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Allelopathic potential of *Chrysanthemoides monilifera* subsp. *monilifera* (boneseed): A novel weapon in the invasion processes

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ABSTRACT

Natural ecosystems and primary production industries are threatened by invasive plant species, and allelopathy is one of the attributes that may assist in the invasion process. We studied the allelopathic potentiality of *Chrysanthemoides monilifera* subsp. *monilifera* (boneseed), one of the seven priority weeds identified for the development of predictive modeling in the world. A series of bioassays compared dose–response to aqueous extracts of boneseed as well as the impact of leachate on model (*Lactuca sativa*) and associated species (*Isotoma axillaris* and *Acacia mearnsii*) with particular reference to biometric, physiological and biochemical parameters. We found total phenolics in the order of leaf > root > stem > infested soil > outside soil. Acetone extracted more phenolics than other solvents, and air-dried double-centrifuged dominates over oven-dried single-centrifuged processing methods. Generally, aqueous extracts of boneseed organs showed ranked inhibition similar to phenolic content on germination indices and biometric parameters of both model and associated species, although, the hypocotyl length and weight response were varied. Dose response studies showed a strong correlation of aqueous extract concentration with both hypocotyl and radical length of *I. axillaris* even at low concentrations providing evidence of the allelopathic potential of boneseed. *I. axillaris* was the most susceptible species showing LC_{50} of 0.46%, 0.89% and 0.86% in response to leaf, stem and root extract respectively. Water uptake and carbohydrate metabolism of *L. sativa* seeds were gradually decreased with increasing extract concentrations. Hydrogen peroxide was increased with increasing extract concentration along with acceleration of electrolytic leakage and lipid peroxidation in *L. sativa* seedlings, providing evidence of cellular fragmentation suggesting a mechanism of allelopathic impact through excessive reactive oxygen species (ROS) production. Overall, leaf extracts showed more phytotoxicity when compared with other organs of boneseed. These findings help to explain the mechanism of invasion by boneseed and emphasize the importance of mitigating the effects of allelopathy by boneseed to protect native and crop species.

1. Introduction

Invasion of exotic species is one of the most important global scale problems experienced by natural ecosystems. More than 40% of the species on the list of threatened and endangered species in the world are listed due to the impact of invasive species on their habitat (Wilcove et al., 1998). Invasive species may pose a threat to the economy of a nation (Pimentel et al., 2005). Introduction of non-native species may occur either through accidental introduction or purposeful import for human utility. Success of invasion is controlled by the characteristics of the invaded habitat (Sakai et al., 2001) and biological attributes of the invader including allelopathy (Adkins and Sowerby, 1996), fitness homeostasis (Matthew, 1971), seed number, size and weight (Rejmánek and Richardson, 1996), animal dispersal (Binggeli, 1996), geographical range (Forcella and Wood, 1984), alternative mode of reproduction (Groves et al., 1995), competitive ability (Noble and Slatyer, 1980), and phenotypic plasticity (Dorken and Barrett, 2004).

The novel weapon hypothesis suggests that 'allelopathy' is one of the powerful mechanisms that permits plants to invade and establish in new ecosystems and ultimately determines the structure and composition of the invaded plant community (Bais et al., 2003; Callaway and Ridenour, 2004; Hierro and Callaway, 2003). Plants that negatively affect other plants through the production of secondary metabolites are considered allelopathic. In an ecosystem it is difficult to distinguish the impact of allelochemicals from more easily quantifiable resource competition, however, a number of studies have demonstrated the potential for allelopathic impact (Bais et al., 2003; Lawrence et al., 1991; Nilsson, 1994; Ridenour and Callaway, 2001).

Australia's natural environment and primary production industries are threatened by invasive species as they displace native species, degrade land, and reduce farm and forest productivity (Department of

Abbreviations: ROS, reactive oxygen species; TCA, trichloroacetic acid; TBA, thiobarbituric acid; TNC, total non-structural carbohydrate; MDA, malondialdehyde.

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Environment, 2013). Boneseed, a weed of national significance in Australia and listed on the National Pest Plant Accord in New Zealand, was introduced to Australia from South Africa as an ornamental garden plant in the mid-nineteenth century, with naturalized examples first recorded in Sydney in 1852 and Melbourne in 1858 (Brougham et al., 2006). This perennial shrub of 1–3 m height is one of the two subspecies (subsp. *monilifera* and *rotundata*) of *Chrysanthemoides monilifera* found in Australia. Both subspecies were planted extensively to stabilize coastal sand dunes and control erosion, particularly from the 1940s to the 1960s, with subsp. *monilifera* more commonly planted in Victoria (Burgman et al., 1998). Boneseed was proclaimed a noxious weed in Victoria in 1969 (Parsons, 1973), and soon after, the Australian Institute of Agricultural Science (AIAS) suggested that boneseed could potentially be "the most important weed on public land in southern Victoria" (Australian Institute of Agricultural Science, 1976) because of its prodigious potential for spread and regeneration, absence of natural enemies, competitive capacity, fire hazard and economic and environmental impacts (Parsons, 1973; Rudman, 2001; Thorp and Lynch, 2000; Weiss et al., 1998). Collectively, the two sub-species of *C. monilifera* threaten about 200 indigenous species in Australia (Department of Environment Conservation, 2006) including significant rare species such as *Pterostylis truncate* in Victoria. It was predicted that more than 15% of Australia could be invaded by these two subspecies in the near future (Weiss et al., 2008). Boneseed infestations have also occurred in South Africa, California, Sicily, St Helena and Southern France (Weiss et al., 1998). Globally, *C. monilifera* has been identified as one of the seven priority weeds for the development of predictive modeling (Underwood et al., 2006). There has been research on boneseed management and control (Brougham et al., 2006; Lane and Shaw, 1978), however, the mechanism of boneseed invasion has never been directly investigated, in spite of the severe threat boneseed poses to native species regeneration (McAlpine et al., 2009). Such research is important to determine the impact of the invasive species on biodiversity, to predict rates of invasion, and to devise control and restoration processes (Dorning and Cipollini, 2006). Several studies on allelopathic potential of *C. monilifera* subsp. *rotundata* (bitou bush) lead the suspicion of the presence of allelochemicals in boneseed as a weapon of invasion (Ens et al., 2009b; Lindsay and French, 2004). Thus, there is a need to demonstrate the allelopathic potential of boneseed as a mechanism in the invasion processes.

Plant–plant negative interactions through the release of allelochemicals in the mode of volatilization (Halligan, 1975), root exudation (Uddin et al., 2014a; Yu et al., 2003), decomposition of residues in soil (Bonanomi et al., 2005; Uddin et al., in press), and leaching (Amoo et al., 2008) are well established. Most of the allelopathic research into the mechanism of the invasion process are based on germination and growth impact studies (Dorning and Cipollini, 2006; Javaid et al., 2006). A number of phytotoxicity studies had been conducted using solvents (methanol, acetone, ethanol) rather than water as an extraction method (Hill et al., 2007; Yamane et al., 1992). The findings of these studies are ecologically questionable as water is the only medium that mimics what would happen in nature. Phenolic content and phytotoxicity of the extracts may vary with extraction solvents (Ens et al., 2009b; Jefferson and Pennacchio, 2003). Additionally, allelopathic impact studies using oven dried samples extract (Morgan and Overholt, 2005; Omezzine et al., 2011) are ecologically unrealistic as the properties of the samples including phenolics may be changed with temperature (Janas et al., 2000; Ju and Howard, 2003). This study includes the influences of extraction media and drying methods in conjunction with centrifuging mechanisms on phenolic content of boneseed, and air dried samples with water extraction was used for bioassay. The physiological responses of plants to allelochemicals are particularly complex since resource competition, allelopathy, nutrient immobilization and microbial influence operate in parallel (Bhowmik, 2003; del Moral, 1997). There is clear evidence that allelochemicals can affect germination, growth, physiology and even genetic factors of neighboring plants (Bais et al., 2003). Secondary metabolites affect cellular processes of target species leading a measurable changes in electrolyte leakage (Galindo et al., 1999), lipid peroxidation (Batish et al., 2006) cell division (Anaya and Pelayo-Benavides, 1997) and stomata opening-closure (Barkosky et al., 2000). Affecting electrolyte leakage, and lipid peroxidation of target species through excessive ROS production, a mode of allelopathic action suggested by Weir et al. (2004) is rarely considered in allelopathic studies. Seed imbibition, the first phase of seed germination, is an important step in germination as no seeds germinate until water uptakes by seeds reach a critical level. With some exception (Chon et al., 2004), the impact of allelochemicals on seed imbibition, leading to a delay or decreasing rates of seed germination, is rarely addressed in allelopathic studies. Another important factor affecting seed germination, seed reserve mobilization (carbohydrate), is also hardly ever studied, or where investigated it is generally over long timescales (once after few days), while in reality there might be significant effects in hours (Uddin et al., 2013). Criticism has also been raised about some bioassay experiments using high concentrations of aqueous extracts in allelopathic studies, as the impact of osmotic potential may be conflated in such studies (Anderson and Loucks, 1966), although dose–response studies remain one of the most effective ways to study phytotoxic potential of allelopathic species (Sunmonu and Van Staden, 2014).

The current study aimed to investigate allelopathy in boneseed by integrating ecological, physiological, and biochemical approaches based on the hypothesis: "boneseed is allelochemically invasive".

2. Methods

2.1. Field Sampling and seed collection

You Yangs Regional Park, Victoria (37° 59′ 44″ S, 144° 24′ 39″ E) was selected as the study area as it is the home, since 1940, of one of the Australia's densest boneseed populations (Roberts, 2008). In September 2012, boneseed plants, and soil samples from the rhizosphere and adjacent boneseed unoccupied areas were collected, sealed in plastic bags and immediately transported to the laboratory. The plant samples were separated into leaves, stems, and roots after cleaning to remove extraneous organic matter and soil. All organs were chopped into 1–2 cm pieces, separately. The samples (plant, soil and litter) were dried either in air or oven (40 °C), as necessary, to constant weights. Dried samples were ground in a grinder, passed through a 0.5 mm mesh sieve and stored in sealed plastic vials until chemical analyses and experiments were conducted. Fresh boneseed leaves were collected, sealed in plastic bags and transferred to the laboratory in refrigerated boxes to preserve freshness for specific leaf leachate impact experiments. Seeds of *Lactuca sativa* and *Acacia mearnsii* were purchased from Bunnings Warehouse Australia and Seed World Australia, respectively, and seeds of other associated species *Isotoma axillaris* were collected from the wild.

2.2. Determination of phenolics concentration

We integrated different mechanisms in extraction of total phenolics from boneseed organs, e.g., drying (air and oven dried), extracting solvents (acetone, methanol, ethanol and water) and centrifuging (single and double centrifuged). Total phenolic concentrations were measured using the Folin–Ciocalteu assay (Singleton and Rossi, 1965) with slight modifications using gallic acid as the standard (Bärlocher and Graça, 2005). Approximately 100 mg of sieved powder of leaves, stems, roots, infested rhizosphere soil and boneseed unoccupied soil was transferred to Eppendorf tubes. To powdered materials, 5 mL of 70% acetone was added, and the samples were incubated at 4 °C for 1 h to extract phenolics. The extracted materials were centrifuged at 4 °C for 15 min at 15,000 rpm (Beckman Avanti 30 High Speed Compact Centrifuge 364105, Beckman Coulter Inc., USA), after which 0.5 mL

supernatant was mixed with distilled water (dH_2O) to 1 mL. 5 mL of "2% Na_2CO_3 in 0.1 N NaOH" was added and mixed. After 5 min, 0.5 mL of the Folin–Ciocalteu reagent was added, mixed and incubated at room temperature for 2 h. Finally, absorbance was measured at 760 nm in a spectrophotometer (Libra S12, manufactured by Biochrom Ltd., England) and phenolic concentrations were determined based on a standard curve of gallic acid. A similar procedure was repeated for measuring total phenolics in water, methanol and ethanol extracts for different drying and centrifuging methods.

2.3. Bioassay with aqueous extract of boneseed

To prepare 5% (w/v) aqueous extracts, 50 g of dry powder of boneseed organs was mixed in 1 L dH_2O and agitated for 24 h on an orbital shaker (Orbital Mixer EOM5, Ratek Instruments Pty. Ltd, Australia) at room temperature. The extract was centrifuged at 3000 rpm (Econospin 120010, Sorvall Instruments, Germany) for 15–20 min, and the supernatant was passed through a 0.22 μm filter before storage at −80 °C. Aqueous extracts of 0.155, 0.31, 0.62, 1.25 and 2.5% (w/v) were prepared for bioassay experiments. To control for possible extraneous effects, pH (Pocket digital pH meter, 99559, Dick-smith electronics, Australia), chloride concentration (Shimadzu Ion Chromatograph, Kyoto, Japan) and electrical conductivity (EC) (TPS Digital conductivity meter, 2100, TPS Pty Ltd., Australia) of all extracts were measured and pH was neutralized using 1 N NaOH solution (Fu and Viraraghavan, 2002). Osmotic potential was calculated following the equation proposed by McIntyre (1980).

All seeds were surface sterilized with 1.5% (v/v) sodium hypochlorite for 1 min before washing in dH_2O (Jefferson and Pennacchio, 2003). Twenty five seeds of *L. sativa* (model species), and *I. axillaris* (associated species) and 10 seeds of *A. mearnsii* (associated species) were placed in 90 mm Petri dish lined with two Whatman no. 1 filter paper moistened with 5 mL of different concentrations of aqueous extracts (0.155% to 5% for *I. axillaris*, and 1.25% to 5% for *L. sativa* and *A. mearnsii*) of each organ. Distilled water was used as a control (0%). Five replicates were maintained for model species and 4 replicates were maintained for associated species in a completely randomized design (CRD) for each treatment. The Petri dishes were sealed with parafilm and incubated in a growth chamber (Westinghouse, Electrolux home products, Australia) at 25 °C/15 °C (day/night) temperature for *A. mearnsii* and 30 °C/20 °C (day/night) temperature for *I. axillaris* while for *L. sativa* constant 25 °C temperature was maintained. The associated species were grown with a 12 h photoperiod, whereas, model species was grown in darkness. The number of germinated seedlings (radicle protrudes by ≥1 mm) in all petri dishes were counted daily until cumulative germination leveled off (7, 15 and 17 days for *L. sativa, I. axillaris* and *A. mearnsii* respectively). Germination indices e.g., total germination (TG), speed of germination (SpG), speed of accumulated germination (SpAG) and coefficient of the rate of germination (CRG) were calculated along with biometric parameters including hypocotyl and radicle length and weight (Chiapusio et al., 1997; Jefferson and Pennacchio, 2003). The lethal concentration (LC_{50}) was calculated from TG.

2.4. Bioassay with boneseed leachate

Leaf leachate of 2.5% (dry equivalent) was extracted by submerging fresh leaf of boneseed, equivalent to the 25 g dry leaf, in 1 L dH_2O for 24 h at room temperature. The leachate was membrane filtered and preserved at −80 °C. Bioassays were conducted as described for the aqueous extract samples (Section 2.3) but were limited to *L. sativa* and *I. axillaris* seeds and preceded for 7 days for *L. sativa* and 15 days for *I. axillaris* after which all the above mentioned parameters (Section 2.3) were measured.

2.5. Seed imbibition

L. sativa, a model test species was used for the evaluation of the physiological and biochemical impact of aqueous extracts (1.25, 2.5 and 5%) of boneseed organs. Effects of extracts on seed imbibition were measured soaking 100 mg seeds in either aqueous extracts of various concentrations of each organ or dH_2O for control. Treatments were arranged in a complete randomized design in triplicate in a growth chamber (Westinghouse, Electrolux home products, Australia) at 25 °C in darkness. The seed imbibition (%) was determined from the weight differences at 0, 4, 8 and 24 h.

2.6. Seed reserve mobilization

Total non-structural carbohydrate (TNC), a measurement of seed reserve mobilization was measured using the phenol sulphuric acid method (Kabeya and Sakai, 2005) with slight modification. Briefly, seeds (~6 mg) after 0, 4, 8 and 24 h imbibition were collected and placed in 50 mL Schott bottles filled with 40 mL of 0.4 N H_2SO_4 acid and refluxed for 1 h in a boiling water bath. The hot solution was filtered through Whatman No. 42 filter paper and diluted to volume with dH_2O after cooling. 2 mL extraction was transferred to test tube and 0.05 mL phenol (80%) and 5 mL H_2SO_4 (95%) were added and mixed thoroughly. After 10 min, the test tubes were placed in a 30 °C water bath for 15 min. Finally, the carbohydrate content of the filtrate was determined spectrophotometrically at 485 nm using glucose solution as a calibration standard.

2.7. Oxidative stress evaluation

A dose response bioassay experiment on *L. sativa* was conducted using 1.25, 2.5, and 5% aqueous extracts of leaf, stem and root to evaluate the impact on ROS production, and consequently, on electrolyte leakage and lipid peroxidation. Three replicates were arranged in a CRD and harvested after 7 days.

2.7.1. Hydrogen peroxide

Hydrogen peroxide (H_2O_2), a member of ROS was measured following the method of Velikova et al. (2000) to investigate whether the aqueous extracts produced excessive ROS. Briefly, 100 mg plantlets was homogenized with 5 mL 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 rpm for 15 min. From the centrifuged material, 0.5 mL supernatant was transferred to test tube to which 0.5 mL 10 mM phosphate buffer (pH 7.0) and 1 mL 1 M potassium iodide were added. The absorbance was read at 390 nm and H_2O_2 concentrations were determined based on extinction coefficient of 0.28 $\mu M^{-1}\ cm^{-1}$.

2.7.2. Electrolyte leakage

To measure electrolyte leakage, seedlings with equal weights (~100 mg) for both control and treatment were placed in 15 mL dH_2O at room temperature in darkness. EC was measured after 2 h incubation, followed by as second EC measurement after 20 min boiling in a water bath. Results were expressed as a percentage of total leakage (Bogatek et al., 2006).

2.7.3. Lipid peroxidation

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content as it is used as an index of lipid peroxidation, and expressed as nmol g^{-1} fresh weight (Jambunathan, 2010). Plantlets of 200 mg was homogenized with 4 mL of 0.1% TCA, and centrifuged at 15,000 rpm for 15 min. 1 mL of supernatant was transferred to a test tube to which 2 mL each of 20% TCA, and 0.5% thiobarbituric acid (TBA) were added and heated at 95 °C in a fume hood followed by water cooling. The absorbance was read at 532 nm and 600 nm using a spectrophotometer and lipid peroxidation was calculated using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$.

2.8. Data analysis

Statistical analysis was conducted using IBM SPSS 21.0. All data are presented as mean ± standard error (SE). Significant differences between the means were determined at a 5% level of probability ($p \leq 0.05$) using independent *T*-test, one-way ANOVA followed by post hoc Dunnett's test or two-way ANOVA followed by LSD based on the structure of data set. Linear regression was adopted to express the relationship between extract concentration and hypocotyl/radical length of germinated species.

3. Results

3.1. Phenolics and physicochemical properties in boneseed extract

High concentrations of total phenolics were found in boneseed organs but little in soil. The highest concentrations of phenolics were measured from air dried, double centrifuged, acetone extracts of leaf (96.86 mg/g), followed by root (74.39 mg/g) and stem (52.15 mg/g) respectively. Leaf leachate, boneseed infested soil and outside (boneseed unoccupied) soil contained total phenolics of 9.43, 3.04 and 0.03 mg/g, respectively. Acetone extracts contained 53, 52 and 41% more phenolics compared with water, methanol and ethanol respectively (Fig. 1). On average, air dried sample extracts contained 30% more phenolics than the oven dried samples, and double centrifuged processing method extracted 21% more phenolics compared with single centrifuged (Fig. 1). pH (before adjustment), EC, osmotic potential and chloride values for different concentrations (0.155 to 5%) of all extracts and leaf leachate were 4.8 to 6.48, 0.06 to 3.22 ms cm^{-1}, −0.02 to −1.16 bar and 26–1013 ppm, respectively. ANOVA test indicated significant individual and interactive effects of boneseed organs, extracting media and drying and centrifuging mechanisms on total phenolic concentrations with the exception of the interactive effect of drying and centrifuging methods which was not significant (Table 1).

3.2. Impact of aqueous extracts on germination of model and associated species

There was a severe impact of extracts on *I. axillaris* even at very low concentrations, with inhibition of TG by 100% in 5% concentration of all extracts (Table 2). Additionally, TG of *I. axillaris* was inhibited by 0.155, 0.31, 0.62, 1.25 and 2.5% extracts of leaf (6.0, 41.7, 58.3, 90.5, 97.7%, respectively), stem (3.6, 16.7, 34.5, 65.5, 86.9%, respectively) and root (4.8, 33.3, 27.4, 77.4, 92.9%, respectively). SpG was inhibited by 18.6, 67.4, 77.5, 95.6, 99.2% (leaf extracts), 8.7, 42.8, 51.0, 73.0, 92.1% (stem extracts), and 10.9, 52.1, 45.5, 85.1, 96.9% (root extracts) respectively, along with inhibition of other germination parameters (Table 2). Leaf extracts of 0.15 to 2.5% showed 5–54%, 15–39% and 7–8%, and 18–91%, 0–15% and 0–2% more inhibition to hypocotyl and radical length of *I. axillaris* compared with control, stem and root extracts, respectively, with a similar impact on hypocotyl and radical weight (Fig. 2). Boneseed organ and extract concentrations had strongly significant impacts ($p < 0.001$) both individually and interactively on germination indices and biometric parameters of *I. axillaris* (Table 3). Inhibition was found for all measured parameters with the exception of hypocotyl length and weight with stem extract (0.155, 0.31 and 0.62%) that perversely caused stimulation. Regression analyses showed that concentration of aqueous extracts had a strong correlation with hypocotyl and radical length of *I. axillaris* (R^2 ranges from 0.97 to 0.72) (Fig. 3). *I. axillaris* was the most susceptible among the tested species with an LC_{50} of 0.46%, 0.89% and 0.86% in response to leaf, stem and root extracts, respectively. Similar to *I. axillaris*, all parameters were significantly affected in the bioassay of *A. mearnsii* with the exception of a non-significant interactive effect on CRG (Tables 2 & 3 and Fig. 4). Despite the observed inhibition to all parameters for all doses of leaf extract, 1.25% stem extract stimulated TG, 2.5% stem extract stimulated hypocotyl and radical length and radical weight, and 1.25% root extract stimulated SpG, SpAG, CRG, hypocotyl length, and hypocotyl and radical weight (Table 2 and Fig. 4).

The individual and interactive impact of boneseed organs and extract concentrations on germination indices and biometric parameters of model species, *L. sativa* was significant with the exception of non-significant interactive and individual (boneseed organs) impact on TG (Table 3). 1.25% and 2.5% leaf extracts showed no inhibition of TG, but it was inhibited by 5% leaf extract by 12, 7.6 and 4.3% respectively when compared with control, stem extracts and root extracts (Table 2). SpG was inhibited by 3.1, 10.1, 69.5% (1.25% leaf extract), 2.0, 8.2, 66.6% (2.5% leaf extract), and 1.2, 5.9, 63.7% (5% leaf extract) when compared to control, stem extract and root extract, respectively, along with similar impacts on SpAG and CRG (Table 2). 5% leaf extract inhibited hypocotyl length by 30.0, 20.5 and 11% compared with control, stem extract and root extract, although 1.25 and 2.5% leaf extracts showed stimulatory effects by 44.5 and 41.4%, and 13.8 and 35.2% compared with control and root extracts (Fig. 5). However, they inhibited hypocotyl length by 8.2 and 23.2% respectively compared with 1.25 and 2.5% stem extracts. Leaf extracts showed stronger inhibition to radical length compared with control and other extracts. 1.25, 2.5 and 5% leaf extracts inhibited radical length by 79.8, 81.8, 86.3%, and 58.7, 23.3, 14.1%, and 53, 26.8, 4.8% compared with control, stem extracts and root extracts (Fig. 5).

Table 1
ANOVA output (*F* − ratios) displaying the effects of various organs of boneseed (O), extraction media (M), drying condition (D) and centrifuging condition (C) on total phenolics concentration.

Parameters	Total phenolic concentration	Sig. (*p* value)
O	$F_{2, 96} = 7135.2^{***}$	.000
M	$F_{3, 96} = 2024.9^{***}$	.000
D	$F_{1, 96} = 3094.45^{***}$	.000
C	$F_{1, 96} = 1605.6^{***}$	.000
O × M	$F_{6, 96} = 90.84^{***}$	.000
O × D	$F_{2, 96} = 169.77^{***}$	.000
O × C	$F_{2, 96} = 63.77^{***}$	.000
M × D	$F_{3, 96} = 9.02^{***}$	.000
M × C	$F_{3, 96} = 125.68^{***}$	.000
D × C	$F_{1, 96} = 0.268$	.606
O × M × D	$F_{6, 96} = 44.95^{***}$	.000
O × M × C	$F_{6, 96} = 21.79^{***}$	.000
O × D × C	$F_{2, 96} = 6.18^{**}$	.003
M × D × C	$F_{3, 96} = 8.27^{***}$	.000
O × M × D × C	$F_{6, 96} = 7.61^{***}$	.000

***Strongly significant ($p < 0.001$). **Significant ($p \geq 0.001$ to < 0.01). *Poorly significant ($p = 0.01$ to ≤ 0.05), and blank means non-significant.

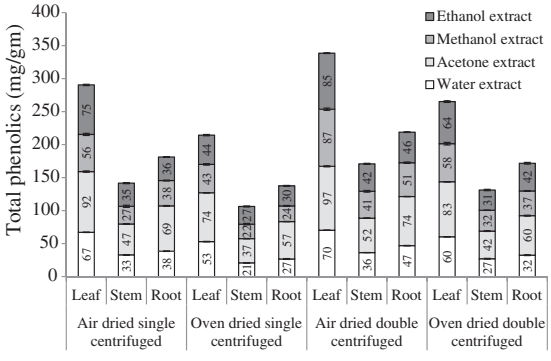

Fig. 1. Total phenolic concentration in boneseed leaf, stem and root extracted using different solvents, drying and centrifuging mechanisms.

Table 2
Impact of boneseed aqueous extracts (dose response) on germination indices of model and associated species. Data presented as average ± SE.

Treatment	*L. sativa*				*I. axillaris*				*A. mearnsii*			
	TG	SpG	SpAG	CRG	TG	SpG	SpAG	CRG	TG	SpG	SpAG	CRG
Control	100 ± 0.00	24.8 ± 0.12	64.42 ± 0.24	24.96 ± 0.03	84 ± 2.83	2.98 ± 0.09	16.58 ± 0.45	9.51 ± 0.03	97.5 ± 2.5	1.18 ± 0.07	6.92 ± 0.51	8.22 ± 0.10
Leaf Extract												
0.155%	–	–	–	–	79 ± 3	2.55 ± 0.10	13.49 ± 0.53	9.21 ± 0.03	–	–	–	–
0.31%	–	–	–	–	49 ± 3.42	1.26 ± 0.08	5.40 ± 0.39	8.54 ± 0.09	–	–	–	–
0.62%	–	–	–	–	35 ± 1.91	0.89 ± 0.06	3.72 ± 0.34	8.47 ± 0.07	–	–	–	–
1.25%	99.2 ± 0.80	24 ± 0.12	62.7 ± 0.30	24.82 ± 0.06	8 ± 1.63	0.19 ± 0.04	0.73 ± 0.20	8.14 ± 0.08	80 ± 4.08	0.88 ± 0.06	4.84 ± 0.36	8.04 ± 0.09
2.5%	98.4 ± 0.98	22.28 ± 0.24	58.55 ± 0.62	24.37 ± 0.07	2 ± 1.15	0.04 ± 0.03	0.14 ± 0.08	7.56 ± 0.25	67.5 ± 2.50	0.67 ± 0.04	3.49 ± 0.29	7.75 ± 0.08
5%	88 ± 2.83	7.6 ± 0.29	23.82 ± 0.84	19.97 ± 0.06	0	–	–	–	50 ± 4.08	0.48 ± 0.03	2.36 ± 0.18	7.70 ± 0.11
Stem extract												
0.155%	–	–	–	–	81 ± 1.91	2.78 ± 0.06	15.13 ± 0.31	9.40 ± 0.02	–	–	–	–
0.31%	–	–	–	–	70 ± 2.58	1.99 ± 0.07	9.48 ± 0.31	8.84 ± 0.04	–	–	–	–
0.62%	–	–	–	–	55 ± 1.91	1.63 ± 0.06	8.12 ± 0.34	8.95 ± 0.03	–	–	–	–
1.25%	100 ± 00	24.5 ± 0.18	63.8 ± 0.79	24.89 ± 0.04	29 ± 1.91	0.88 ± 0.08	4.48 ± 0.50	9.02 ± 0.08	100 ± 0.00	1.18 ± 0.03	6.77 ± 0.27	8.22 ± 0.06
2.5%	99.2 ± 0.80	24.27 ± 0.25	63.20 ± 0.59	24.88 ± 0.04	11 ± 1.91	0.29 ± 0.05	1.31 ± 0.26	8.60 ± 0.18	92.5 ± 2.50	0.98 ± 0.02	5.36 ± 0.14	7.96 ± 0.04
5%	95.2 ± 1.5	22.65 ± 0.37	59.24 ± 0.88	24.71 ± 0.10	0	–	–	–	77.5 ± 2.50	0.82 ± 0.01	4.48 ± 0.11	7.96 ± 0.08
Root extract												
0.155%	–	–	–	–	80 ± 2.83	2.73 ± 0.09	14.78 ± 0.47	9.40 ± 0.01	–	–	–	–
0.31%	–	–	–	–	56 ± 1.63	1.63 ± 0.05	7.95 ± 0.30	8.89 ± 0.03	–	–	–	–
0.62%	–	–	–	–	61 ± 1.91	1.81 ± 0.08	9.04 ± 0.48	8.94 ± 0.04	–	–	–	–
1.25%	99.2 ± 0.8	24.3 ± 0.2	63.3 ± 0.48	24.9 ± 0.05	19 ± 1.91	0.53 ± 0.06	2.47 ± 0.30	8.76 ± 0.11	95 ± 2.89	1.22 ± 0.06	7.19 ± 0.47	8.40 ± 0.06
2.5%	98.4 ± 0.98	23.67 ± 0.32	61.63 ± 0.84	24.75 ± 0.07	6 ± 1.15	0.14 ± 0.03	0.52 ± 0.15	8.04 ± 0.15	80 ± 4.08	0.90 ± 0.08	5.04 ± 0.50	8.09 ± 0.10
5%	92 ± 2.53	20.85 ± 0.41	54.74 ± 1.05	24.4 ± 0.11	0	–	–	–	67.5 ± 2.50	0.69 ± 0.02	3.65 ± 0.15	7.82 ± 0.04

– Not investigated. – No germination occurred.

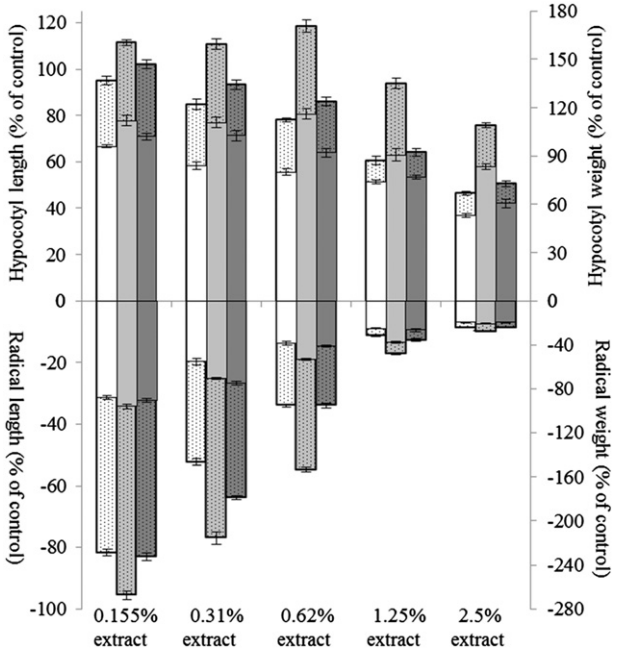

Fig. 2. Effect of different concentrations of boneseed leaf (white), stem (light ash) and root (deep ash) extracts on hypocotyl (upward) and radical (downward) length (plain) and weight (dotted) on *I. axillaris*. Data presented as the percentage of control.

3.3. Impact of leachate on germination of test species

Impact of leaf leachate on model and associated species was negligible compared with similar dose of aqueous extract derived from boneseed leaves (Table 2 and Fig. 6). Inhibition of TG, SpG, SpAG, CRG, hypocotyl length, hypocotyl weight, radical length and radical weight of *L. sativa* and *I. axillaris* by leachate was 2, 7, 6, 2, 6, 19, 15 and 29%, and 67, 57, 51, 15, 72, 71, 24 and 34% reduced (% of control) when compared to impact of 2.5% aqueous extract respectively (Table 2 and Fig. 6).

Table 3
ANOVA output (F — ratios) displaying the effects of various organs of boneseed (O) and extract concentration (C) on germination of *I. axillaris*, *A. mearnsii* and *L. sativa*.

Species	Parameter	O	C	O × C
I. axillaris	TG	$F_{2, 54} = 44.86^{***}$	$F_{5, 54} = 588.33^{***}$	$F_{10, 54} = 7.88^{***}$
	SpG	$F_{2, 54} = 63.97^{***}$	$F_{5, 54} = 791.38^{***}$	$F_{10, 54} = 8.78^{***}$
	SpAG	$F_{2, 54} = 74^{***}$	$F_{5, 54} = 868.72^{***}$	$F_{10, 54} = 9.75^{***}$
	CRG	$F_{2, 54} = 104.03^{***}$	$F_{5, 54} = 130.88^{***}$	$F_{10, 54} = 46.35^{***}$
	HL	$F_{2, 54} = 286.41^{***}$	$F_{5, 54} = 284.62^{***}$	$F_{10, 54} = 18.17^{***}$
	HW	$F_{2, 54} = 94.15^{***}$	$F_{5, 54} = 90.49^{***}$	$F_{10, 54} = 6.89^{***}$
	RL	$F_{2, 54} = 223.48^{***}$	$F_{5, 54} = 4898^{***}$	$F_{10, 54} = 38.64^{***}$
	RW	$F_{2, 54} = 24.56^{***}$	$F_{5, 54} = 736.53^{***}$	$F_{10, 54} = 5.81^{***}$
Amearnsi-i	TG	$F_{2, 36} = 39.37^{***}$	$F_{3, 36} = 72.55^{***}$	$F_{6, 36} = 4.84^{**}$
	SpG	$F_{2, 36} = 24.61^{***}$	$F_{3, 36} = 60.93^{***}$	$F_{6, 36} = 3.27^{*}$
	SpAG	$F_{2, 36} = 19.43^{***}$	$F_{3, 36} = 54.04^{***}$	$F_{6, 36} = 2.75^{*}$
	CRG	$F_{2, 36} = 6.78^{**}$	$F_{3, 36} = 17.51^{***}$	$F_{6, 36} = 1.50$
	HL	$F_{2, 36} = 40.67^{***}$	$F_{3, 36} = 123.78^{***}$	$F_{6, 36} = 12.68^{***}$
	HW	$F_{2, 36} = 32.15^{***}$	$F_{3, 36} = 105.69^{***}$	$F_{6, 36} = 4.75^{**}$
	RL	$F_{2, 36} = 61.71^{***}$	$F_{3, 36} = 699.57^{***}$	$F_{6, 36} = 35.84^{***}$
	RW	$F_{2, 36} = 115.38^{***}$	$F_{3, 36} = 407.92^{***}$	$F_{6, 36} = 63.10^{***}$
L. sativa	TG	$F_{2, 48} = 2.84$	$F_{3, 48} = 26.21^{***}$	$F_{6, 48} = 14.31$
	SpG	$F_{2, 48} = 365.53^{***}$	$F_{3, 48} = 639.35^{***}$	$F_{6, 48} = 252.04^{***}$
	SpAG	$F_{2, 48} = 315.37^{***}$	$F_{3, 48} = 573.37^{***}$	$F_{6, 48} = 219.31^{***}$
	CRG	$F_{2, 48} = 540.21^{***}$	$F_{3, 48} = 617.92^{***}$	$F_{6, 48} = 408.56^{***}$
	HL	$F_{2, 48} = 211.19^{***}$	$F_{3, 48} = 720.15^{***}$	$F_{6, 48} = 76.97^{***}$
	HW	$F_{2, 48} = 105.6^{***}$	$F_{3, 48} = 342.6^{***}$	$F_{6, 48} = 29.67^{***}$
	RL	$F_{2, 48} = 73.36^{***}$	$F_{3, 48} = 3506^{***}$	$F_{6, 48} = 38.28^{***}$
	RW	$F_{2, 48} = 11.04^{***}$	$F_{3, 48} = 1335^{***}$	$F_{6, 48} = 6.75^{***}$

*** strongly significant, ** significant, * poorly significant, and blank means non-significant. HL = hypocotyl length, RL = radical length, HW = hypocotyl weight, RW = radical weight.

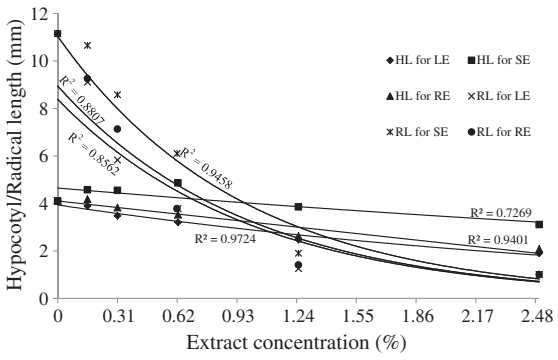

Fig. 3. Correlation between aqueous extract concentrations of leaf (LE)/stem (SE)/root (RE) and hypocotyl (HL)/radical (RL) length of *I. axillaris*.

Independent T-test (2-tailed) showed that the mean differences of control and treatment were significant for all parameters of both model and associated species ($p < 0.001$) while the germination indices of *L. sativa* were not significantly affected.

3.4. Effect on seed imbibition

The water uptake by *L. sativa* seeds was decreased with increasing concentration of aqueous extracts, with the greatest impact caused by leaf extracts. Initial seed imbibition was identical for both extracts and control, however, at 4, 8 and 24 h incubation in leaf extracts of 1.25 to 5% water uptake was reduced by 3.9–10.4%, 3.9–10.3%, 8.0–22.0% (compared to control), 1.5–1.4%, 2.1–1.3%, 2.9–4.1% (compared to stem extracts) and 0.3–1.2%, 1.3–1.7%, 1.4–5.6% (compared to root extracts), respectively (Fig. 7). The greatest differences in water uptake (%) compared to previous measurements were observed at 4 h with 65, 62, 60 and 58% for control, 1.25, 2.5 and 5% of all extracts respectively. The water uptake rates were gradually reduced after 4 h. Finally, after 24 h *L. sativa* seeds gained 95% of the fresh weight for control, while 5% leaf extract gained 74%. The effects of boneseed organs, extract concentrations and time on water uptake of *L. sativa* were strongly

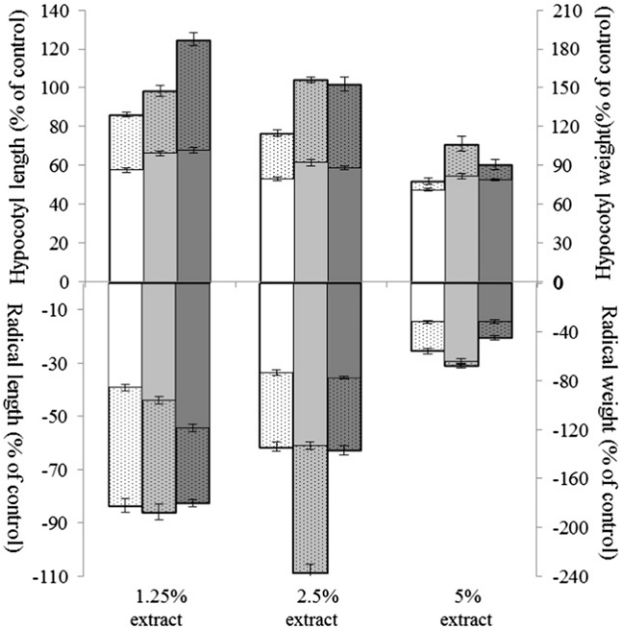

Fig. 4. Effect of different concentrations of boneseed leaf (white), stem (light ash) and root (deep ash) extracts on hypocotyl (upward) and radical (downward) length (plain) and weight (dotted) on *A. mearnsii*. Data presented as the percentage of control.

Table 4
ANNOVA output (F — ratios) displaying the effects of various organs of boneseed (O), extract concentrations (C) and time (T) on (a) seed imbibition and total non-structural carbohydrate content, and (b) H_2O_2, electrolyte leakage and lipid peroxidation of *L. sativa*.

4a

Parameter	O	C	T	O × C	O × T	C × T	O × C × T
SI	$F_{2, 96} = 23.85^{***}$	$F_{3, 96} = 726.16^{***}$	$F_{3, 96} = 84{,}498^{***}$	$F_{6, 96} = 3.62^{**}$	$F_{6, 96} = 7.29^{***}$	$F_{9, 96} = 164.97^{***}$	$F_{18, 96} = 1.46$
TNC	$F_{2, 96} = 35.47^{***}$	$F_{3, 96} = 796.92^{***}$	$F_{3, 96} = 3764.3^{***}$	$F_{6, 96} = 13.75^{***}$	$F_{6, 96} = 6.38^{***}$	$F_{9, 96} = 175.68^{***}$	$F_{18, 96} = 1.8^{*}$

4b

Parameter	O	C	O × C
H_2O_2	$F_{2, 24} = 153.6^{***}$	$F_{3, 24} = 289.62^{***}$	$F_{6, 24} = 17.87^{***}$
EL	$F_{2, 24} = 74.82^{***}$	$F_{3, 24} = 632.25^{***}$	$F_{6, 24} = 13.82^{***}$
LPO	$F_{2, 24} = 90.87^{***}$	$F_{3, 24} = 98.66^{***}$	$F_{6, 24} = 11.3^{***}$

*** strongly significant, ** significant, * poorly significant, and blank means non-significant.
SI = seed imbibition, TNC = total non-structural carbohydrate, EL = electrolyte leakage, LPO = lipid peroxidation.

significant with the exception of non-significant interactive effect of all components (Table 4a).

3.5. Effect on seed reserve mobilization

TNC metabolism in *L. sativa* seeds exposed to dH_2O were 13, 28.3 and 36.8% at hours 4, 8 and 24 respectively compared with initial (0 h) (Fig. 8). The TNC metabolism was decreased incrementally with increasing extract concentrations. The rate of TNC metabolism for 1.25 to 5% leaf extracts was 9.5–2.1%, 21.2–5.4% and 31.0–11.2% respectively at hours 4, 8 and 24. The usage of TNC for stem extracts was 5.1–1.3%, 7–2.3% and 7.6–2.4% more than the leaf extracts respectively, while root extracts showed impacts in between leaf and stem extracts. Highest differences in TNC metabolism (%) occurred during 4 to 8 h for control and 1.25% extracts (all organs), while it occurred during 8 to 24 h for 2.5 and 5% of all organs extracts. ANOVA test showed all individual and interactive significant effects of organs, extract concentrations and time on carbohydrate metabolisms (Table 4a).

3.6. Oxidative stress evaluation

All extracts produced increased H_2O_2 levels in *L. sativa* seedlings compared with control, and consequently, the electrolyte leakage and lipid peroxidation were also increased, concomitantly with increasing extract concentrations (Fig. 9). H_2O_2 concentrations in *L. sativa* seedlings were increased by 294, 171 and 152% when treated with 1.25% leaf extract compared to the control and the same concentrations of stem and root extracts, respectively (Fig. 9). These values were 350, 167 and 158% for 2.5% leaf extract and 403, 157 and 152% for 5% leaf extracts, respectively. 1.25% to 5% leaf extracts stimulated electrolyte leakage by 213–299%, 112–130% and 113–129% when compared to the control, stem extracts and root extracts, respectively. Similar comparisons showed 159–236, 152–149 and 148–147% stimulation for lipid peroxidation, respectively (Fig. 9). The effects on all three parameters by different concentrations of leaf, stem and root extracts were found to be highly significant ($p < 0.001$) both for individual and interactive impacts (Table 4b).

4. Discussion

Our study demonstrated that boneseed aqueous extracts and leachate negatively affected germination indices, biometric, physiological and biochemical parameters of both a model species and two native species. The impact of boneseed aqueous extracts on *I. axillaris*, even at very low concentrations suggests that allelopathy is likely involved in the invasion processes of boneseed. However, the high level of osmotic potential observed in more concentrated extracts might contribute to the phytotoxic impact (Anderson and Loucks, 1966; Robinson

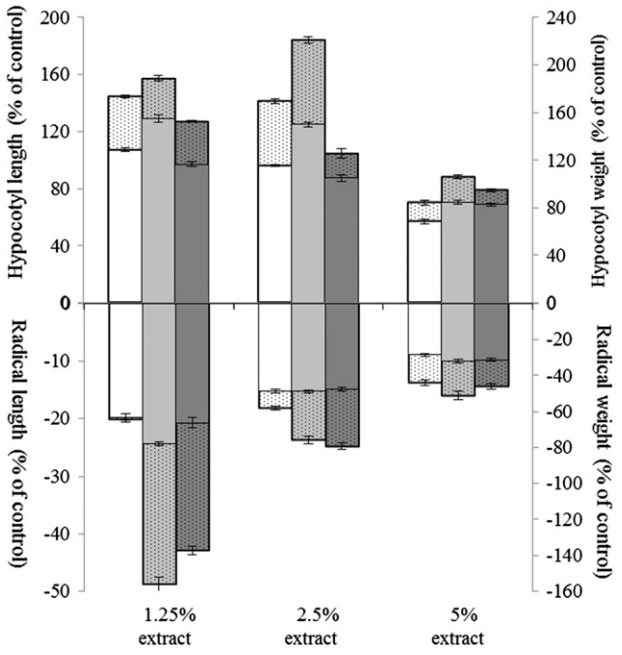

Fig. 5. Effect of different concentrations of boneseed leaf (white), stem (light ash) and root (deep ash) extracts on hypocotyl (upward) and radical (downward) length (plain) and weight (dotted) on *L. sativa*. Data presented as the percentage of control.

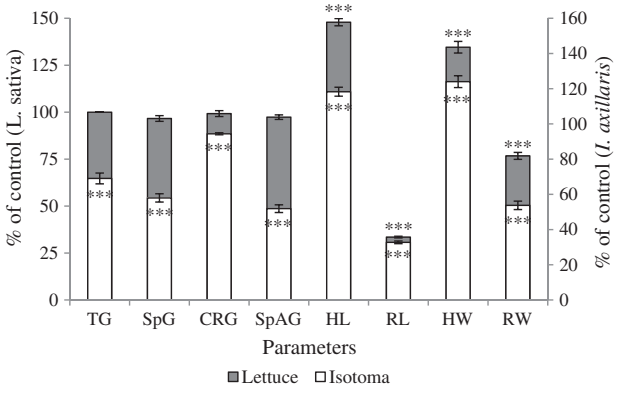

Fig. 6. Germination and biometric parameters of *L. sativa* and *I. axillaris* exposed to boneseed leaf leachate. Data presented as the percentage of control. ***Strongly significant, and blank means non-significant variation compared with means.

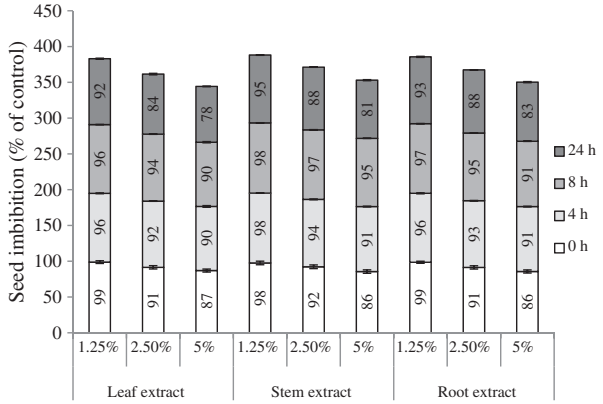

Fig. 7. Dynamics of seed imbibition of *L. sativa* exposed to various concentrations of boneseed leaf, stem and root extracts. Data presented as the percentage of control.

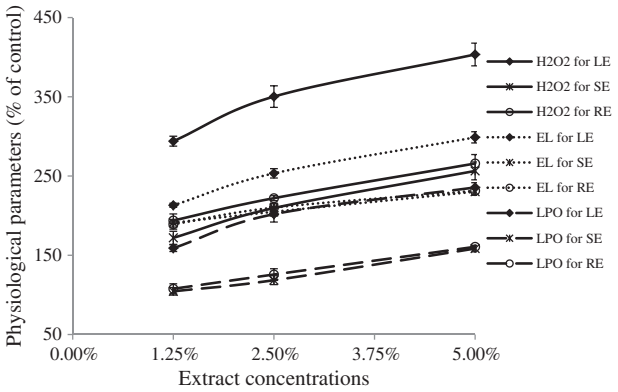

Fig. 9. Impact of boneseed extracts on hydrogen peroxide (H_2O_2), electrolyte leakage (EL) and lipid peroxidation (LPO) of *L. sativa*. Data presented as the percentage of control. LE = leaf extracts, SE = stem extracts, RE = root extracts.

et al., 2006). The evidence of high concentration of total phenolics in boneseed in the order of leaf > root > stem > leachate > infested soil > outside soil when compared to other allelopathic species (Rashid et al., 2010; Uddin et al., 2012) indicates the potential of boneseed allelopathy. With the exception of an earlier study that partially addressed this issue (Xu and Chang, 2007), our investigation into the impact of drying and centrifuging methods in conjunction with the use of different extraction solvents on total phenolic concentration are novel in allelopathy research. Acetone extracts were found to contain more phenolics than ethanol, methanol and water extracts, in agreement with the study of Ens et al. (2009b). This methodological approach of extracting phenolics might contribute in identifying individual phenolics through high performance liquid chromatography (HPLC), essential for ecological, herbicidal and medicinal perspectives of allelopathic research.

Leachate exhibited less effect compared with aqueous extract, and overall, leaf extract impacted more than the other organ's extracts on both model and associated species similar to the findings for other allelopathic species (Dorning and Cipollini, 2006; Uddin et al., 2012). Although the current study didn't specifically identify active components in boneseed tissue, the identification of several allelochemicals in bitou bush and infested soils gives some notion of allelochemicals being active components in boneseed (Ens et al., 2009a). Speed of germination has been generally considered as the key indicator among germination indices in allelopathic studies (Chiapusio et al., 1997). In this study, the SpG, particularly for *L. sativa*, was significantly affected even though other indices had negligible change compared with control. Dorning and Cipollini (2006) found that number of seed germinations decrease

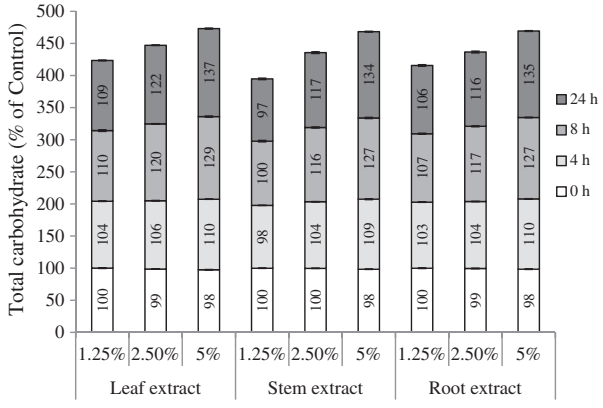

Fig. 8. Reduction of carbohydrate metabolism of *L. sativa* exposed to various concentrations of boneseed leaf, stem and root extracts. Data presented as the percentage of control.

with increasing extract concentrations, similar to the results of our dose response study. We found stronger impact on both radical length and weight compared with hypocotyl for all model and associated species which is similar to the other allelopathic studies (Kobayashi et al., 2008). Aqueous extracts of some doses exhibited stimulatory effects on hypocotyls, which is not completely unexpected as allelochemicals at low concentrations have been found to be stimulatory in other reports (Chon and Kim, 2002; Tefera, 2002). Our findings suggested species-specific and varied seed size impacts of aqueous extracts which are similar to the literature (Al-Khatib et al., 1997; Ens et al., 2009b).

The decreasing water uptake by *L. sativa* seeds with increasing concentration of aqueous extracts might suggest the impact of boneseed allelopathy on seed imbibition, the leading factor in delaying or decreasing germination of test plants. This is in agreement to reports in the literature (Chon et al., 2004) that suggested the inhibition of water uptake by allelopathic species. The delay and decrease in water uptake observed in the *L. sativa* seeds in response to aqueous extracts of boneseed, may significantly impact metabolism and initiation of germination (Bewley, 1997). We found that TNC metabolism at all of the investigated time points was reduced with increasing concentration of extracts, with more impact shown by leaf, in agreement with other studies (Singh et al., 2009). In stressed condition like drought, salinity, nutrient and oxygen scarcity and presence of toxins, seed carbohydrate metabolism is decreased that may adversely affect cellular respiration and germination processes (Fritz and Braun, 2006) threatening the plant species growing favorably in that area (Guglielminetti et al., 1995).

In favorable conditions, ROS production in plant species is optimal that drastically increases during stressed condition and create oxidative stress. Hydrogen peroxide as a potential species of ROS enhances electrolyte leakage and lipid peroxidation, damages macromolecules like protein, nucleic acids, etc., and has been identified as a mechanism of action of allelochemicals (Batish et al., 2006; Weir et al., 2004). In our findings, boneseed aqueous extracts led to increasing H_2O_2 and resulted electrolyte leakage and lipid peroxidation that correlates with the phenolic content of the organs (leaf > root > stem) and germination impacts that clearly suggest the mechanism of allelopathic impact through excessive ROS production. However, investigating the impacts of boneseed volatile matters on test species physiology is imperative as the volatiles contribute significantly in the plants' invasion processes (Halligan, 1976). Experiments with lower concentrations of aqueous extracts to observe the impact on physiology could dissect out the allelopathic impacts from other extraneous effects, particularly osmotic potential that has been identified in our earlier study (Uddin et al., 2013). ROS may also influence the mobilization of seed reserve (Gomes and Garcia, 2013), a novel finding that supports our study of the increase in ROS and TNC with increasing extract concentrations.

The study suggests that allelopathy might be involved in invasion processes of boneseed, as impacted on other plants (Callaway and Aschehoug, 2000; Inderjit and Dakshini, 1994) along with other mechanisms suggested for boneseed propagation like high seed production, post-fire regeneration capacity, absence of natural enemies and competitive capacity (Parsons, 1973; Rudman, 2001; Thorp and Lynch, 2000; Weiss et al., 1998). These combined impacts have led to predictions that boneseed and bitou bush may invade more than 15% of Australia in the near future (Weiss et al., 2008). These findings complement the study conducted by Ens et al. (2009b) who suggested the community composition changing due to the allelopathy of bitou bush. Although our findings indicate allelopathic potential of boneseed, field evidence is imperative to demonstrate allelopathic impact more authentically as edaphic and environmental factors work together in influencing allelopathic effect (Inderjit and Duke, 2003). We started with this work to assess whether allelopathy in boneseed may be one of the important mechanisms in its invasion processes, however, a detailed study on volatile impact, root exudation, litter decomposition, etc. might be imperative along with identification of individual allelochemicals in boneseed to advance the study on allelopathy as one of its invasion mechanisms.

This preliminary study helps to explain the boneseed allelopathy, one of the potential invasion mechanisms and emphasize the importance of mitigating the effects of allelopathy by boneseed to protect native species and crop species.

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