potential, its highest inhibition was approximately 70% at the highest concentration tested. In addition, E1 and E2 showed a maximum 60% inhibition of the complement activity. The extracts E3, E4, E5 and E6 however, as well as the standards, dextran sulphate and inulin showed a strong, maximum inhibition between 80 and 100%.

The IC50 results obtained for dextran sulphate, was very close to the value (5.3 μg/ml) obtained by Bloksma *et al* (1980) in a similar *in vitro* experiment using human complement. Although the organic extract E3, showed less potency ($P<0.05$) than that of dextran sulphate, its complement inhibitory potency via CP was greater ($P<0.05$) than that of inulin. Hence the results of this investigation have provided a useful starting point for activity-guided isolation of immunoactive compounds from *Euphorbia peplus*.

6.3.1.2 *In vitro* Screening of *Euphorbia peplus* extracts for inhibition of human complement activity via Alternative Pathway (AP)

The results obtained for anticomplement activity of *E. peplus* extracts via alternative pathway (AP) are given in Figure 46. The boiling water extract, EB was not tested in the AP experiment.

Figure 46 shows that dextran sulphate, a sulphated polysaccharide, possesses the highest ($P<0.05$) inhibitory potential (IC50= 40±4 μg/ml) among the samples tested, for complement activation via alternative pathway (AP). All the other samples tested for AP, gave IC50 values, >100 μg/ml.

Comparison of results of Wagner and Yamada's research groups showed a good congruence for most of the anticomplement polysaccharides. These were found to be pectic heteroglycans with a relatively high proportion of galacturonan backbone. Most of the most active anticomplement polysaccharides from algae were found to be sulphated polysaccharides such as carrageenan, fucoidans and heparin (Wagner *et al*, 1987). Bloksma *et al* (1980), reported potent anticomplement activity of polyanions such as carrageenan, dextran sulphate, and polyanethosulphate (3.6-5.5 μg/ml) whereas suramin had a relatively lower anticomplement activity (50 μg/ml). The ε-aminocaproic acid (50 mM),

heparin (15 U/ml), L-lysine (20 mM) and suramin (0.02%) have been used as known complement inhibitors of classical pathway (Van Dijk *et al* , 1980). Hence the anticomplement activity showed by the aqueous extracts of *E peplus* may be due to polysaccharide derivatives present in the extracts.

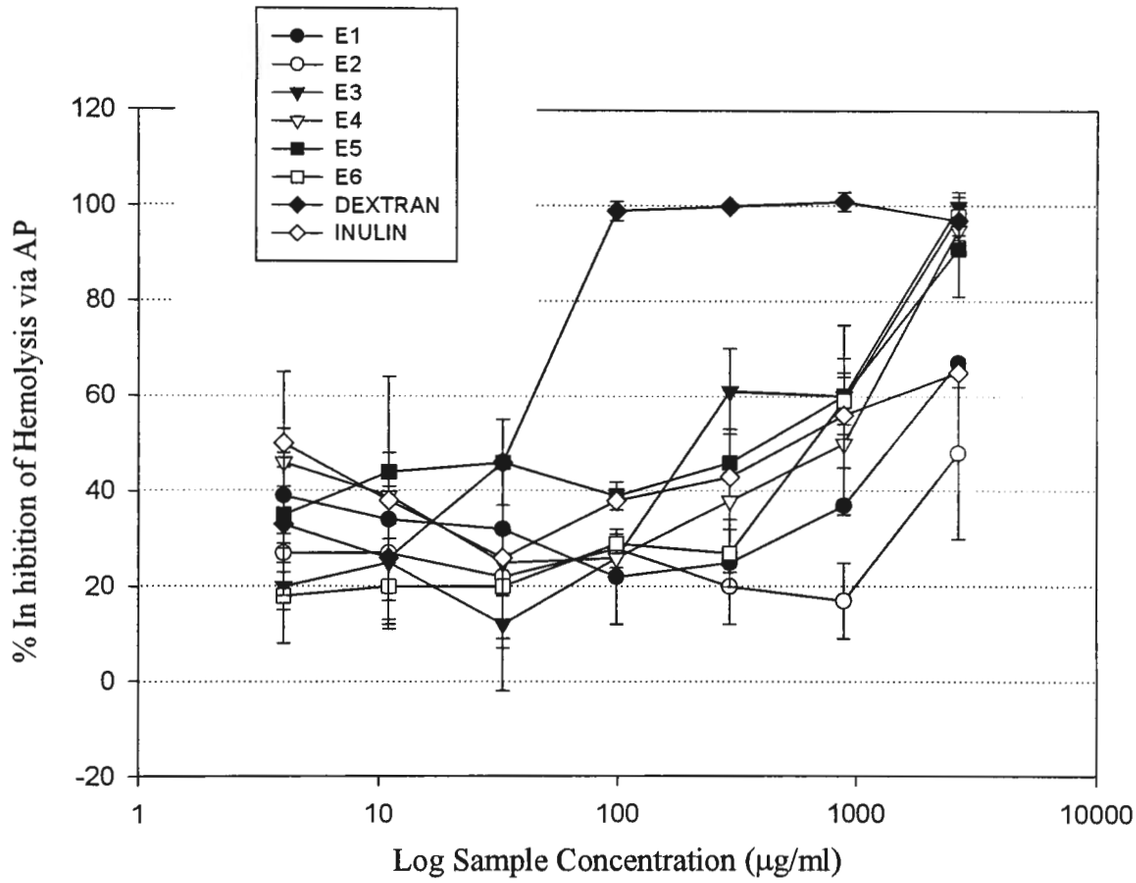

Figure 46 Percent inhibition of haemolytic complement activity via alternative pathway (AP) against log sample concentration of the sequential extracts, E1 to E6 and boiling water extract of *Euphorbia peplus* compared with dextran sulphate and inulin. Data points shown are the mean values with the error bars showing standard error of the mean (SEM) value of four replicate determinations.

Comparatively lower IC50 value among the extracts was shown by the E3 extract (31±9 μg/ml), when tested for inhibition of complement activity via classical pathway (CP). This effect was reflected in the AP results obtained for E3 (234 ±50 μg/ml). The extracts, E4, E5 and E6 gave AP-IC50 values of 889±133, 465±70 and 722±51 μg/ml respectively. As in the case of CP, both E1 and E2 gave IC50 values >1000 μg/ml when tested via AP.

Aqueous plant extracts (Nores *et al*, 1999. Ivanovska *et al*, 1995) as well as EtOH, MeOH and non-polar extracts dissolved in 1% DMSO in the concentration range of 0.1 to 1000 μg/ml have shown *in vitro* anticomplement activity (Labadie, 1993). However, according to our results, the non-polar and relatively less polar extracts, E1 and E2 (Table 24) prepared by dissolving in 1% DMSO did not show inhibitory potentials below 1000 μg/ml via both AP and CP. Hence the anticomplement activity of *Euphorbia peplus* was confined to the polar extracts.

The relatively higher IC50 values in AP results could also be due to the autolysis of rabbit erythrocytes as higher absorbance values were shown in the results of the control experiment carried out using heat-inactivated serum, or serum blank.

According to Ivanovska *et al* (1995), the activation of alternative pathway results in the generation of anaphylatoxins (C3a, C5a and C3b), required for many immune reactions. Activation of complement via non-antibody dependent alternative pathway has been suggested by Unsworth *et al* (1993) for very rapid onset of clinical relapse observed in patients with coeliac disease treated, with wheat gluten extracts.

Although the inhibitory potential of the E3 extract was higher ($P<0.05$) than the other extracts of *Euphorbia peplus L* against the *in vitro* activation of human complement via both CP as well as the AP, the classical pathway inhibitory potential of E3 was more pronounced than that via alternative pathway. The sequential extract E3, was therefore selected for fractionation using HPLC for further testing. Due to very small quantities being available of fractionated material, the fractions were tested only for the classical pathway inhibition.

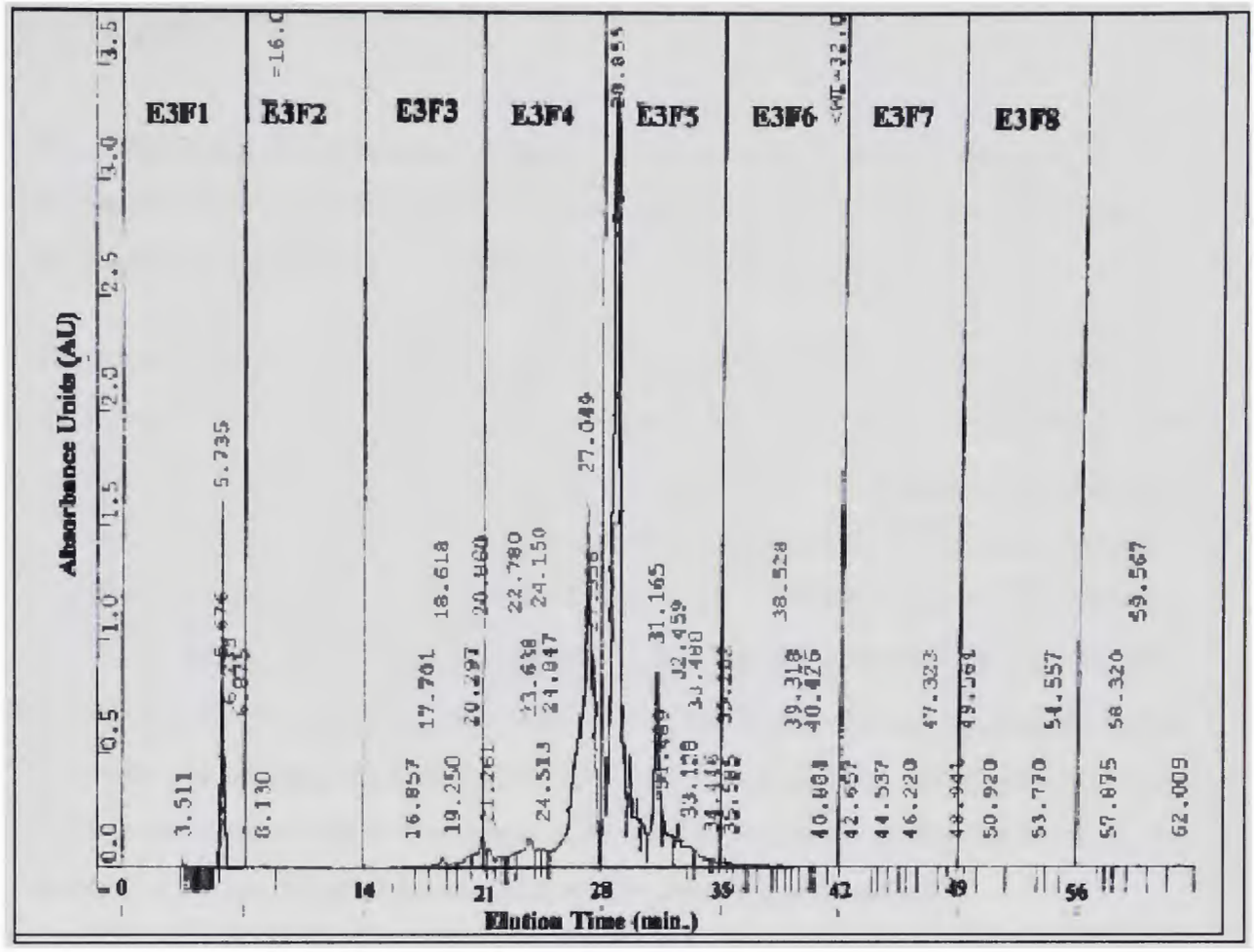

Figure 47 The HPLC profile of the E3 extract of *Euphorbia peplus* using a PDA-UV detector wavelength of 254 nm. The fractions of E3 extract, separated using a C18 column and collected every 7 min. using a 100 to 0% Milli Q water (0.05% TFA)-ACN gradient over 63 min., at a flow rate of 0.5 ml/min, are shown.

6.3.1.3 *In vitro* Screening of HPLC Fractions of the E3 extract of *Euphorbia peplus* for inhibition of human complement activity via classical pathway (CP)

The HPLC trace of the fractions of the E3 sequential extract of *Euphorbia peplus*, detected at 254nm using a photo diode array detector is given in Figure 46. The procedure used for the fractionation of E3 extract, is given in Section 2.5 of Chapter 2.

The results of the anticomplement activity of the HPLC fractions of the E3 extract of *Euphorbia peplus*, are illustrated in Figure 48. According to data shown in Figure 48, dextran sulphate showed the highest inhibitory potential (IC50 <2 μg/ml) against *in vitro* activation of human complement via the classical pathway. The HPLC fractions, E3F1, E3F2, E3F3, E3F4, E3F5, E3F6, and E3F7 indicated in Figure 47, gave IC50 values of 74, 43, 10, 15, 2, 126, 223 μg/ml, respectively, whereas E3 gave an IC50 value of 30±9 μg/ml. The fractions E3F3, E3F4, and E3F5 of E3 extract showed significantly higher CP inhibitory potential than that of E3 ($P<0.05$, $r^2>0.8$) whereas E3F5 gave the lowest IC50 value. On the basis of the lowest IC50 value being shown among the fractions, the fraction, E3F5 was selected for chemical studies discussed in Chapter 7.

In addition, further experiments were required to identify the structure of the complement inhibiting immunomodulatory compound in the E3F5 fraction. One approach that could have been used was to create a monoclonal antibody (MAb) and then to use this antibody to ascertain whether the MAb would cause complement-mediated cytolysis of the cancer cells *in vivo*. Such a strategy would ascertain the anticancer activity of the putative anticancer plant like *Euphorbia peplus*. As suggested by Asghar and Pasch (2000) therapeutic inhibition of complement would provide one strategy for combating many human diseases including cancer.

The sequential methanol-$CHCl_3$ (1:1) extract (E3) and its fraction E3F5 of *Euphorbia peplus* showed a dose-dependant inhibition of *in vitro* human complement activation via the classical pathway. The IC50 values given by E3 and E3F5 were 30±9 and 2 μg/ml, respectively. The sequential extracts E1 and E2 that showed *in vitro* anticancer activity (Chapter 3) did not show a marked dose dependent inhibition of complement activation via both classical and alternative pathways, below a concentration of 1000 μg/ml.

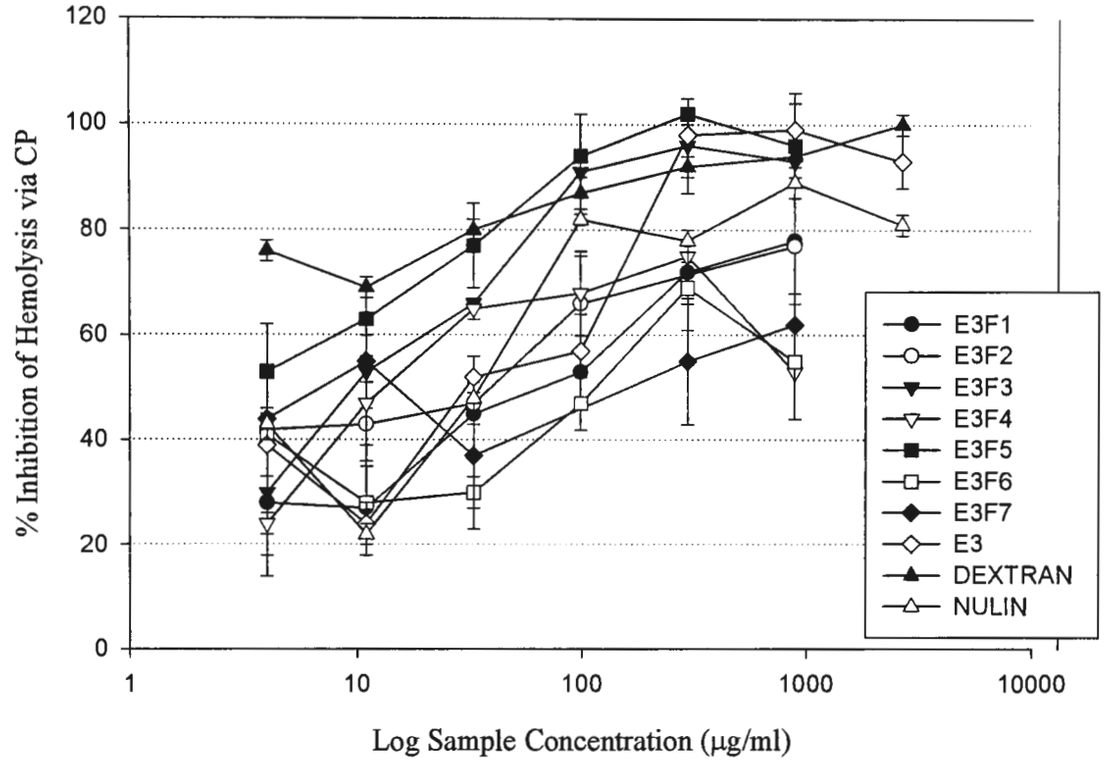

Figure 48 Percent inhibition of haemolytic complement activity via classical pathway (CP) against log sample concentration of the HPLC fractions of the sequential extracts, E3 *of Euphorbia peplus* compared with E3, dextran sulphate and inulin. Data points shown are the mean values of four replicate determinations and the error bars are standard error of the mean value.

In a similar study, Simons *et al* (1990) reported a dose-dependent inhibition of complement activation via both classical and alternative pathways by the extracts of the Ayurvedic medicinal plant, *Picrorhiza kurroa.* Simons *et al* (1990) suggested interference with the complement cascade rather than on a direct interaction with target erythrocytes or chelation of essential bivalent cations as the mode of action of the plant extracts.

The inhibitory activities of the plant extracts on the complement system may be due to complement consumption or direct blockage of the complement cascade (Thabrew *et al*, 1991). Either of these mechanisms reduces the available complement components for an interaction with the possible target. Decreased complement activity according to Thabrew *et al* (1991) leads to reduced complement-elicited responses such as anaphylaxis, chemotaxis, opsonization, stimulation of macrophages and PMNs. If the plant extracts activate the complement *in vivo*, an anticancer effect mediated through the above immune responses is possible.

Asghar and Pasch (2000), in their review on the therapeutic inhibition of the complement system suggested that the C1 esterase inhibitor may be used to avoid cytotoxicity caused by IL-2 therapy in cancer and for inflammatory conditions. Ito (1986) suggested that C3 activation might provide a possible mechanism for the inhibition of sarcoma 180 solid tumour in mice, treated with plant polysaccharides. Several studies have shown expression of human protectin (CD59) *in vivo* on cells of breast, ovarian, central nervous system, melanoma and prostate cancers (Hakulinen and Meri, 1994; Bjorge *et al.*, 1997; Maenpaa *et al.*, 1996; Brasoveanu et al, 1995; Jarvis *et al.*, 1997). Protectin is an inhibitor of complement activation which was thought to protect cancer cells from immunological responses leading to the complement-mediated cytolytic activity, by blocking the full assembly of the membrane attack complex (Jarvis *et al*, 1997).

The *in vitro* inhibition of complement activation by *Euphorbia peplus* extracts, observed in this study supports the view that the putative anticancer activity, of the plant *Euphorbia peplus*, may be partially, due to its influence on the immune system. However, the present experimental findings are not sufficient to suggest any *in vivo* anticomplement or complement activity until further *in vivo* experiments are undertaken.

6.3.2 *In vitro* screening for anti-inflammatory activity

In order to reduce the number of extracts tested in the screen, and to ascertain whether the antioxidant extracts also possess anti-inflammatory activity, only the extracts that showed the highest antioxidant activity of each plant were used for anti-inflammatory activity assays. The results obtained for general (anti-platelet aggregatory) and enzyme specific (inhibition of COX-2 enzyme) anti-inflammatory activity of the extracts, E4 and

V3, of *Euphorbia peplus* and *Viola odorata*, together with their boiling water extracts, EB and VB, are discussed in the following sections.

6.3.2.1 *In vitro* screening of *Euphorbia peplus* and *Viola odorata* for inhibition of platelet aggregation

The results of the *in vitro* inhibition of arachidonic acid induced platelet aggregation, as measured by the impedance method described in Chapter 2, are given in Table 25.

As mentioned in Table 25, the 70% EtOH extract, E4 of *Euphorbia peplus* showed a 47% inhibition of the human platelet aggregation *in vitro*. The crude, boiling water extract, EB, however, indicated only a 6% inhibition, which was not significantly different ($p>0.05$) from the control.

In the case of *Viola odorata*, its sequential MeOH-$CHCl_3$ extract, V3, indicated 73±9 % inhibition of the platelet aggregation, whereas its boiling water extract, VB, showed a 19±8 % inhibition significantly higher ($P<0.01$) compared to the control. The platelet aggregation inhibitory activity of the V3 extract of *Viola odorata* was significantly higher ($P<0.05$) than that of the E4 extract of *Euphorbia peplus*.

Table 25 Percent inhibition of platelet aggregation by extracts *of Euphorbia peplus* and *Viola odorata*

Sample	% inhibition of platelet aggregation	SEM
E4	47	6
EB	06	3
V3	73	9
VB	19	8
Control	0	0

Each value represents the average inhibition with the standard error of the mean (SEM) of three replicate determinations.

Thus the E4 and V3 extracts of *Euphorbia peplus* and *Viola odorata* showed the highest anti-platelet aggregation t activity among the sequential extracts of each plant. As discussed in

Chapter 5, these extracts also showed a considerable antioxidant activity. The relatively higher ferric reducing antioxidant activity, however shown by the E4 extract, compared to the V3 extract did not show an anti-platelet aggregation activity that was higher than the V3 extract. The results indicate the importance of using both the antioxidant and anti-platelet aggregation activities in a broad spectrum or multi-assay monitoring system.

The maximum rate of arachidonic acid induced human platelet aggregation declined significantly (i.e. from 9.1 to 7.3 to 3.5 ohms/min) as the concentration of V3 extract was increased from 100 to 200 to 500 μg/ml, respectively. However, an inverse dose-response relationship for platelet aggregation, was observed by Carr *et al* (1995) for the antileukaemic agent, bryostatin-1. Hence, bryostatin-1 was reported to be the first antineoplastic agent, with human platelet activating activity. Carr *et al* (1995) however, did not use a standard aggregation agent such as arachidonic acid as in this study, to investigate the effect of bryostatin-1 on platelet aggregation.

On the other hand, Steiner and Lin (1998) reported potent inhibition of epinephrine- and collagen-induced platelet aggregation by an aged garlic extract. However, the same garlic extract failed to inhibit adenosine diphosphate (ADP)-induced human platelet aggregation thus indicating the importance of using appropriate aggregation-inducer for *in vitro* platelet aggregation assays (Steiner and Lin, 1998). Saeed *et al* (1995), in a previous study, have used arachidonic acid, ADP, collagen and PAF as inducers for *in vitro* platelet aggregation assay of eugenol.

Chen *et al* (1997) reported that the antiplatelet drugs, pentoxifyline, dipyridamole and ticlopidine-HC showed *in vitro* antineoplastic property

6.3.2.2 *In vitro* screening of *Euphorbia peplus* and *Viola odorata* extracts for COX-2 inhibition

The TLC-radiochromatograms of the arachidonic acid (AA*) which remained after incubating with the COX-2 enzyme in the presence of E4 extract of *Euphorbia peplus* and V3 extract of *Viola odorata*, was compared with the standard inhibitor, indomethacin and the control containing AA* and COX-2 without an inhibitor (Figure 49).

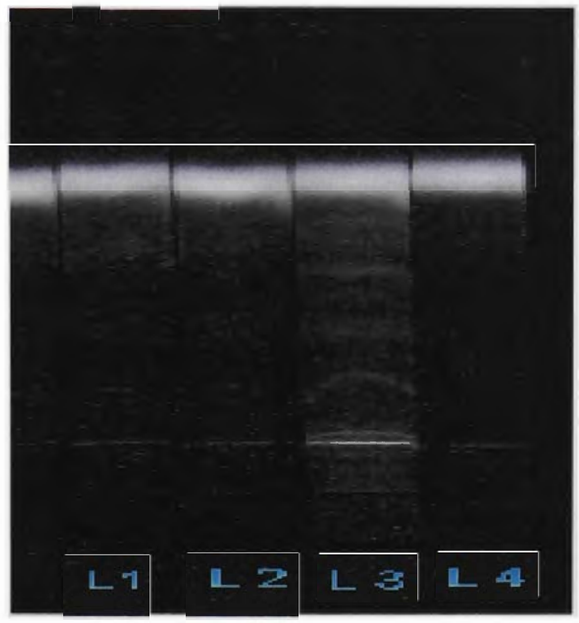

Figure 49 The TLC-radiochromatograms showing the upper white bands of unmetabolised radio-labelled arachidonic acid (AA*) following incubation with the COX-2 enzyme in the presence of the extracts, V3 (L1), E4 (L2), the inhibitor, indomethacin (L4) and the control (L3). The image is a three-fold reduction of the original.

The control is shown in lane three (L3) and the standard inhibitor indomethacin in lane four (L4). The upper prominent white band at R_f 0.70 in the chromatogram of the inhibitor, indomethacin (L4) is due to the radioactive arachidonic acid (AA*) as determined by comparing the R_f value (0.70) of a separate chromatogram of AA* run under the same conditions. In the TLC-radiochromatogram of the control (L3), the weak white bands shown below the strong AA* band are due to the radioactive metabolic products of AA* effected by the COX-2 activity in the reaction mixture. Both E4 and V3 extracts, at the concentration of 200 μg/ml, showed a very weak band just below the strong band of AA* in the radiogram L1 and L2, respectively, which was not detectable in the radiogram of indomethacin (L4).

Quantification of the intensity of the bands of the radiochromatogram showed complete inhibition of COX-2 activity by a known COX-2 inhibitor, indomethacin at a concentration of 40 μg/ml. The COX-2 inhibition activity for the E4 and V3 extracts,

calculated on the basis of the AA* band intensity of the control are given in Table 26. Each measurement represents the mean of duplicate values.

Table 26. Percent Inhibition of COX-2 activity by the extracts E4, V3, and indomethacin

Sample	% COX-2 inhibition	SEM
E4	90*	3
V3	85*	4
Control	69*	7
Indomethacin	94*	5

* Significantly higher inhibition ($P<0.05$) compared to the control. Each measurement represents the mean of duplicate values.

According to Table 26, significant differences between the control and V3, E4 and indomethacin were observed for inhibition of COX-2 activity ($P<0.001$). For both the extract, E4 of *Euphorbia peplus* and the extract, V3 of *Viola odorata*, the COX-2 inhibitory activity was close to that of indomethacin.

Although the anti-platelet aggregation results of extract, E4 (47±6%) was significantly lower ($P<0.05$) than that of V3 (73±9%), both the E4 and V4 extracts showed a considerably higher COX-2 inhibition activity of 85±4% and 90±5%, respectively. The COX-2 inhibition activities of E4 and V3 extracts were significantly higher than that of the control (69±7%, $P<0.05$) and very close to that of indomethacin (94±5%).

Both antiplatelet aggregation and COX-2 inhibition assays represent the anti-inflammatory activity of the extracts. If the platelet activating factor, PAF, is a mitogen that contributes to the known increase risk of malignancies associated with chronic inflammatory conditions (Bennett and Birnboim, 1997), then the activation of the PAF m RNA receptor may induce production of arachidonic acid and activation of protein kinase C (PKC). The activation of the PFA mRNA receptor in human lung fibroblasts followed by the induction of arachidonic acid production and activation of protein kinase C has been reported (Bennett and Birnboim, 1997). Hence inhibition of platelet aggregation may be due to the inhibition of PAF activation followed by the inhibition of arachidonic

production and PKC. Despite the mechanistic differences of the two assays, the above results indicate the importance of using both anti-platelet aggregation as well as the COX-2 inhibition assays to represent a broad spectrum of assays for monitoring anticancer properties of a putative medicinal plant.

The above results indicate that the extracts, E4 and V3 which showed the highest ferric reducing antioxidant activity (Chapter 5), also showed antiplatelet aggregating and COX-2 inhibiting anti-inflammatory activities.

Although it is suggested that the growth of malignant cancer cells of the intestine may be due to the COX-2 enzyme (Gorman, 1998), it is not established whether the presence of COX-2 may be due to an inflammatory response from the surrounding tissue. The induction of COX-2 may have been due to an inflammatory reaction arising from cancer cells themselves. Specific inhibition of COX-2 has led to a significant *in vivo* tumour reduction in murine lung cancer model (Stolina *et al*, 2000). In the case of human lung cancer (non-small cell) involvement of COX-2 over expression in the pathogenesis has been suggested by Hosomi *et al* (1999). According to Reinemachers and Everson (1994), epidemiological studies have indicated that a decrease in the incidence of lung cancer in subjects may be due to the regular use of aspirin, which would support the hypothesis of Schreinemachers and Everson (1994). More recently, Dohadwala *et al* (2001) reported that genetic inhibition of COX-2 expression reverses the immunosuppression induced by non-small cell lung cancer. In addition to the cancers already mentioned, expression of COX-2 in prostate (Gupta *et al*, 2000), skin (Higashi *et al*, 2000), pancreatic (Kokawa *et al*, 2000), pulmonary, colonic and mammary (Sosllow *et al*, 2000) tumours has been reported, indicating the commonality of COX-2 expression or over-expression in cancer cells. Thus COX-2 may be an important target for gene or pharmacological therapy for cancer, utilizing natural COX-2 inhibitors found in putative anticancer plants.

6.4 Discussion

The findings of this study have shown that although the sequential extracts of both plants, *Euphorbia peplus* and *Viola odorata* failed to show a potent cytotoxic activity in the NCI's *in vitro* screens, the E4 and V3 extracts of both plants showed a higher antiplatelet aggregation and COX-2 inhibition activities *in vitro*. In addition, E3 extract of *Euphorbia peplus* indicated a higher *in vitro* anticomplement activity via CP. The *in vitro* results thus

raise the possibility that putative anticancer effect of *Euphorbia peplus* and *Viola odorata* may be due to their immunomodulatory activities mediating platelet activating factor, COX-2 and also complement in case of *Euphorbia peplus*, although further studies will be needed to confirm these preliminary observations.

Chapter 7 Chemical Studies of the extracts of *Euphorbia peplus* and *Viola odorata*

ABSTRACT

Sequential extracts containing potential antioxidant and anticancer activities were analysed using the techniques of electrospray ionisation-mass spectrometry (ESI-MS), GC-MS and HPLC-UV.

The HPLC profiles obtained for the E4 extract, E4F1 fraction and for the V3F5 fraction are also presented for chemical analysis. ESI spectra were obtained for the E4F1 fraction of *Euphorbia peplus* that showed antioxidant activity (chapter 6) and a sub fraction, E4F1F6. In addition GC-MS spectra of E4F1F6 fraction are presented.

The V3 extract, and its fraction V3F5, from *Viola odorata* that showed *in vitro* cytotoxic and antioxidant activity were analysed using both positive and negative mode ESI and GC-MS spectra and TLC.

The ESI+ spectra of both E4F1F6 and V3F5 sub fractions gave a mass ion that indicated the presence of quercetin aglycone. Further separation and analysis using FTIR, NMR and X-ray diffraction studies will need to be carried out to confirm the presence of quercetin in these sub fractions.

carbon skeleton together with ingenanes and jatrophanes was reported by Jakupovic *et al* (1998). The structure of pepluane was revised by Hohmann *et al* (1999).

Quercetin, the presence of which was also reported in *Euphorbia peplus*, has been reported to attack different targets and different phases of the cell cycle, which may explain its effectiveness in inhibiting the growth of OVCAR-5 ovarian cancer cells (Weber *et al*, 1997). According to Weber *et al* (1997), quercetin is of interest because the drug crucially inhibits the neoplastic program of cells, which is thought to lead to induce differentiation and apoptosis.

Krol *et al* (1996) reported anti-inflammatory activity of ferulic acid, caffeic acid phenylethyl ester and three flavonols (galangin, kaempferol and kaempferide) measured by the inhibition of luminol-enhanced chemiluminescence of neutrophils between 66-100%. The same type activity was inhibited to an extent of 44% by quercetin at the concentration of 10 μM. When tested at 100 μM of these compounds, the inhibition was 95-100%.

7.1.2. Reported chemical constituents of *Viola odorata*

Watt and Breyer-Brandwijk (1962) reported the presence of the phenolic glycoside, gaultherin in *Viola odorata*, which yields methyl salicylate following hydrolysis. In addition, violutoside, myrosin, violin, rutin, violarutin, odoratine, and 2-nitroproprionic acid were reported from the leaves and flowers of *Viola odorata* (Hoffman, 1990).

Ladwa and Dutta (1969) reported the presence of a triacetonamine, odoratine, in the plant and isolated a triterpene ketone, friedelin, and β-sitosterol from leaves. Kroutil and Kroutilova (1968) attributed the anti-inflammatory effect of *Viola odorata* extract to the voline, an alkaloid resembling emetine. A list of constituents of *Viola odorata,* according to Grieve (1992, and http://chili.rt66.com/hrbmoore/Constituents/Viola_odorata.txt) is given in Appendix II.

Recently Lindholm *et al* (2002), reported a potent cytotoxic activity of the macrocyclic peptide, cycloviolacin 02 isolated from *Viola odorata*, against drug resistant human tumour cell lines.

7.2 Results and Discussion

7.2.1 DAD profiles of the HPLC fractions of *Euphorbia peplus* extracts

The HPLC profile of the E4 extract as monitored by the photodiode array detector at 254 nm is shown in Figure 50. When the various fractions were assayed using the ferric-TPTZ reducing antioxidant assay, two of the E4 fractions, E4F1 and E4F2, showed significant ($P < 0.001$) antioxidant activity. Since the E4F1 fraction, shown in Figure 50, contained a number of peaks, this meant that further separation of this fraction was necessary before a characterisation of constituents could be undertaken.

Further sub-fractionation of E4F1, carried out using a different gradient described in section 2.5 of chapter 2, gave UV traces shown in Figure 51. The highest ($P < 0.05$) antioxidant activity was shown by the E4F1F6 sub fraction. Hence the E4F1F6 sub fraction was further investigated using ESI and GC-MS analysis.

7.2.2 The electrosprayionisation (ESI) mass spectrum of the E4F1F6 sub fraction of *Euphorbia peplus*

The positive mode ESI spectrum of the E4F1F6 sub fraction is given in Figure 52. Positive mass ions were detected at *m/z* = 137, 163, 179, 215,231, 241, 275, 287, 303, 309, 325, 419, 497, 574 and 652. The ESI+ profile of the E4F1F6 fraction (Figure 52) indicated a mass ion at *m/z* =303, which may be due to the mass ion of quercetin aglycone ([M+H]+; MW=302).

7.2.3 The GC-MS spectra of the E4F1F6 sub fraction of *Euphorbia peplus*

The GC-MS total ion chromatogram of the E4F1F6 subfraction is given in Figure 53. The total ion chromatogram (Figure 53) indicates 12 major peaks. The GC-MS fragments of some of the peaks are given in Appendix I. Although the MS library search resulted in a close match for some of these fragmentograms, further separation and studies using IR, NMR analysis are required to confirm the chemical structures of the constituents in the active sub fractions.

The mass ion at *m/z* = 303 shown in the ESI+ spectrum (Figure 52), of the sub fraction, E4F1F6 may be due to the presence of quercetin aglycone ([M+H]+, MW=302 Dalton) of the quercetin-3-glucoside as reported previously by Dumkow and Pohl (1973).

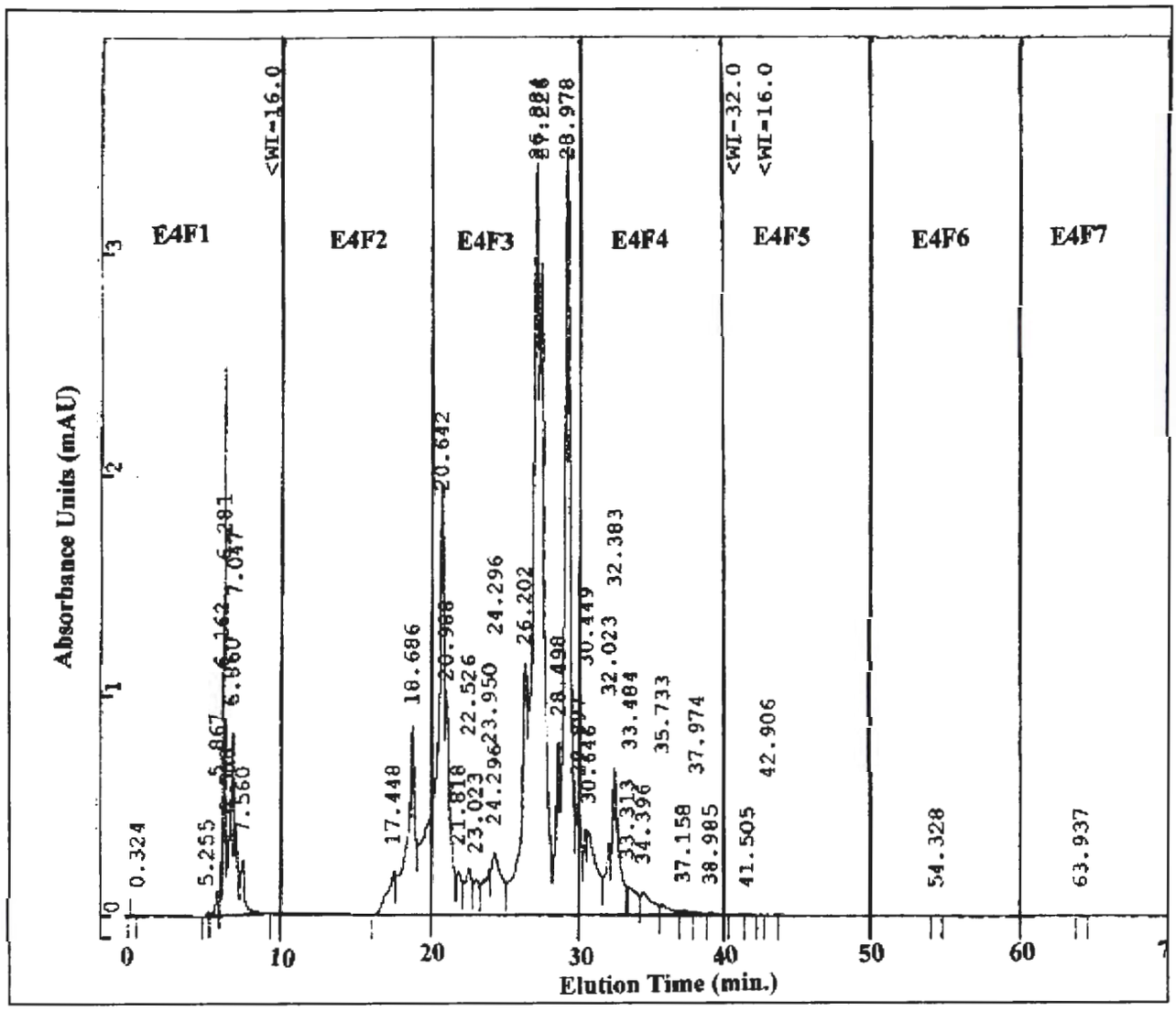

Figure 50 **The HPLC profile of the E4 extract of *Euphorbia peplus* using a PDA-UV detector wavelength of 254 nm. The fractions of E4 extract were separated using a C_{18} column and collected every 10 min using a 100 to 0% (v/v) Milli Q water (0.05% (v/v) TFA)-ACN gradient over 70 min, at a flow rate of 0.5 ml/min.**

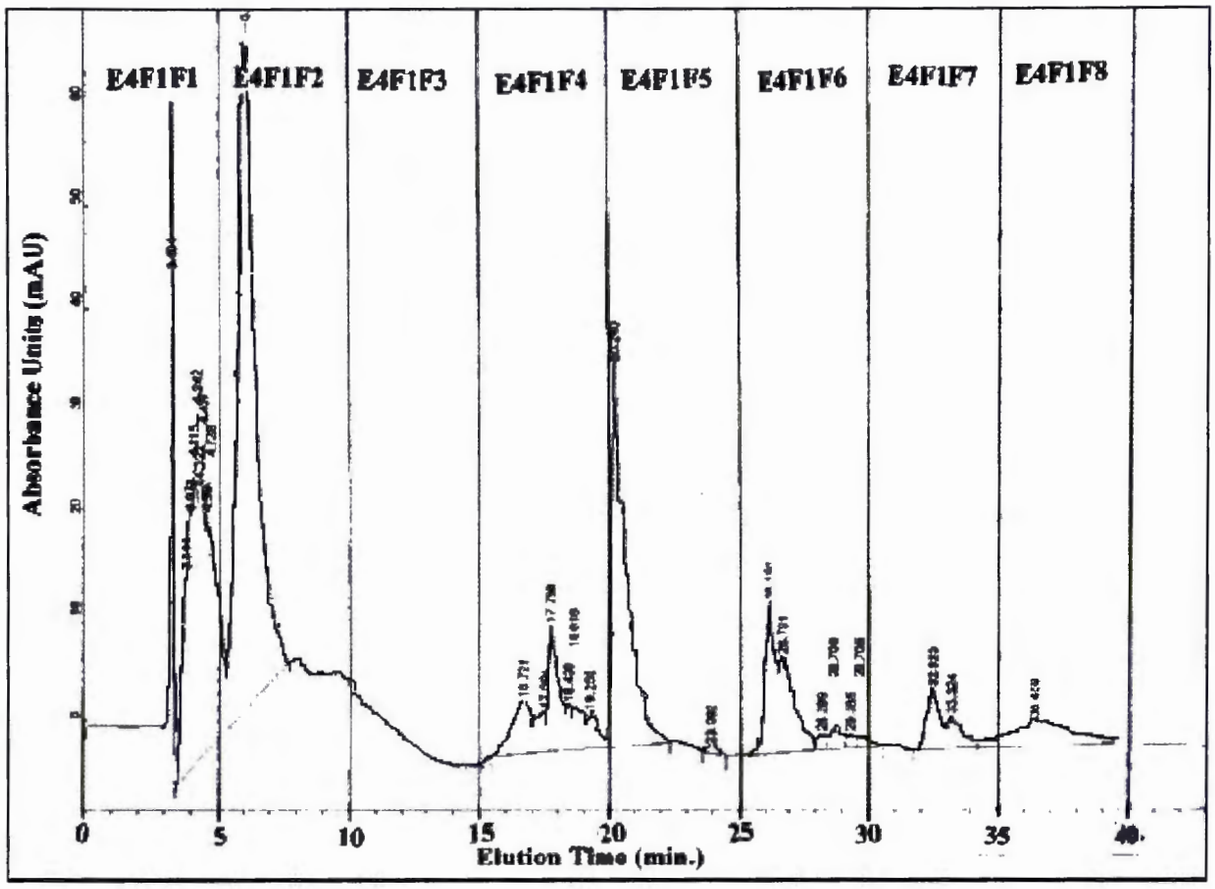

Figure 51 The HPLC profile of E4F1 fraction of *Euphorbia peplus* using a PDA-UV detector wavelength of 254 nm. The sub fractions of E4F1 separated using a C18 column and collected every 5 min using a 100 to 0% Milli Q water (0.05% TFA)-ACN gradient over 40 min, at a flow rate of 0.5 ml/min, are shown.

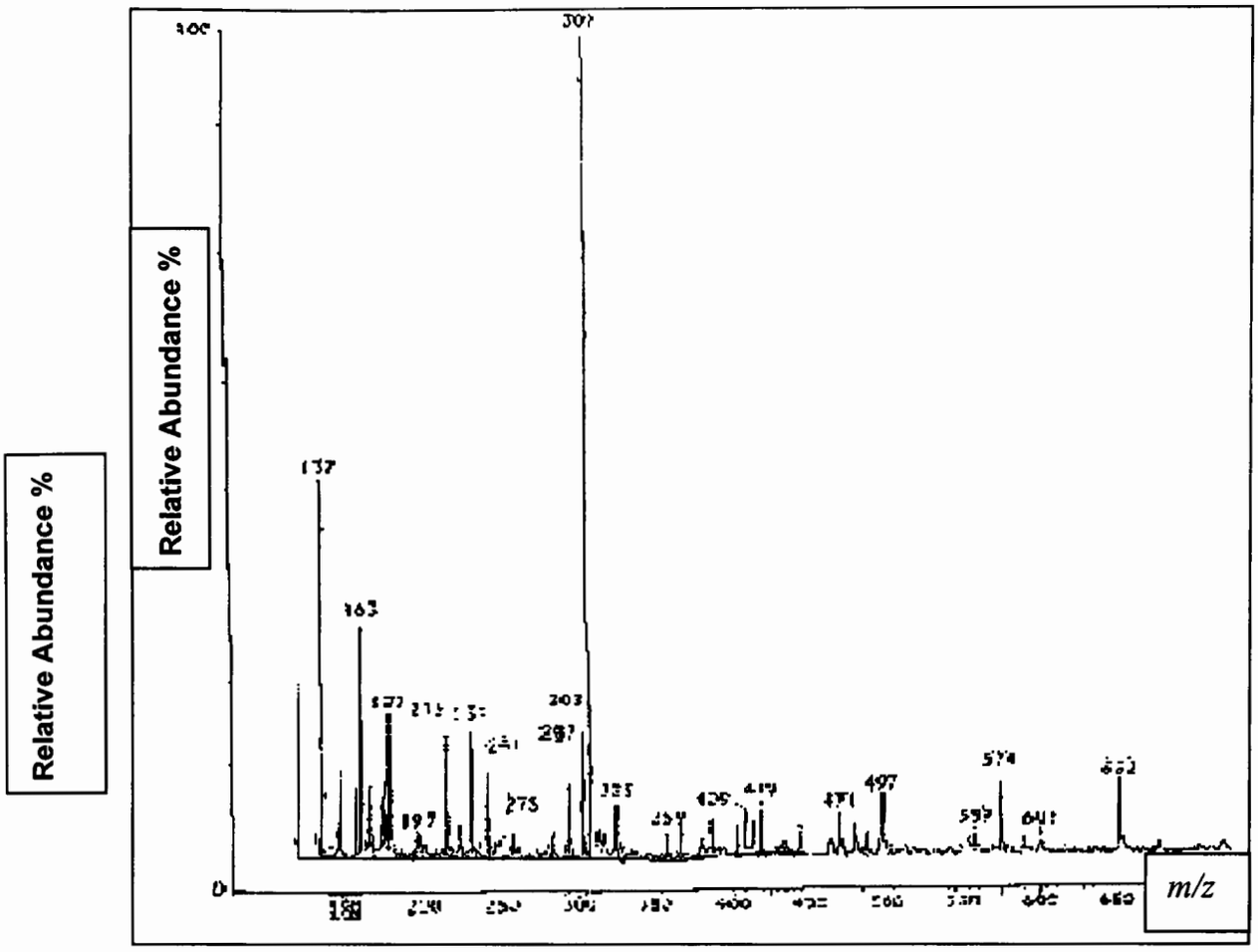

Figure 52 The ESI+ profile of the E4F1F6 sub fraction of *Euphorbia peplus*

On the basis of the previously reported constituents of *Euphorbia peplus*, which were discussed in section 7.1.1, the mass ion 287 may be due to the aglycone of kaempferol ([M+H]+, MW =286). The ESI spectra given in Appendix I for quercetin and kaempferol standards showed the [M+H]+ molecular ions, 303 for quercetin (Appendix I.7), and the protonated molecular ion 287 for kaempferol (Appendix I.8). The weak mass ion signals at 401 and 415 *m/z* (Figure 52) may be due to the [M+H]+ molecular ions of campesterol (M W=400) and β-sitosterol (MW =414) respectively.

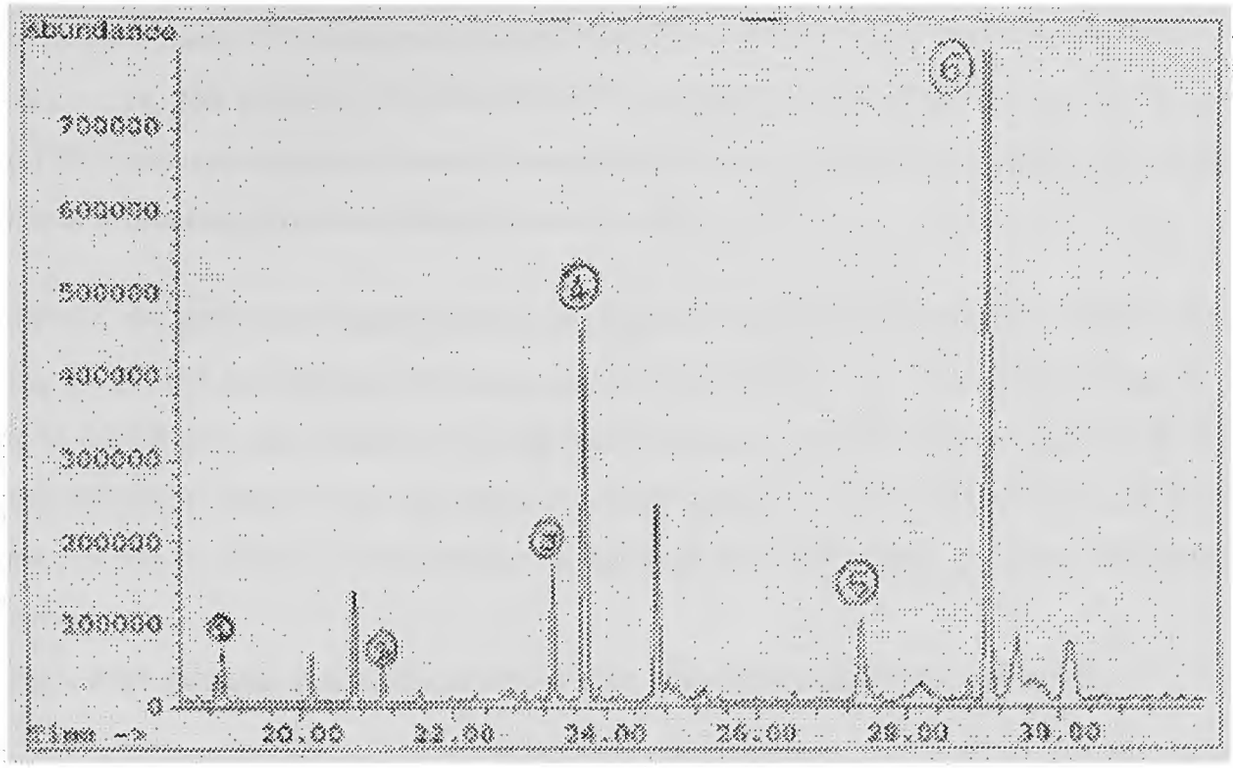

Figure 53 The GC-MS total ion chromatogram of the E4F1F6 sub fraction of *Euphorbia peplus*

The presence of quercetin indicates that this substance may be one of the constituents of *Euphorbia peplus* that would account for the reported anticancer properties of the plant.

7.3 Chemical studies of *Viola odorata*

7.3.1 DAD profiles of the HPLC fractions of *Viola odorata* extracts

According to the results in this study discussed in Chapter 4 and 5, the sequential V3 extract showed the highest *in vitro* anticancer activity as well as the highest antioxidant activity. The HPLC fractionation of the V3 extract, as monitored by the photodiode array detector at 254 nm, is shown in Figure 30 in Chapter 4. The V3F5 fraction, which showed highest antioxidant activity, was further subjected to ESI and GC-MS analysis.

The positive mode ESI+ spectrum of the V3F5 (Figure 54) fraction showed the mass ions at *m/z* = 214, 240, 247, 264, 293, 304, 309, 327, 342, 387, 438, 487, 512, 516 and 588. Since the ESI+ spectrum however showed a number of mass ions, further separation of V3F5 fraction is necessary to isolate the active compounds.

However, the prominent negative mass ions, shown in the negative mode ESI- (Figure 55) were, at 227, 504 and 508 Da. The mass ion *m/z* = 227 of the ESI- mass spectrum may be due to the $CF_3CO_2^-$ ion, since 0.05% trifluoroacetic acid was used as the component A of the mobile phase, used in the separation of compounds using HPLC. The presence of 227 mass ion due to CF_3CO_2Na contamination has been reported previously in the ESI-mass spectra, (http://www.micromass.co.uk/UserNet/knowledge%20Base/contamination.htm).

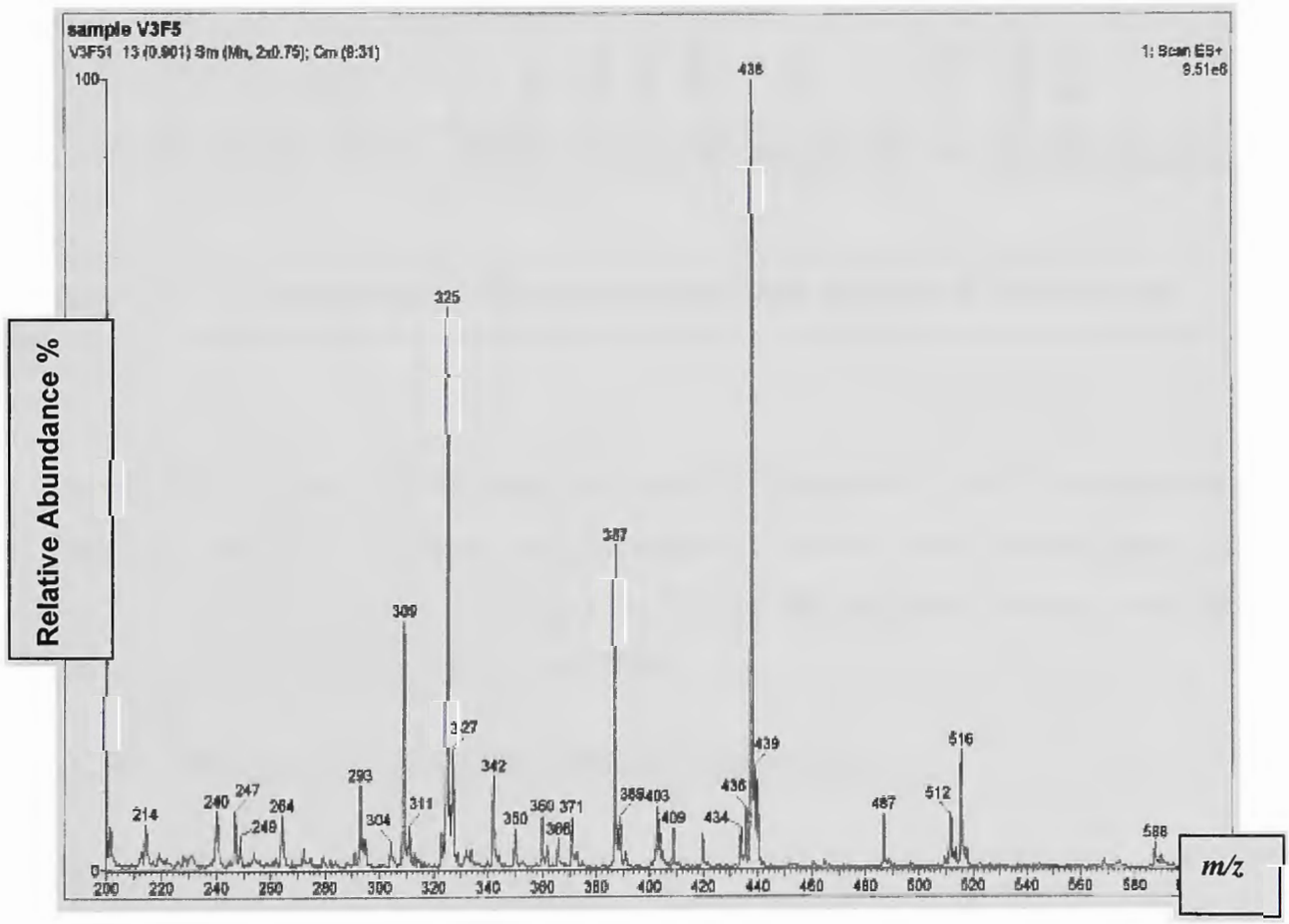

Figure 54 Positive mode ESI+ spectrum of V3F5 fraction of *Viola odorata*

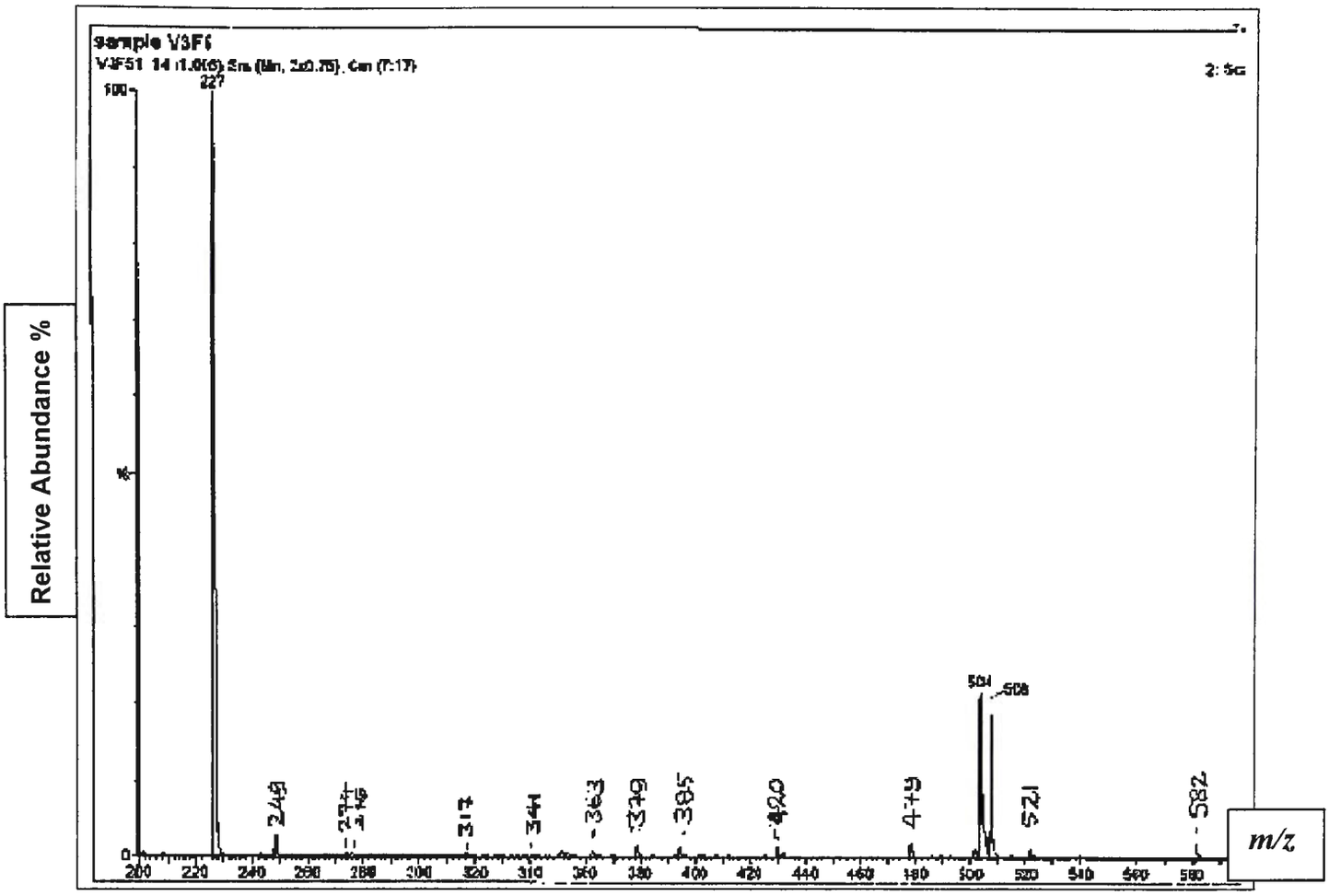

Figure 55 Negative mode ESI- spectrum of V3F5 fraction of *Viola odorata*

The very high abundance of the mass ion at m/z = 227 is shown in the ESI-mass spectrum (Figure 55), next prominent mass ions were at m/z = 504 and 508. However, mass ions, 249, 274, 317, 341, 363, 379, 395, 430, 479, 521 and 582 were also observed in the ESI-mass spectrum at a very low relative abundance.

7.3.3 GC-MS spectrum of V3F5 fraction of *Viola odorata*

The GC-MS spectrum of the V3F5 fraction subjected to the silyl derivatization described in Chapter 2 are given in Appendix III. The presence of the mass ion of m/z = 309 in the ESI+ spectrum (Figure 54), was also shown in one of the GC-MS spectra (Appendix III.1) obtained from V3F5. The base peaks shown by the GC-MS spectra of V3F5 were at m/z = 206, 42, 275, 349, 276, 371 and 341. However, the presented GC-MS results were not

sufficient to characterise the constituents of the V3F5 extract. Isolation of the constituents followed by the IR, UV, NMR and X-ray diffraction spectral studies will be necessary to confirm the identity of the chemical constituents of V3F5 extract.

7.3.4 TLC analysis

The ESI+ profile of the V3F5 fraction (Figure54) indicated a [M+H]+ mass ion at *m/z* = 304, thus raising the possibility that one of the compounds may be quercetin aglycone ([M+2H]+; MW=302). Hence, the TLC analysis of V3F5 fraction was carried out using quercetin aglycone as the reference compound, according to the method described in Section 2.14.

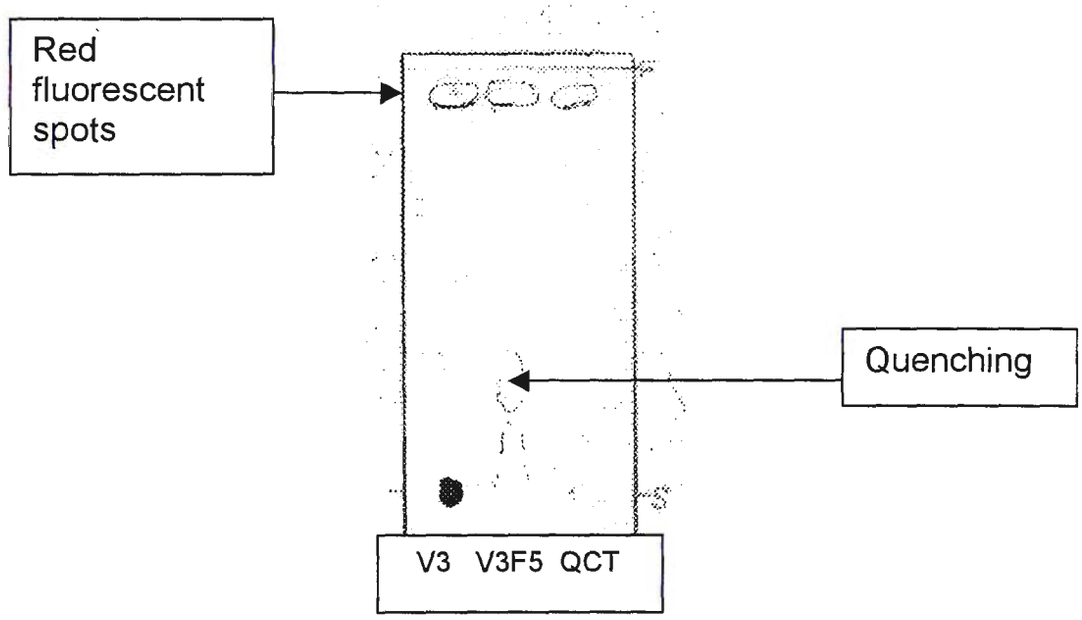

Figure 56 TLC profiles viewed under UV 366 nm light of the V3 extract and V3F5 fraction of *Viola odorata* compared with the quercetin standard

The TLC analysis for lipophilic flavonoids aglycones was carried out using the solvent system, toluene-ethyl acetate (93:7) according to the method of Wagner and Bladt (1996). As shown in Figure 56, red fluorescent spots having the same R_f value (0.93) were detected in the TLC of the V3, V3F5 and the quercetin standard under UV-366 nm light. According to Wagner and Bladt (1996), these spots may be due to the specific substitution pattern containing two adjacent hydroxyl groups in ring B of flavonols such as quercetin (Figure 57).

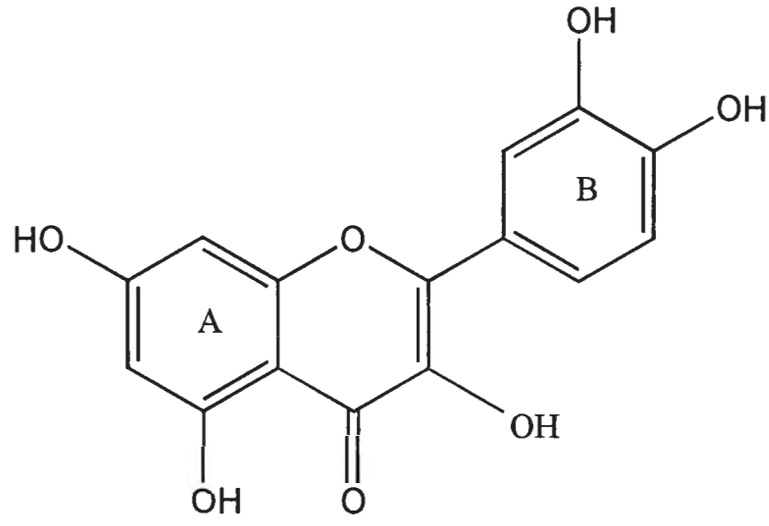

Figure 57 The chemical structure of quercetin

However, further separation of the constituents and studies using FTIR, NMR spectral analysis will be required for the confirmation of their chemical structures.

7.4 Possible anticancer constituents of *Viola odorata*

One of the reported constituents of *Viola odorata*, rutin, a flavonoid has been shown to give a 71% growth inhibition of lung metastasis in induced B16F10 melanoma cells in mice (Menon *et al*, 1995). However, the use of a single ingredient in isolation is not in accordance with the principles of traditional medicine, discussed under "Ayurvedic drugs" in Section 1.4.1. Accordingly, the Ayurvedic attributes of *Viola odorata*, discussed in section 1.7.2, need to be defined in scientific terms, initially by using the isolated constituents and then by testing a combination of different constituents. Methods need to be developed, to identify the unique combination of different chemical constituents that give rise to the specified attributes described as *Rasa*, *Guna*, *Veerya*, *Vipaka* and *Prabhava* of a particular plant part or an extract. Information found in the literature may be used to devise a strategy to best utilize the effective constituents once they are isolated and characterized. Having established the attributes of the extracts, further investigation is necessary to identify a battery of pertinent screening assays to determine the effectiveness of a combination of different substances in plant extracts like *Euphorbia peplus* and *Viola odorata* for further anticancer activity attributable to the aetiology of the disease, cancer.

Chapter 8 Conclusions and Further Research

8 General Conclusions

8.1 Anticancer activity of *Euphorbia peplus* L. and *Viola odorata* L.

Although the extracts of *Euphorbia peplus*, have failed to show any significant anticancer activity using *in vitro* human KB cells and *in vivo* mouse LE, PS, LL, SA and rat WA tumours in the previous screening programme of the US National Cancer Institute (NCI), according to our results the petroleum ether (E1), $CHCl_3$ (E2) and $CHCl_3$-MeOH (E3) extracts obtained from whole plant of *Euphorbia peplus*, showed a selective but weak *in vitro* growth inhibition of human leukaemia, non-small cell lung, and breast cancer cell lines of the NCI's 60-human cell panel. This may be due to the improved exhaustive sequential solvent extraction method, the use of different cell lines and the sulfarhodamine B assay method.

The highest GI50 inhibitory potency was shown by the E3 extract at a concentration of 0.15 μg/ml against the HOP-92 cell line of non-small lung cancer sub panel, which was more than 100-fold higher than that (24μg/ml) obtained by the NCI against KB cells *in vitro*, for the aqueous-EtOH extract using protein synthesis assay. Although this result was slightly higher than the activity criteria of <0.1 μg/ml the NCI, it was very close to the acceptance criterion.

The total growth inhibition of A498 and UO-31 cell lines was effected by E1 extract at 26 and 28 μg/ml respectively whereas that of TK-10 was exerted by E3 at 37 μg/ml. Thus with respect to the total growth inhibitory potential, E1 showed the highest *in vitro* cytotoxic activity followed by E2. Higher cytotoxic activity of the less polar organic extracts like E1 and E2 was reflected in the results of the [^{3}H]-thymidine uptake assay and the brine shrimp lethality assay.

Although, the sequential V3 and V4 extracts of *Viola odorata* (aerial parts) showed a weak growth inhibitory potential (GI50=28, 41 μg/ml) against the 60 human cell line panel, $CHCl_3$-insoluble fraction of V3 showed a considerably higher cytotoxicity (GI50=1.9-3.7 μg/ml) than V3 against the 60human cell panel. The sequential root extract VRT3 also showed a moderate inhibitory potential (GI50=1.8-16.2 μg/ml).

However, when *Viola odorata* (aerial parts) extracts were screened against sensitive CEM leukaemia cell line using ^{3}H-thymidine uptake assay, the sequential V3 extract gave the highest inhibitory potential of 3.2±1.5 μg/ml, which was considerably higher than the activity shown in NCI's both *in vitro* screens.

The [^{3}H]-thymidine CEM cell assay indicated a 10-fold higher *in vitro* anticancer activity when compared with the sulpharhodamine B assay against the RPMI-8226 cells and previous results obtained from testing direct solvent extracts and protein synthesis assay against the KB cells. Hence it appears that the detectability of cytotoxic anticancer activity can be increased 10-fold by modifying the conventional *in vitro* method to include sequential solvent extracts, sensitive CEM cell line and appropriate assay technique such as ^{3}H-thymidine uptake method.

Due to the wide variation in the results, the brine shrimp assay appears not suitable for the assay of moderately toxic extracts. However, [^{3}H]-thymidine uptake assays appeared more sensitive to the cytotoxicity detection of crude extracts than the assays involving optical density measurement. The MTS results need to be further evaluated, before a comparison is made.

8.1.2 Antioxidant activity of *Euphorbia peplus* and *Viola odorata*

The E4 and V3 extracts were found to effect 81±13% & 96±1% linoleic peroxidation inhibition, 31±7% & 14±4% DPPH free radical scavenging and 31±1% & 11±1% TPTZ-ferric reducing antioxidant activities, respectively.

The modified TPTZ method yielded results comparable with those of DPPH while the FTC and TBARS results for inhibition of linoleic peroxidation, by plant extracts E4 and V3, were significantly higher ($P>0.001$) than those given by DPPH and FRAP assays. The modified TPTZ method gave a greater precision (within-run CV, 5%, n=25) and reproducibility (between-runs CV, 6%, n=5) than the other methods investigated while DPPH method showed an acceptable precision (CV<9.5%) but a poor (CV<27%) in reproducibility. Findings of the present research suggest that a convenient free radical generator such as DPPH may be useful in determining the free radical scavenging antioxidant activity of a plant extract.

Although V3 and E4 extracts were found to be inactive in the NCI's 60-cell line screen, it showed a relatively higher linoleic peroxidation inhibitory, ferric-TPTZ reducing and DPPH free radical scavenging antioxidant activities among respective sequential extracts.

8.1.3 Immunomodulatory activities of *Euphorbia peplus* and *Viola odorata*

The findings of this study indicate that although the sequential extracts of both plants, *Euphorbia peplus* and *Viola odorata* failed to show a high anticancer activity, in the NCI's *in vitro* screens, the E4 and V3 extracts of both plants showed antiplatelet aggregation (47±6% & 73±9%) and COX2 inhibition (90±3% & 85±4%) activities *in vitro*. In addition, E3 extract of *Euphorbia peplus* indicated *in vitro* anticomplement activity IC50=30±9 μg/ml). The *in vitro* results thus raise the possibility that putative anticancer effect of *Euphorbia peplus* and *Viola odorata* may be due to their immunomodulatory activities mediating platelet activating factor, COX2 and also complement in case of *Euphorbia peplus*, although further studies will be needed to confirm these preliminary observations.

Although the plant, *Euphorbia peplus* is known as a toxic plant due to the presence of toxic 3-*O*-acyl esters of ingenol, its E3 extract at low concentrations (31±9 μg/ml), as shown by our results, probably exerts an anticancer activity by a total or partial immune-induced mechanism of action. The sequential extracts E1 and E2 that showed *in vitro* anticancer activity (Chapter 3) did not show a marked dose dependent inhibition of complement activation via both classical and alternative pathways, below a concentration of 1000 μg/ml.

On the other hand, in an adjuvant therapy anticancer drugs are being administered in combination with immuno potentiating drugs. Thus the use of a crude plant extract, such as a combined E2 and E3 extracts, having both cytotoxic and immunomodulatory activities may be useful in cancer therapy. The immunomodulatory activity may be manipulated to target cancer cells *in vivo* resulting in a differential cytotoxic effect. The *in vitro* inhibition of complement activation by *Euphorbia peplus* extracts, observed in this study supports the view that the putative anticancer activity, of the plant *Euphorbia peplus*, may be partially, due to its influence on the immune system. However, the present experimental findings are not sufficient to suggest any *in vivo* anticomplement or complement activating until further *in vivo* experiments are undertaken. It is plausible to

assume that many cancer drugs probably exert their antitumour activities by a total or partial immune-induced mechanism of action including complement modulation.

The crude anticancer drugs probably exert their antitumour activities by a total or partial immune-induced mechanism of action including complement modulation.

Our *in vitro* results raise the possibility that broad-spectrum approach comprising battery of assays system including the antioxidant, immunomodulatory (anticomplement), anti-inflammatory (anti-platelet aggregatory & COX-2 inhibitory) activity screens, may help to explain putative *in vivo* anticancer activity of the extracts of *Euphorbia peplus* and *Viola odorata*.

In order to investigate them effectively, the 'broad spectrum' approach entailing immunomodulators and antioxidant screens presented in this thesis is proposed. Not only novel therapeutic agents, but also novel biological targets would be revealed in such an investigation. With the revelation of the involvement of oncogene suppression and apoptosis gene activation in cancer, a broad-spectrum approach would encompass pathways and drug targets that influence such genes.

8.2 Further research directions

8.2.1 Screening assays

Selection criteria for complex extracts such as plant extracts need to be modified if the method of detection is not extremely sensitive.

If cytotoxic compounds need to be developed from crude extracts, then the NCI's selection criteria for the crude extracts need to be revised probably by increasing the minimum GI50 inhibitory potential criterion at least by 10-fold to 1.0 μg/ml.

A battery of assays, need to be developed to evaluate the putative herbal anticancer drugs. The attributes of natural materials and the aetiology of a disease as described in Ayurvedic system of medicine and other traditional systems of medicine must be subjected to a thorough scientific investigation. This by no means an easy task as many concepts related to the attributes of a drug and the aetiology of a disease need to be transformed into scientifically measurable parameters.

On the other hand, literature based studies would enable researchers to identify the appropriate assays that possibly measure the relevant attribute of a plant drug. Immunomodulatory and antioxidant activities may be included in a battery of assays for screening plant extracts for anticancer activity.

As a prescreen, the possibility of adopting screening assays such as COX-2 inhibitory activity, antiplatelet aggregation activity, immunomodulatory, and antioxidant activity which indirectly related to the anticancer activity need to be investigated.

After having been subjected to the toxicity evaluations, effective ranges for the antioxidant constituents based on their activity levels need to be developed, for the natural antioxidant preparations intend for medicinal use.

Based on the findings of this work, it is proposed that the DPPH method may be further developed by maintaining a constant temperature, i.e. 25^0C in a medium of which, the pH is adjusted to 6.5 according to Blois (1958). In order to further reduce the variation in results, covering of the reaction flask with aluminium foil and keeping the reaction mixture under nitrogen in a stoppered flask are suggested.

8.2.2 Chemical studies

The future research on active components of natural products may well be directed towards, standardization to ensure the identity, quality, purity and potency of the plant materials and to understand the mechanism of action of the active compounds in synergy rather than isolating a single active compound for mass scale drug manufacture.

The ESI-MS spectra and UV spectra acquired during HPLC fractionation using the photodiode array detector can be developed into profiles for recognition of patterns attributed to the chemical nature of certain active compounds. In addition, ESI technique may be used to screen the fractionation of active extracts, until one or two active compounds are detected in the positive as well as the negative mode ESI spectra. Having isolated the active compounds, the GC-MS, NMR, and FTIR analysis can be performed to characterise the compounds.

8.2.3 Utilising resources from traditional medical systems for future drug development

Two major areas needed to be further investigated, in order to utilise the herbal medical preparations described in the traditional medical systems, such as Ayurveda, for current diseases.

Firstly the aetiology of a disease, need to be defined in accurate scientific terms with the development of suitable parameters that measure the attributes of a disease state. Secondly, measurable parameters need to be developed to scientifically evaluate the attributes (Rasa, Guna, Veerya, Vipaka and prabhava) of Ayurvedic drugs in order to determine which plant extract is suitable for which disease state of the body. Such a field of research has economical benefits with the growing awareness of the use of natural products as promotive, preventive and curative medicaments, dietary supplements, and nutraceuticals.

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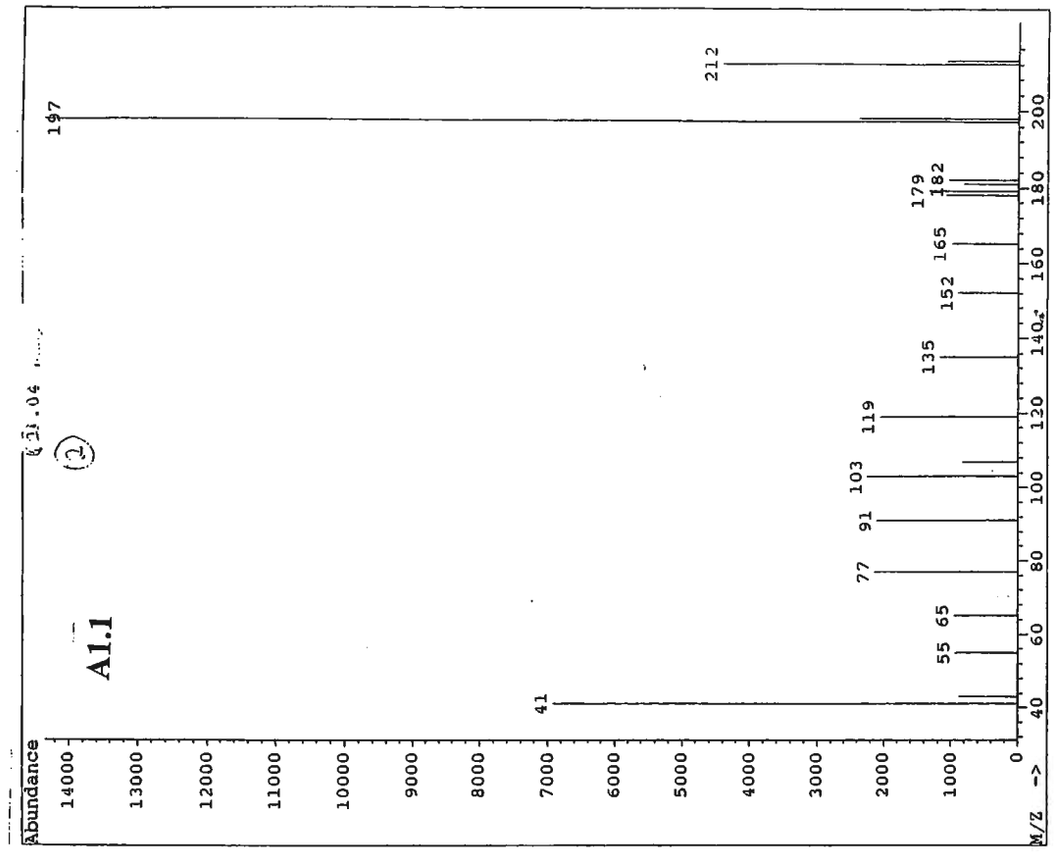
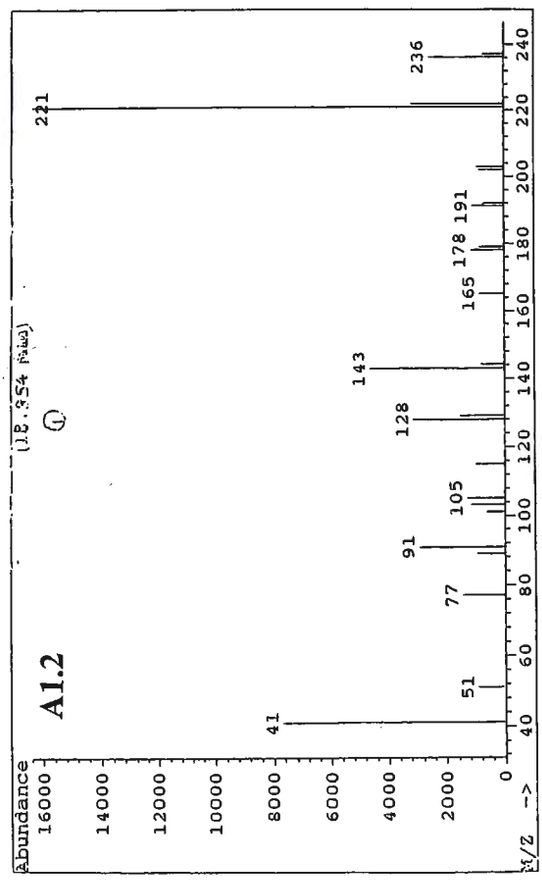
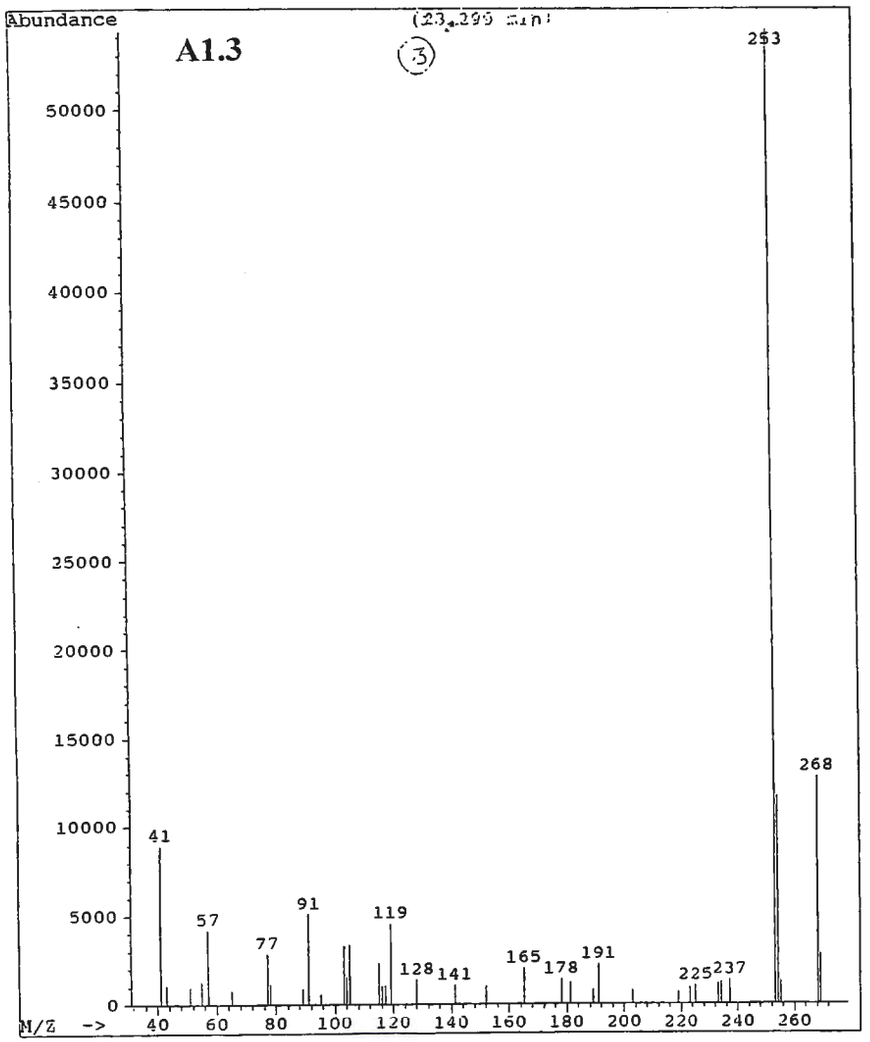
APPENDIX 1

GC-MS Spectra of the E4F1F6 fraction of *Euphorbia peplus* L.



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(23.299 min)
③
253
268
41
57
77
91
119
128
141
165
178
191
225
237
M/Z ->
0
5000
10000
15000
20000
25000
30000
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40000
45000
50000
40
60
80
100
120
140
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200
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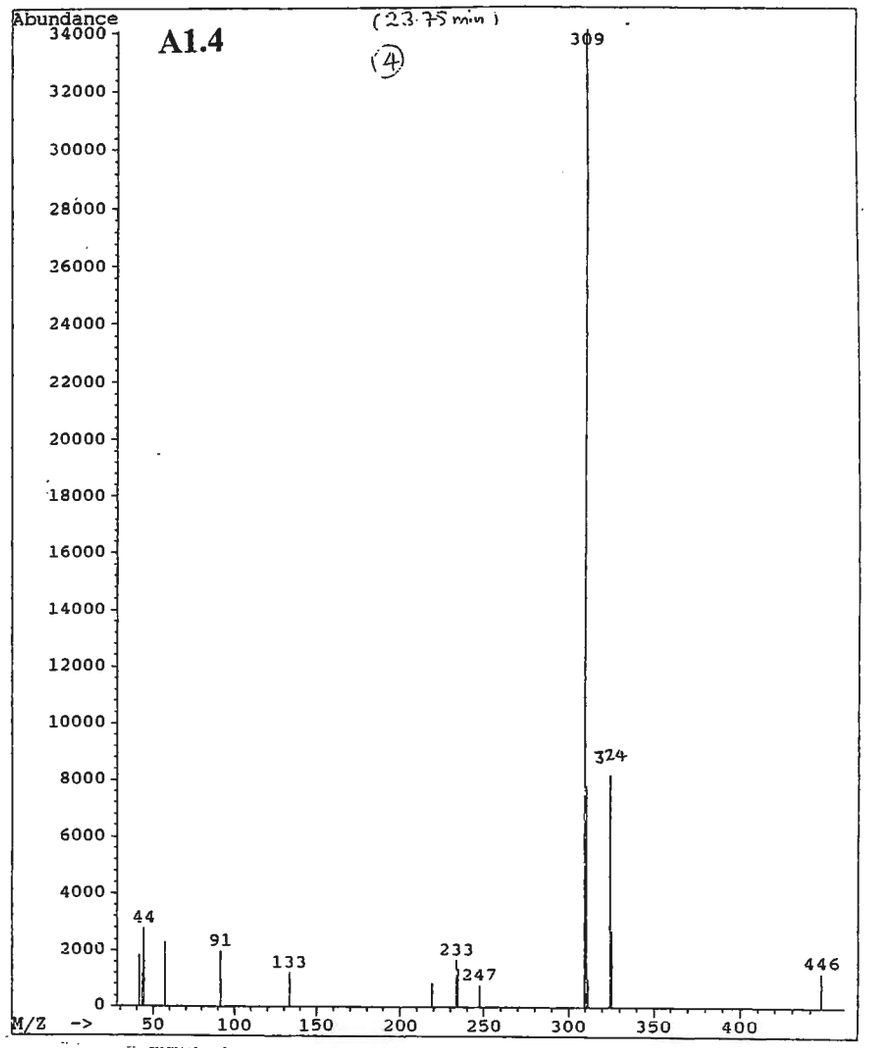
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(23.75 min)
④
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324
44
91
133
233
247
446
M/Z ->
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
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24000
26000
28000
30000
32000
34000
50
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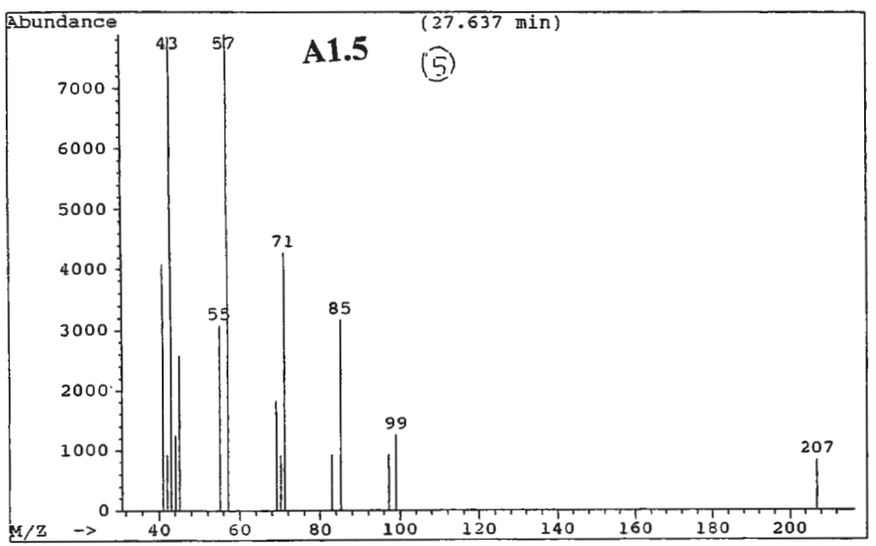
350
400


Abundance
(27.637 min)
A1.5
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57
71
55
85
99
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140
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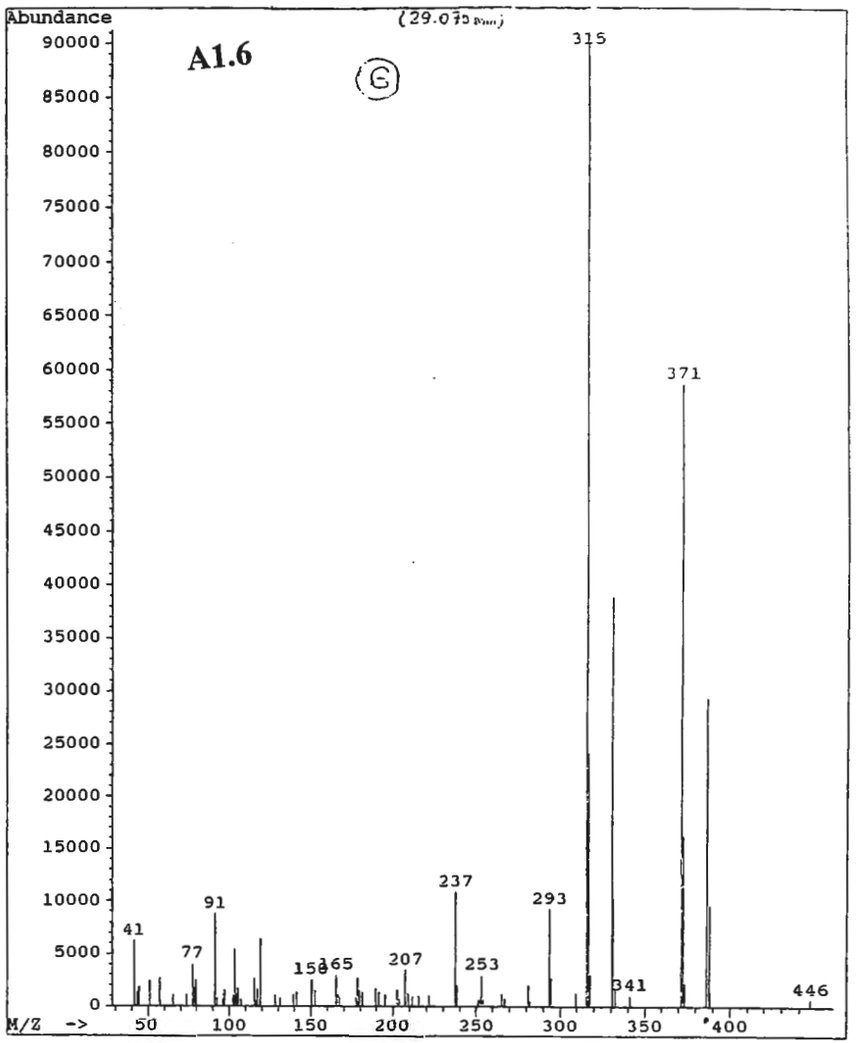
180
200


Abundance
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315
371
237
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91
41
77
165
207
253
341
446
M/Z ->
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80000
75000
70000
65000
60000
55000
50000
45000
40000
35000
30000
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10000
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0
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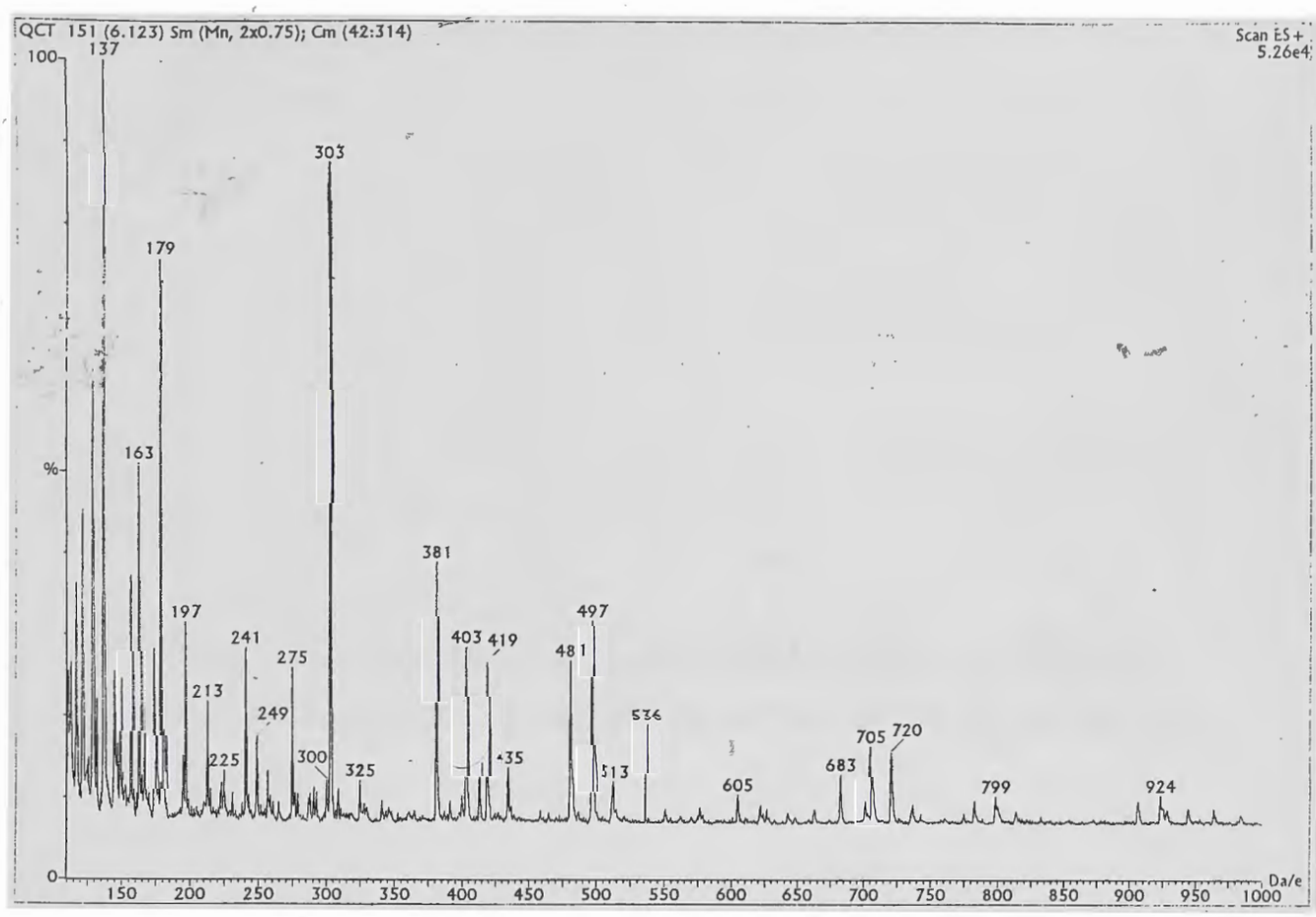
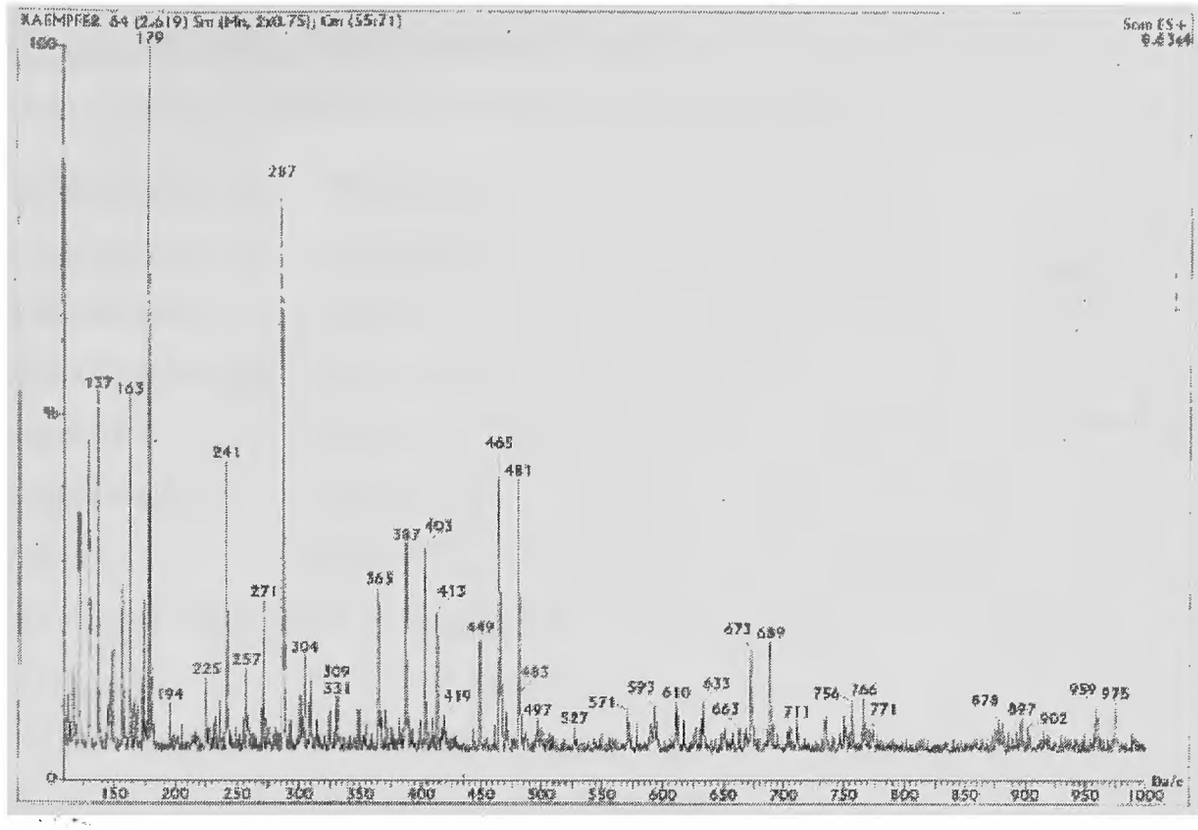
250
300
350
400

A. 1. 7 ESI+ spectrum of the quercetin standard



A. 1. 8 ESI+ spectrum of the kaempferol (aglycone) standard



APPENDIX 2

Constituents of *Viola odorata* L., according to,

Http://chili.rt66.com/hrbmoore/Constituents/Viola_odorata.txt;

Viola odorata L. (Violaceae) "Common Violet, Sweet Violet"

2,6-nanodiene-1-al	Flower; Leaf
2,6-nonadiene-1-ol	Flower; Leaf
3-β-friedelanol	Flower
aliphatic aldehydes	Flower 30,000 - 50,000 ppm
α-ionone	Flower
benzyl alcohol	Flower
β-ionone	lower
β-nitropropionic acid	Root 10 - 20 ppm
β-sitesterol	Plant 330 ppm
cyanin	Flower 53,000 ppm
eugenol	Flower 147 - 357 ppm; Leaf
D-dihydro-α-ionone	Flower
D- dihydro-β-ionone	Flower
diethylphthalate	Flower
enanthic acid	Leaf, Essential Oil, 900 - 1,200 ppm; Flower 300 - 1,700 ppm; Root 380 ppm
eugenol	Flower 147 - 357 ppm; Leaf
ferulic acid	Plant
friedlin	Leaf 160 ppm
gualtherin	Plant
heptenol	Flower
isoborneol	Flower
kaempferol	Plant
L-zingiberene	Flower
malic acid	Flower
methyl salicylate	Root
myrosin	Seed

n-2-octanol	Leaf
n-heptynol	Leaf
n-heptyl acid	Plant
n-hexanol	Flower; Leaf
n-hexenol	Leaf
n-octenol	Leaf
octadienol	Flower
octenoic acid	Leaf
octylic acid	Leaf
odoratine	Root 14,100 ppm
palmitic acid	Leaf
parmone	Flower
piperonal	Flower
propionic acid	Leaf;
quercetin	Plant
rutin	Flower 20,000 ppm
salicylic acid	Leaf
methyl salicylate	Root
saponins	Root 1,000 - 25,000 ppm
scopoletin	Plant
sinapic acid	Plant
triacetoamine	Root
undecanone-2	Flower
vanillin	Flower
violaquercetrin	Flower
violarutin	Flower 20,000 ppm
violin	Flower; Root
violutoside	Plant

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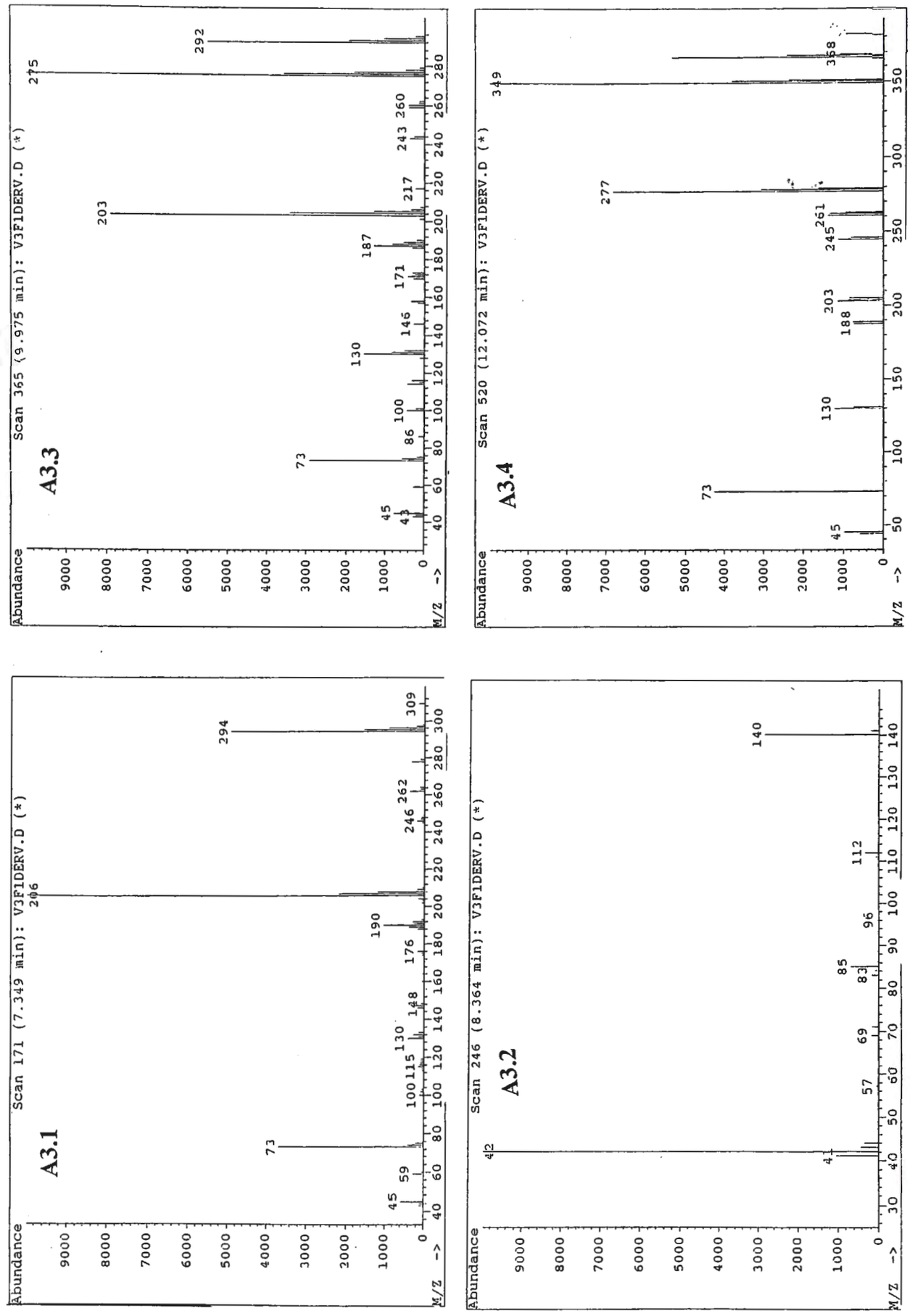
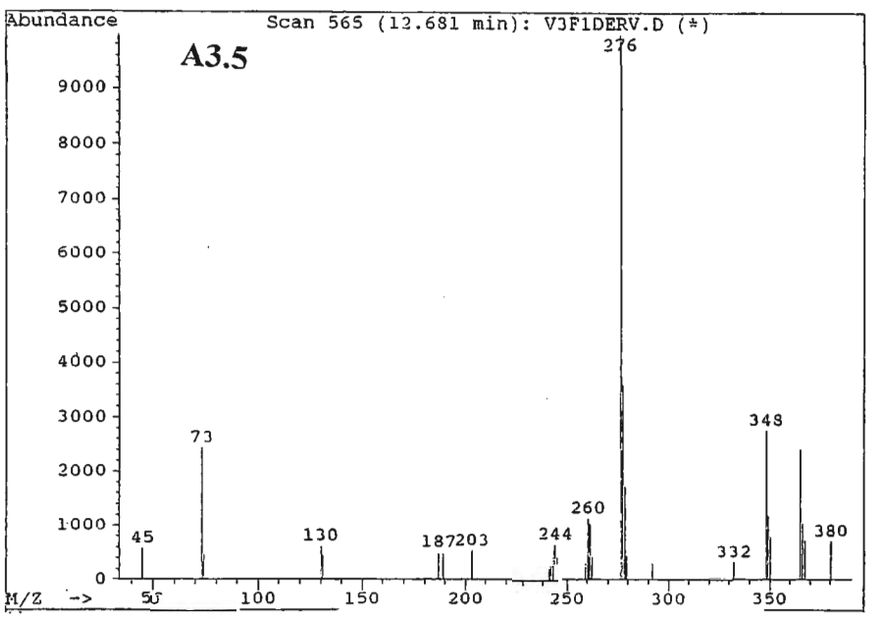
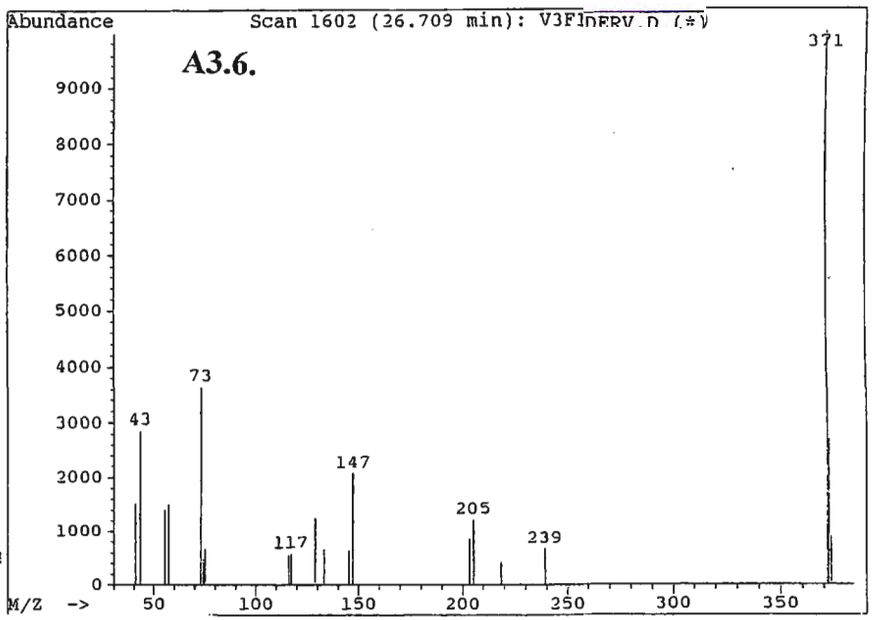
ppm = parts per million tr = trace

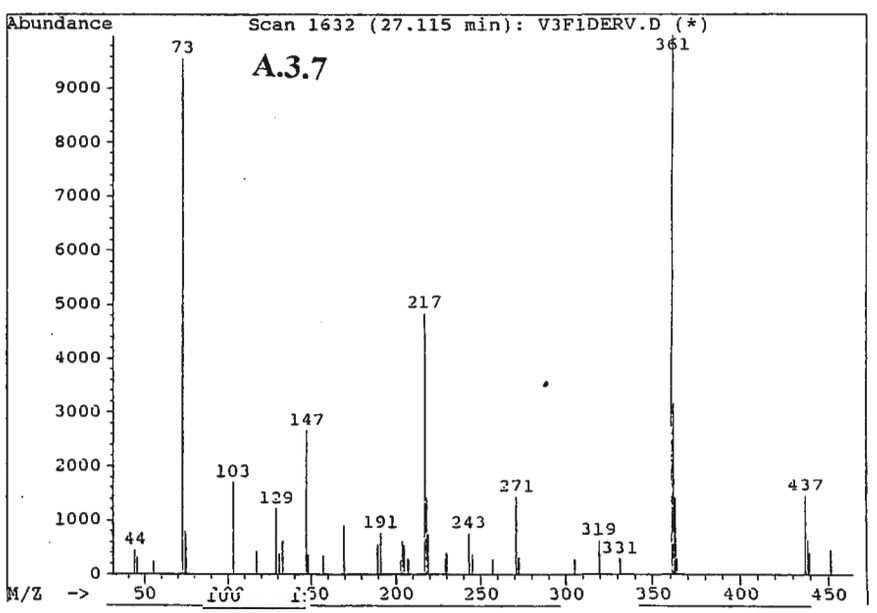
APPENDIX III

GC-MS Spectra of the V3F5 fraction of *Viola odorata* .




A3.5
Abundance
Scan 565 (12.681 min): V3F1DERV.D (*)
276
73
348
260
45
130
187203
244
332
380
M/Z ->
50
100
150
200
250
300
350


A3.6.
Abundance
Scan 1602 (26.709 min): V3F1DERV.D (*)
371
73
43
147
205
239
117
M/Z ->
50
100
150
200
250
300
350


A.3.7
Abundance
Scan 1632 (27.115 min): V3F1DERV.D (*)
73
361
217
147
103
129
271
437
191
243
319
331
44
M/Z ->
50
100
150
200
250
300
350
400
450