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Do phytotoxic allelochemicals remain in ashes after burning *Chrysanthemoides monilifera* subsp. *monilifera* (boneseed)?

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ABSTRACT

Australia is facing challenges in controlling *Chrysanthemoides monilifera* subsp. *monilifera* (boneseed). However, burning has achieved some success in this regard. We aimed to investigate the comparative phytotoxicity of boneseed dried powder and ashes (burnt at 450°C and 250°C). Phenolic compounds in powder and ashes were measured using Folin–Ciocalteu assay and HPLC. The phytotoxicity of boneseed powder and ash extracts was assessed through germination bioassay on *Lactuca sativa* and the phytotoxicity of litter and ashes was evaluated using field soil, both in growth chamber. Burning of boneseed reduced total phenolics in ashes of boneseed organs by 99% and 100% both at high and low temperatures. The four phenolic compounds that were detected in boneseed were either absent or at negligible levels in the ashes, with inversely related to temperature. Both boneseed ash extracts and litter ash-mediated soil significantly reduced phytotoxicity displaying increased germination, biometric and biochemical parameters of test species compared with unburnt powder extracts and litter powder-mediated soil respectively, with greater reduction of phytotoxicity found for ashes produced at the lower temperature. Interestingly, the ash extracts and litter ash-mediated soil were found to stimulate some of those parameters of the test species compared to control. There was no excessive reactive oxygen species (ROS) produced in test species exposed to ash extracts compared with unburnt powder extracts. These findings suggest that burning of boneseed is an appropriate method of weed control and that this approach will reduce phytotoxicity of this species on native plants.

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Introduction

Australia is facing challenges in controlling boneseed (Brougham et al., 2006; Lane and Shaw, 1978), a Weed of National Significance in Australia and listed on the National Pest Plant Accord in New Zealand. Boneseed infestations have occurred in South Africa, USA and Southern France (Weiss et al., 1998), and is one of the two subspecies (subsp. *monilifera* and *rotundata* (bitou bush)) of *Chrysanthemoides monilifera* found in Australia that were originally

introduced from South Africa during the mid-19th century (Brougham et al., 2006). Boneseed was proclaimed a noxious weed in Victoria in 1969 (Parsons, 1973), and soon after, the Australian Institute of Agricultural Science suggested that boneseed could potentially be “the most important weed on public land in southern Victoria” (Australian Institute of Agricultural Science, 1976). Boneseed and bitou bush collectively threaten about 200 indigenous species in Australia (Department of Environment Conservation, 2006) including significant rare species such as *Pterostylis truncate*. It has been predicted that

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more than 15% of Australia could be invaded by these two subspecies in the near future (Weiss et al., 2008). McAlpine et al. (2009) postulated that boneseed posed a severe threat to native species regeneration. Previous work (Ens et al., 2009) investigated the suppression of native species in Australia by bitou bush (subsp. *rotundata*) through the release of allelochemicals. We recently confirmed that allelopathy is one of the mechanisms underpinning the rapid invasion of boneseed in Australia (Harun et al., 2014, 2015). However, further investigations are needed to determine how this allelopathic nature should inform the management strategies of boneseed.

Several weed control techniques have been implemented to control and manage boneseed including hand weeding (Brougham et al., 2006), slashing (Thomas et al., 2000), biological control (Adair and Edwards, 1996), and herbicides (Brougham et al., 2006). However, none of these strategies address the issue of the seedbank in the soil, despite each boneseed plant producing around 50,000 seeds (viable 800–3000 m⁻²) in Australia (Weiss et al., 2008), with 15% of them found to be viable after 3 years and some even viable after 10 years (Parsons and Cuthbertson, 2001; Weiss, 1986). Although these individual approaches in boneseed control have achieved some success at the landscape scale, none of them have had significant impact on controlling boneseed at the field level. Recently, Melland and Preston (2008) reported that controlled burning (200–300°C) killed and removed all standing boneseed plants and killed all the viable seeds in soil. In the light of the promise shown by the use of burning as an effective approach to the control of boneseed, it is important to assess how burning impacts the allelochemicals and consequent phytotoxicity produced by this species.

Efforts to reduce the allelopathic impact of species have included applying activated carbon (AC) in soil (Jandová et al., 2014), cutting (Uddin et al., 2014) and crop rotation (Mamolos and Kalburtji, 2001). In contrast, the impact of burning on the allelopathic properties and the phytotoxicity of ashes has rarely been studied (Inderjit et al., 2004), despite burning being widely used to control weeds, including boneseed (Cudney et al., 1992; Melland and Preston, 2008). Among the allelochemicals identified in invasive species, phenolic compounds represent a class of great importance and are ubiquitous in all plant organs that have been studied in chemical, biological, agricultural, and medical research over recent decades (Gallardo-Williams et al., 2002; Li et al., 2010; Liu et al., 2013). Despite the understanding that phytotoxicity depends on the types and quantity of allelochemicals released by the donor plants (Li et al., 2010), there is a lack of information about the presence of specific allelochemicals in ashes of allelopathic species after burning. A comparison of the phytotoxicity of equivalent masses of ash and unburnt plant extracts could provide useful information in this regard.

The physiological responses of plants to allelochemicals are particularly complex since resource competition, allelopathy, nutrient immobilization and microbial influence operate in parallel (Bhowmik and Inderjit, 2003; Inderjit and del Moral, 1997). Despite these challenges there is clear evidence that allelochemicals can affect plant physiology (electrolyte leakage, lipid peroxidation, etc.) through excessive ROS production, a mode of allelopathic action suggested by Weir et al. (2004) that has rarely been investigated in allelopathic studies. Particularly, the question: does ash phytotoxicity (if it exists) inhibit growth of neighbouring species through excessive ROS production? —

has not been explicitly studied, although ROS production has been identified in plants affected by allelochemicals (Batish et al., 2006) and aqueous extracts of boneseed (Harun et al., 2014).

Further, the incorporation of soil in allelopathic studies and particularly, field studies is imperative to demonstrate the allelopathic impact more authentically, as edaphic and environmental factors work together in influencing the allelopathic effect (Inderjit and Duke, 2003). The findings by Inderjit et al. (2008) addressing the breakdown of soil allelochemicals by microorganisms and consequent reduction of their phytotoxicity reinforce the importance of allelopathic phytotoxicity studies in soil systems. To our knowledge, ash phytotoxicity in a soil system has never been studied before despite such an approach better representing the real field conditions, including microbial interactions.

This study hypothesised that “burning does not leave phytotoxic allelochemicals in boneseed ashes”. To test this hypothesis, we aimed to identify and quantify phenolic compounds in boneseed powder and ashes, compare the phytotoxicity of boneseed powder and ashes in terms of germination and physiological impact on a test species. Furthermore, our study aimed to assess the impact of litter powder and ashes on early growth of target species in a soil system.

1. Materials and methods

1.1. Sample collection and processing, and seed collection

The samples for identification of phenolics including fresh boneseed organs and litter were collected in June 2014, while samples (boneseed plants, and boneseed non-infested soil) for other experimental purposes were collected during July–September 2013, both from the You Yangs Regional Park, Victoria (37°59'44" S, 144°24'39" E), as it is the home, since 1940, of one of the Australia's densest boneseed populations (Roberts, 2008). The samples were sealed into plastic bags and immediately transported to the laboratory. The root samples were cleaned with water to remove soil. All organs were chopped into 1–2 cm pieces, separately. The samples (boneseed organs, soil and litter) were dried in air to constant weights. Dried boneseed organs were ground in a grinder, passed through a 0.5 mm mesh sieve and stored in sealed plastic vials until chemical analyses and growth experiments were conducted. We used air dried samples at ambient temperatures to avoid temperature-dependant alteration of the phenolics as previously described (Harun et al., 2014; Janas et al., 2000; Ju and Howard, 2003). Extraneous materials were removed from the dried litter which was then chopped into <0.5 cm pieces and preserved in sealed plastic bags until used. Air dried soil was passed through a 1 mm mesh sieve and stored in sealed plastic vials until all experiments were executed. Seeds of *Lactuca sativa* (model species) were purchased from Bunnings Warehouse, Australia.

1.2. Burning boneseed organs and litter

Two distinct temperatures, 450°C and 250°C were used to burn boneseed organs and litter. The rationale for selecting these

temperatures and times was based on literature that suggested that 450°C was required to turn plant material into complete ash (Peacock, 1992) and 250°C was the temperature that boneseed was exposed to when it burned as part of its management (Melland et al., 1999). Boneseed organs (leaf, stem and root) and litters (500 g, air dried) were put into pestles separately and burned inside a muffle furnace (Model F635, Scientific Equipment Manufacturers Pty Ltd., Magill, Adelaide, Australia) for 8 hr at 450°C and 2 hr at 250°C. The furnace was allowed to cool for 8 hr before collection of ash, which was subsequently stored in plastic vials prior to analysis.

1.3. Phenolic compounds in boneseed powder and ash

Total water soluble phenolics (WSP) concentration in unburnt powder and burnt ashes of boneseed organs and litter were measured using the Folin–Ciocalteu assay (Singleton and Rossi, 1965) with slight modifications using Gallic acid as the standard (Bärlocher and Graça, 2005). Phenolic compounds were identified using high performance liquid chromatography (HPLC). Briefly, 300 mg of powder or ash from boneseed leaf or root was mixed with 8 mL 50% acidified methanol (prepared with 1.2 mol/L HCl) to homogenize on a roller mixer at 4°C for 24 hr. The extract was centrifuged at 4°C and 17,404 g for 12 min. The supernatant was double filtered through 0.2 µm phenomenex regenerated cellulose (RC) membrane to make it ready for HPLC analysis. Known phytotoxic phenolic compounds, gallic acid, catechin, epicatechin, p-coumaric acid, ferulic acid, phloridzin, arbutin, hydroxymethylfuraldehyde (HMF), gentisic acid, chlorogenic acid and rutin, that fell within the capacity of the HPLC column (Uddin et al., 2013) were included for potential identification in boneseed. Initial trials revealed the presence of catechin, p-coumaric acid, ferulic acid and phloridzin and subsequent analysis was limited to those four compounds. Standard phenolic compounds were purchased from Sigma-Aldrich, Australia to prepare standard stock solutions of 12.5, 25, 50 and 100 µg/mL. A Kinetex PEP 5 µm 100 Å LC Column 250 × 4.6 mm equipped in HPLC system (Shimadzu, Tokyo, Japan) was used in the identification processes. The mobile phases A (0.1% phosphoric acid in water) and B (0.1% phosphoric acid in acetonitrile) were fixed at a flow rate of 1 mL/min and 65 min run-time. The gradient elution profile was: 0–30 min, 90:10 (A:B); 30.01–40 min with a linear decrease of A and increase of B to 50:50 (A:B); 40.01–45 min, 10:90 (A:B), 45–55 min with a linear increase of A and decrease of B to 50:50 (A:B), and then back to initial condition. The chemical analysis was conducted in triplicate at ambient temperature with injection volumes of 10 µL. The analysis was monitored with a photodiode array detector at 220 and 271 nm. The presence of the phenolic compounds was confirmed with retention time in samples compared with standard. Based on the standard curve, the quantity (µg/mg powder or ash) of each phenolic compound was calculated.

1.4. Bioassay with powder and ash aqueous extract

To prepare 4% (W/V) aqueous extracts, 4 g of dry powder of boneseed organs was mixed in 100 mL dH₂O and agitated for 24 hr on an orbital shaker (Orbital Mixer EOM5, Ratek Instruments Pty. Ltd., Boroona, Victoria, Australia) at room temperature.

The extract was centrifuged at 941 g (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 2 and 0.5% (W/V) were prepared for bioassay experiments. Similarly, the ash extracts of boneseed leaf (0.5%, 0.25% and 0.06% for ashes burnt at 450°C, and 0.6%, 0.3% and 0.075% for ashes burnt at 250°C), stem (0.24%, 0.12% and 0.03% for ashes burnt at 450°C, and 0.5%, 0.25% and 0.06% for ashes burnt at 250°C) and root (0.2%, 0.1% and 0.025% for ashes burnt at 450°C, and 0.5%, 0.25% and 0.06% for ashes burnt at 250°C) ashes were prepared that were equivalent to the boneseed powder extract concentrations (4%, 2% and 0.5%). To control for possible extraneous effects, pH (Pocket digital pH metre, 99559, China, made for Dick-Smith electronics, Australia) and electrical conductivity (EC) (TPS Digital conductivity metre, 2100, TPS Pty Ltd., Brendale, Queensland, Australia) of all extracts were measured, and pH was adjusted to 6.5 with 1 mol/L NaOH or HCl solution, as pH may influence the phytotoxicity (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 dH₂O (Jefferson and Pennacchio, 2003). Twenty five seeds of *L. sativa* were placed in 90 mm Petri dish lined with two Advantec filter paper moistened with 5 mL of different concentrations of aqueous extracts of each organ (powder and ash). Distilled water was used as a control (0%). Five replicates (for a total of 140 petri dishes) were sealed with parafilm and incubated in a growth chamber (Model RI250SG, Thermoline Scientific, Wetherill Park, New South Wales, Australia) at 25°C in darkness. The number of germinated seedlings (radicle protrudes by ≥1 mm) in all petri dishes was counted daily until cumulative germination levelled off. 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. After 8 days seedlings were harvested to measure biometric parameters including hypocotyl length (HL), radicle length (RL), hypocotyl weight (HW) and radicle weight (RW) (Chiapusio et al., 1997; Jefferson and Pennacchio, 2003).

1.5. Oxidative stress evaluation

To investigate whether the impact of boneseed ashes on test species involved excessive ROS production, we measured H₂O₂ of germinated (7 days) *L. sativa* seedlings (exposed to dried powder and ash extracts) following the method of Velikova et al. (2000). Briefly, 100 mg plantlets were homogenized with 5 mL 0.1% trichloroacetic acid (TCA) and centrifuged at 17,404 g for 15 min (Beckman Avanti 30 High Speed Compact Centrifuge 364,105, Beckman Coulter Inc., Atlanta, USA). From the centrifuged material, 0.5 mL supernatant was transferred to a test tube to which 0.5 mL 10 mmol/L phosphate buffer (pH 7.0) and 1 mL 1 mol/L potassium iodide were added and mixed. The absorbance was measured at 390 nm in a spectrophotometer (Libra S12, manufactured by Biochrom Ltd., Cambridge, England) and H₂O₂ concentrations were determined based on an extinction coefficient of 0.28/µM⁻¹·cm⁻¹.

Lipid peroxidation that increases with excessive ROS production was measured in terms of malondialdehyde content

as it is used as an index of lipid peroxidation, and expressed as nmol/g fresh weight (Jambunathan, 2010). Plantlets of 200 mg were homogenized with 4 mL of 0.1% TCA, and centrifuged at 27,193 g for 15 min. Supernatant (1 mL) 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 $155 \text{ m} \cdot (\text{mol/L})^{-1} \cdot \text{cm}^{-1}$.

Electrolyte leakage that increases in seedlings with increasing H_2O_2 was measured following the method of Bogatek et al. (2006). Briefly, seedlings with equal weights (100 mg) for both control and treatments were placed in 15 mL dH_2O at room temperature in darkness. Electrical conductivity was measured after 2 hr incubation, followed by a second EC measurement after 20 min boiling in a water bath. Results were expressed as a percentage of total leakage.

1.6. Allelopathy of litter and ash-mediated soil

Growth media consisted of boneseed litter (2 g) mixed with 100 g boneseed non-infested field soil, or ash of equivalent weight (0.20 g ash burnt at 450°C or 0.27 g ash burnt at 250°C) while controls contained non-infested field soil with no boneseed materials, both in plastic pots. Into each pot, 5 pre-germinated (radicle ≤ 1 mm) seedlings of *L. sativa* were transplanted. Triplicates with a total of 12 pots were designed in CRD to incubate at 15/25°C (night/day) at 12 hr photoperiod. The experimental plants were harvested after 4 weeks to measure shoot length (SL), root length (RL), shoot weight (SW), root weight (RW), leaf number (LN), leaf relative water content (RWC) and chlorophyll content of leaf. To measure leaf RWC, 1 fresh leaf was picked from the same node of all treatment plants (1 from each pot) and weighed. The leaf was incubated in 10 mL dH_2O in a plastic tube at 4°C for 24 hr. Then the leaf was weighed after blotting. Finally, the dry weight of the leaf was taken after drying in an oven at 60°C for 24 hr. The leaf RWC was calculated following the method of Saura-Mas and Lloret (2007). To measure chlorophyll, small discs of leaf were taken by a hole-punch and placed in 7 mL of *N,N*-dimethylformamide for 24 hr in the dark at 4°C and absorbance at 647 and 664 nm was measured. Chlorophyll *a*, chlorophyll *b* and total chlorophyll content were calculated following the method of Inskeep and Bloom (1985).

1.7. Data analysis

Statistical analysis was conducted using IBM SPSS 21.0. All data were presented as mean \pm standard error (SE). Prior to statistical tests data were transformed as necessary. The impact of boneseed powder and ash aqueous extracts on germination and physiology of *L. sativa* was evaluated using one-way ANOVA followed by *post hoc* Dunnett's test and independent *T*-test (2-tailed) depending on the data structure. The impact of boneseed litter and ash-mediated soil on growth of *L. sativa* was analysed in similar manner. Significant differences between the means were determined at a 5% level of probability ($p \leq 0.05$). Linear regression was adopted to express the relationship among different parameters.

2. Results

2.1. Phenolic compounds in boneseed

Burning of boneseed reduced WSP compounds in ashes of boneseed organs (leaf, stem and root) by 99%–100% both at high and low temperatures (Fig. 1). The WSP content in ashes did not vary significantly with burning temperature. After burning at 450°C, none of the four phenolic compounds (catechin, *p*-coumaric acid, ferulic acid and phloridzin) that had been detected in boneseed organs (leaf and root) were detected in the ashes except phloridzin, where trace amounts were detected in root ash (1% was measured in dried root powder (Fig. 2)). In contrast, at 250°C the leaf ash contained 5% phloridzin compared with leaf powder and root ash contained 12% and 2% of ferulic acid and phloridzin, respectively, compared with root powder (Fig. 2). The HPLC output shows the peaks of identified phenolic compounds in powder materials and ashes (Fig. 3).

2.2. Impact of ash extracts on germination

Leaf ash (burnt at 450°C) extracts of 0.06%–0.5% increased a number of germination and growth parameters including TG (0–8%), SpG (3%–176%), SpAG (3%–128%), CRG (1%–21%), HL (101% in 0.5% extract only), RL (246%–180%), HW (92% in 0.5% extract only) and RW (47%–136%) compared with the impact of equivalent unburnt leaf powder extracts (Table 1). Similarly, the stem ash (burnt at 450°C) extracts of 0.03%–0.24%, increased TG (0–2%), SpG (2%–7%), SpAG (2%–7%), CRG (1%), HL (56% in 0.24% extract only), RL (122%–349%), HW (42% in 0.24% extract only), and RW (17%–145%). The root ash (burnt at 450°C) extracts of 0.025%–0.2% increased the TG (0–5%), SpG (2%–16%), SpAG (2%–15%), CRG (1%–3%), HL (63% in 0.2% extract only), RL (149%–465%), HW (55% in 0.2% extract only), and RW (24%–164%) compared with the impact of equivalent dried root powder extracts. The effects of all ash extracts on germination and growth parameters of *L. sativa* were significant compared with equivalent dried powder extracts with the exception of impacts on TG (by two lower concentrations of all ash extracts), CRG (by 0.12% stem and 0.025% root ash extracts) and HL (by 0.25% leaf extract) which were not significant. Even, when compared with control, ash extracts (both in high and low temperatures) significantly stimulated hypocotyl length and weight for all tested concentrations, though the highly concentrated ash extracts showed significant inhibition to speed of germination and radicle growth.

The extracts of all ashes burnt at 250°C had similar pattern of effects on germination and growth parameters of *L. sativa* (Table 1) as the ashes of 450°C. The ash (burnt at 250°C) extracts significantly increased the abovementioned parameters compared with the equivalent powder extracts with the exception on TG (by 0.075% leaf, all stem, and 0.06% and 0.25% root ash extracts), SpG (by 0.25% stem ash extract), SpAG (by 0.25% and 0.5% stem ash extracts), CRG (by 0.25% and 0.5% stem, and 0.06% root ash extracts), and HL and HW (by 0.3% leaf extracts) that were not significant.

The ash extracts of boneseed organs burnt at 250°C had no significant impact on germination and growth parameters when compared with the impact of equivalent ash extract burnt

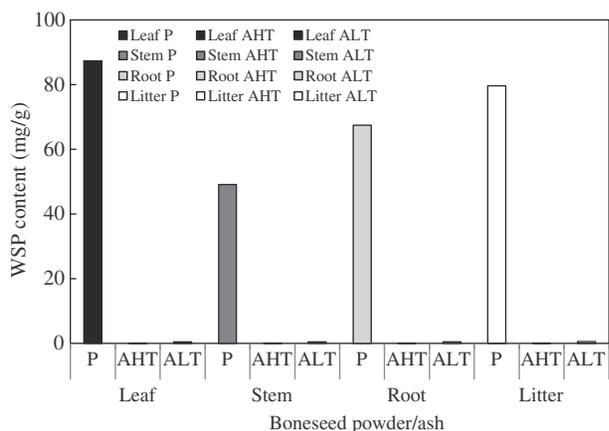


Fig. 1 – Concentration of total water soluble phenolic compounds (WSP) in boneseed powder and ashes. The X-axis denotes the types of boneseed materials e.g., leaf, stem, root and litter powder (P), and leaf, stem, root and litter ash burnt at 450°C Ash burnt at high temperature (AHT) and 250°C Ash burnt at low temperature (ALT).

at 450°C (Table 1) with the exception of impact on SpG, SpAG and CRG (decreased by 0.5% stem ash extract), HL (increased by 0.075% and 0.6% of leaf and 0.06% and 0.5% of stem ash extracts), RL (decreased by 0.6% leaf and 0.5% stem ash extracts), HW (increased by 0.3% and 0.6% leaf, 0.5% stem, and 0.25% root ash extracts), and RW (decreased by 0.5% stem ash extracts) that were significant.

2.3. Oxidative stress evaluation

Lactuca sativa exposed to leaf (0.06%–0.5%), stem (0.03%–0.24%) and root (0.025%–0.2%) ash (burnt at 450°C) extracts had 4%–71%, 10%–58% and 2%–68% less H₂O₂ compared with the equivalent

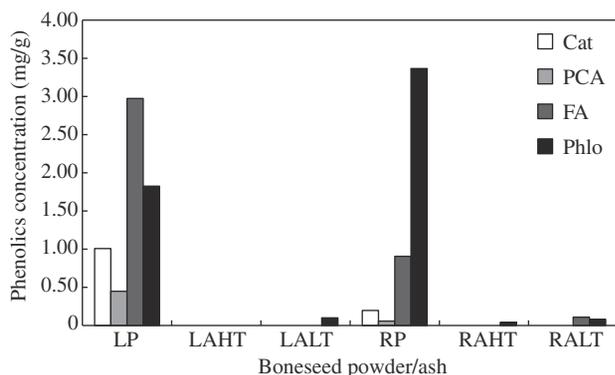


Fig. 2 – Concentration of specific phenolic compounds identified in boneseed powder and ashes. The X-axis denotes the types of boneseed materials e.g., leaf and root powder (LP, RP), leaf and root ash burnt at 450°C (Leaf ash burnt at high temperature (LAHT), Root ash burnt at high temperature (RAHT)), and leaf and root ash burnt at 250°C (Leaf ash burnt at low temperature (LALT), Root ash burnt at low temperature (RALT)). Values on Y-axis denote the concentration of phenolic compounds e.g., catechin (Cat) (no filled), p-coumaric acid (PCA) (light ash), ferulic acid (FA) (deep ash) and phloridzin (Phlo) (Blackshaw and Harker, 2002) in boneseed organs in mg/g.

dried powder extracts (Fig. 4). Although the test plant exposed to lower concentration of ash extracts of boneseed organ had slightly more LPO, the higher concentration of leaf (0.5%), stem (0.24%) and root (0.2%) ash extracts reduced it by 60%, 58% and 65% compared with equivalent dried powder extracts. Similar comparison showed that the electrolyte leakage (EL) of *L. sativa* exposed in leaf, stem and root ash extracts were reduced by 15%–59%, 6%–44% and 11%–55%, respectively. At high temperature the impact of all ash extracts compared with dried powder extracts of boneseed organs were significant with the exception of the impact of lower concentration of leaf and root (on H₂O₂, LPO) and stem (on H₂O₂, LPO and EL) ash extracts that were not significant (Appendix A Table S1). Boneseed organs ash (burnt at 450°C) extracts had no significant impact on the level of H₂O₂, LPO and EL in *L. sativa* seedlings compared with control.

L. sativa exposed to leaf (0.075%–0.6%), stem (0.06%–0.5%) and root (0.06%–0.5%) ash (burnt at 250°C) extracts had 2%–71%, 4%–58% and 11%–68% less H₂O₂ compared with the equivalent dried powder extracts, respectively (Fig. 4). Similar to that observed with ash produced at the higher temperature, the LPO was slightly increased in *L. sativa* exposed to lower concentration of ash extracts but it was decreased by 66%, 58% and 62% when *L. sativa* exposed to the higher concentration of leaf (0.5%), stem (0.24%) and root (0.2%) ash extracts compared with equivalent dried powder extracts. Similar comparison showed that EL of *L. sativa* exposed in leaf, stem and root ash extracts were reduced by 19%–61%, 7%–46% and 7%–54%. The impact of all ash extracts produced at the lower temperature when compared with dried powder extracts of boneseed organs were significant with the exception of the impact of lower concentration leaf (on H₂O₂, LPO), stem (on H₂O₂, LPO and EL) and root (on LPO) ash extracts that were not significant (Appendix A Table S2). Alike to the impact of high temperature, the ash (burnt at low temperature) extracts had no significant impact on the level of H₂O₂, LPO and EL in *L. sativa* seedlings compared with control.

There was no significant variation of physiological parameters in *L. sativa* seedlings exposed to boneseed ash extracts burnt at 250°C compared with equivalent ash extracts burnt at 450°C with the exception of LPO (decreased) in 0.3% and 0.6% leaf ash extracts, and H₂O₂ (decreased) in 0.06% root ash extracts and EL (increased) in 0.5% root ash extract that varied significantly.

2.4. Comparative phytotoxicity of litter and ash-mediated soil

The litter ash (burnt at 450°C) increased SL (27%), RL (84%), SW (92%), RW (49%), LN (27%), leaf RWC (10%), chlorophyll a (58%), chlorophyll b (64%) and total chlorophyll (95%) in *L. sativa* seedlings when compared with the impact of equivalent unburnt litter powder (Fig. 5). While compared with control, the ash increased SL (4%), RL (3%), SW (5%), RW (21%), chlorophyll a (22%) and total chlorophyll (6%), whereas LN (10%), leaf RWC (1%) and chlorophyll b (4%) were decreased. The stimulatory impact of ash (burnt at 450°C), compared with control was significant for RW and chlorophyll a, while all parameters were significantly increased compared with the impact of unburnt litter powder (Appendix A Table S3).

The ash burnt at 250°C increased SL (34%), RL (108%), SW (98%), RW (64%), LN (40%), leaf RWC (13%), chlorophyll a (67%), chlorophyll b (86%) and total chlorophyll (109%) when compared with the impact of equivalent litter powder, however, the

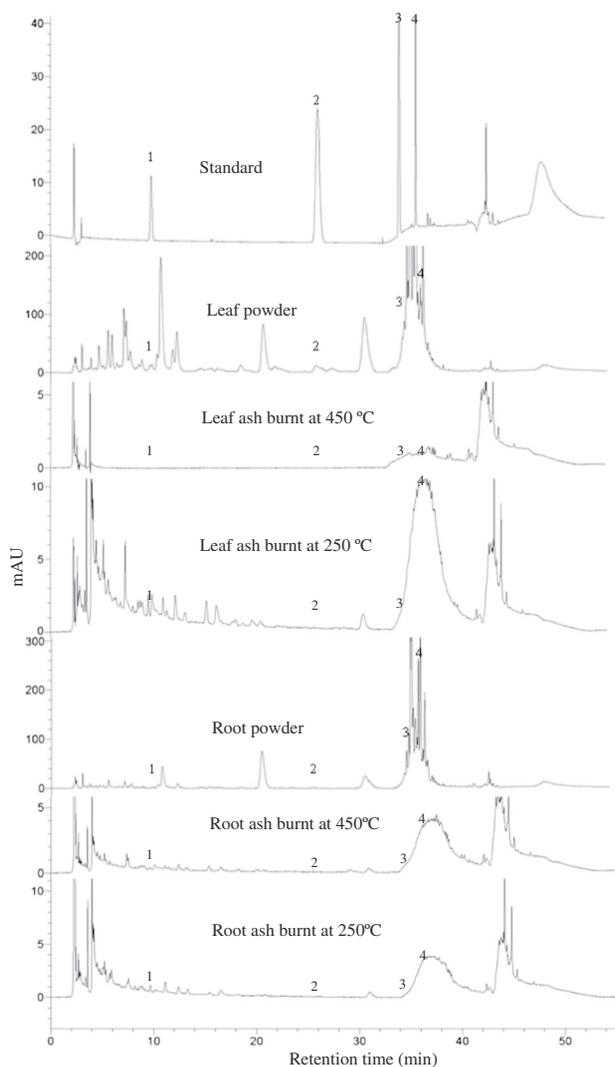


Fig. 3 – HPLC output. Chromatograms of standard phenolic compounds mixer (1 = catechin, 2 = p-coumaric acid, 3 = ferulic acid, 4 = phloridzin), boneseed tissue (leaf and root) and boneseed ash (leaf and root) extracts burnt at 450°C and 250°C.

parameters were increased by 10%, 14%, 9%, 34%, 0, 2%, 29%, 9% and 13% compared with control (Fig. 5). The stimulatory impact of ash (burnt at 250°C), compared with control was significant for RL and RW, and chlorophyll a and total chlorophyll, while all parameters significantly increased compared with the impact of litter powder (Appendix A Table S4).

None of the biometric or biochemical parameters of *L. sativa* exposed to boneseed ash burnt at 250°C were affected significantly compared with equivalent ash burnt at 450°C with the exception of chlorophyll b that was increased significantly.

3. Discussion

Our own unpublished observations and those of other studies (Simões et al., 2008; Zhang et al., 2007) have revealed that the

level of phenolic content observed in plant organs are known to have a phytotoxic effect. The use of burning as a mechanism of controlling the spread of this species in Australia raises the question of the impact that this process has on the allelopathic content and phytotoxicity of the ash produced by this strategy. Our findings revealed that the total water soluble phenolic content measured in boneseed leaf and root was reduced substantially, even completely in some instances, after burning, without any significant variation between ash produced at 450°C and that at 250°C. In contrast, Inderjit et al. (2004) observed about 50% reduction of water soluble phenolics in ash (undefined temperature) of (*Oryza sativa* L.) compared with original powder materials. This variation between our study and the observations of Inderjit et al. (2004) may be due to variation of species, burning temperature and/or time. Although there have been reports of specific allelochemicals in the powdered organs of a number of plant species (Gallardo-Williams et al., 2002; Ossipov et al., 1996; Yuan et al., 2012), to our knowledge this is the first report that identifies the impact that burning has on specific allelochemicals in the ash produced from any plant species. The ferulic acid and phloridzin that was measured in high concentration in boneseed organs were still present, albeit at very low quantities, in ashes burnt at the lower temperature, while ashes produced at the higher temperature contained no ferulic acid and only 1% phloridzin in root ash compared with unburnt root. All other phenolic compounds were not detected in the ash after burning. However, future study identifying other groups of allelochemicals (flavonoids, terpenoids, etc.) in both unburnt and burnt boneseed organs is imperative to draw firm conclusions. The individual and combined impact of the individual phenolics at the concentrations observed in ashes in the present study would be of interest and are currently being investigated in our lab (unpublished).

All of the ash extracts had significantly reduced phytotoxicity when compared with unburnt boneseed powder extracts and actually had stimulatory effects on some biometric parameters of test species compared with control. Similar to our findings, Inderjit et al. (2004) addressed reduced phytotoxicity by ash extracts compared with original powder extracts. The stimulatory effects in conjunction with low concentration of allelopathic phenolics suggest that burning could actually produce ash that provide a source of nutrients and thus stimulate the growth of associated plants, as addressed in other studies (Schmithals and Kühn, 2014). Despite, the negligible concentration of phenolics in the ashes, the more concentrated ash extracts showed inhibition to speed of germination and radicle of *L. sativa*, possibly due to high osmotic potential. The strong correlation ($r = 0.87$) between osmotic potential of boneseed ash extracts and test species radicle length (Appendix A Fig. S1) may indicate the inhibition by higher doses of ash extracts is due to the high osmotic potential. The inhibition of plant growth by treatments with similar osmotic potential is also suggested by other studies (Anderson and Loucks, 1966; Uddin et al., 2013). The ashes may also contain other unidentified phytotoxins that may contribute to the inhibitory effects at the higher concentrations. Despite the ashes burnt at 250°C containing more phenolic compounds than the ashes burnt at 450°C, the ashes burnt at low temperature showed less phytotoxicity than that of high temperature which, again, may due to the reduced OP in the ashes burnt at lower temperature. However, future research to assess

Table 1 – Impact of phenolic compounds (dose–response) on germination indices and biometric parameters of *I. axillaris*. Data presented as average ± Standard error (SE).

Treatment	Parameters							
	TG	SpG	SpAG	CRG	HL (mm)	RL (mm)	HW (mg)	RW (mg)
Control	100 ± 0.0	24.8 ± 0.12	64.4 ± 0.24	25 ± 0.03	29.14 ± 0.52	34 ± 0.52	12.4 ± 0.22	1.99 ± 0.06
<i>Leaf extract</i>								
0.5%	100 ± 0.0	24.1 ± 0.14	62.9 ± 0.29	24.8 ± 0.03	39.5 ± 0.32	10.1 ± 0.31	16.1 ± 0.23	1.39 ± 0.03
2%	97.6 ± 1	22.5 ± 0.35	59.1 ± 0.84	24.5 ± 0.08	41.8 ± 0.52	6.6 ± 0.23	14.3 ± 0.20	1.09 ± 0.04
4%	92 ± 1.26	8.6 ± 0.18	27.2 ± 0.59	20.5 ± 0.09	22 ± 0.51	4.7 ± 0.23	9.7 ± 0.23	0.68 ± 0.01
<i>Stem extract</i>								
0.5%	100 ± 0.0	24.4 ± 0.16	63.5 ± 0.34	24.9 ± 0.04	42.4 ± 0.47	15.1 ± 0.36	19.3 ± 0.30	1.70 ± 0.03
2%	99.2 ± 0.8	24.3 ± 0.15	63.2 ± 0.38	24.9 ± 0.05	50.9 ± 0.77	9 ± 0.27	19 ± 0.22	1.20 ± 0.04
4%	96.8 ± 0.8	22.9 ± 0.25	60 ± 0.57	24.7 ± 0.09	26.3 ± 0.26	6 ± 0.25	11.9 ± 0.29	0.74 ± 0.02
<i>Root extract</i>								
0.5%	99.2 ± 0.8	24.3 ± 0.20	63.3 ± 0.48	24.9 ± 0.05	41 ± 0.66	15 ± 0.54	17.3 ± 0.44	1.70 ± 0.04
2%	98.4 ± 1	23.7 ± 0.32	61.6 ± 0.84	24.8 ± 0.07	32.3 ± 0.78	8.2 ± 0.25	13.8 ± 0.43	1.17 ± 0.03
4%	94.4 ± 1.6	21.2 ± 0.21	55.9 ± 0.59	24.4 ± 0.07	24.4 ± 0.44	5.1 ± 0.15	10.7 ± 0.24	0.73 ± 0.01
<i>Leaf ash (450°C)</i>								
0.06%	100 ± 0.0	24.9 ± 0.10**	64.6 ± 0.20**	25 ± 0.02**	34.1 ± 1.14***	34.9 ± 1.31***	12.6 ± 0.59***	2.04 ± 0.06***
0.25%	99.2 ± 0.8	24.5 ± 0.16***	63.7 ± 0.42***	24.9 ± 0.03***	40.4 ± 1.42	24.4 ± 0.77**	16.8 ± 0.72**	1.77 ± 0.07***
0.5%	99.2 ± 0.8***	23.7 ± 0.21***	61.9 ± 0.47***	24.7 ± 0.06***	44.4 ± 0.63***	13.1 ± 0.52***	18.6 ± 0.35***	1.60 ± 0.03***
<i>Stem ash (450°C)</i>								
0.03%	100 ± 0.0	25.0 ± 0.0**	64.8 ± 0.0**	25 ± 0.0**	29.7 ± 1.21***	33.6 ± 1.45***	12.5 ± 0.43***	1.99 ± 0.05**
0.12%	100 ± 0.0	24.8 ± 0.11*	64.3 ± 0.22*	24.9 ± 0.02	33.3 ± 1.5***	33.9 ± 1.03***	13.6 ± 0.41***	1.99 ± 0.04***
0.24%	99.2 ± 0.8*	24.6 ± 0.19***	63.9 ± 0.47***	24.9 ± 0.03*	41 ± 1.31***	27 ± 1.52***	16.9 ± 0.58***	1.82 ± 0.08***
<i>Root ash (450°C)</i>								
0.025%	99.2 ± 0.8	24.8 ± 0.20	64.3 ± 0.52	25 ± 0.03	28.8 ± 1.55***	37.3 ± 1.76***	12.3 ± 0.29***	2.11 ± 0.04***
0.1%	100 ± 0.0	24.9 ± 0.10**	64.6 ± 0.20**	25 ± 0.02**	36.5 ± 1.75*	34.4 ± 1.32***	14.4 ± 0.50	2.02 ± 0.06***
0.2%	99.2 ± 0.8*	24.7 ± 0.20***	64.1 ± 0.51***	25 ± 0.02***	39.8 ± 1.49***	28.7 ± 0.76***	16.5 ± 0.51***	1.85 ± 0.05***
<i>Leaf ash (250°C)</i>								
0.075%	100 ± 0.0	25 ± 0.0***	64.8 ± 0.0***	25 ± 0.0***	29.1 ± 0.52***,b	35.6 ± 2.02***	12.4 ± 0.22***	2.10 ± 0.06***
0.3%	100 ± 0.0*	24.8 ± 0.12***	64.4 ± 0.24***	25 ± 0.03***	39.8 ± 1.61	34.2 ± 1.29***	15.8 ± 0.72 ^a	1.99 ± 0.06***
0.6%	98.4 ± 1**	24 ± 0.16***	62.5 ± 0.42***	24.9 ± 0.05***	51.8 ± 0.88***,a	18.5 ± 0.56***,a	20.6 ± 0.68***,c	1.60 ± 0.03***
<i>Stem ash (250°C)</i>								
0.06%	100 ± 0.0	25 ± 0.0**	64.8 ± 0.0**	25 ± 0.0**	34.3 ± 1.24***,c	33.9 ± 1.35***	13.6 ± 0.42***	2.00 ± 0.07**
0.25%	99.2 ± 0.8	24.7 ± 0.20	64.1 ± 0.51	25 ± 0.02	33.7 ± 1.6***	33.2 ± 1.62***	13.2 ± 0.39***	1.96 ± 0.06***
0.5%	96.8 ± 1.5	22.6 ± 0.35 ^b	59.2 ± 0.9 ^b	24.6 ± 0.03 ^a	47.7 ± 0.71***,b	17.1 ± 0.39***,a	19.1 ± 0.31***,c	1.45 ± 0.02***,b
<i>Root ash (250°C)</i>								
0.06%	100 ± 0.0	24.9 ± 0.10*	64.6 ± 0.20*	25 ± 0.02	31 ± 1.41***	36 ± 1.5***	13.2 ± 0.32***	2.08 ± 0.05***
0.25%	100 ± 0.0	24.9 ± 0.10**	64.6 ± 0.20**	25 ± 0.02**	40.5 ± 1.76**	34.1 ± 1.33***	16.1 ± 0.61** ^b	2.03 ± 0.05***
0.5%	99.2 ± 0.8*	23.7 ± 0.22***	62 ± 0.58***	24.7 ± 0.02***	51.9 ± 0.65***	18.2 ± 0.37***	22.1 ± 0.53***	1.60 ± 0.05***

TG = total germination, SpG = speed of germination, SpAG = speed of accumulated germination, CRG = coefficient of rate of germination, HL = hypocotyl length, RL = radicle length, HW = hypocotyl weight, RW = radicle weight.

*** Strongly significant ($p < 0.001$).

** Significant ($p = 0.001$ to <0.01).

* Poorly significant ($p = 0.01$ to ≤ 0.05), blank means not significant compared with equivalent unburnt powder extracts.

^a Strongly significant ($p < 0.001$).

^b Significant ($p = 0.001$ to <0.01).

^c Poorly significant ($p = 0.01$ to ≤ 0.05) compared with high temperature.

phytotoxicity of other groups of allelochemicals (if identified in boneseed and ashes) on associated species is important to draw more rigorous conclusions.

The non-significant increases of H₂O₂, LPO and EL (compared with control) in *L. sativa* seedlings exposed to all doses of ash

extracts suggest that the mechanism of adverse impact showed by the highly concentrated boneseed ash extracts to germination and growth of test species didn't involve excessive ROS production, in contrast with the impact that the unburnt powder extracts had on these parameters. The non-significant increase

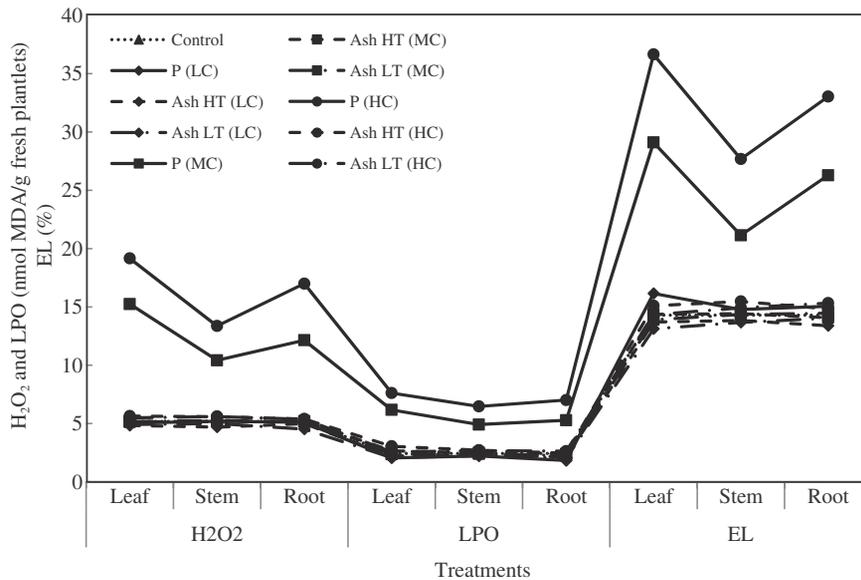


Fig. 4 – Phytotoxicity of ash aqueous extracts on physiology of *L. sativa* compared with powder extracts. The X-axis denotes the types of parameters e.g., hydrogen peroxide (H₂O₂), lipid peroxidation (LPO) and electrical conductivity (EC) in *L. sativa* seedlings exposed to boneseed powder and ash extracts while values on Y-axis denote their concentrations (H₂O₂ and LPO in nmol MDA/g fresh plantlets, and EL in %). Square dot, solid lines, dash and long dash dot denote the values for control, boneseed powder, ash burnt at high temperature (HT, 450°C) and ash burnt at low temperature (LT, 250°C) respectively. Diamond, rectangular and circle markers denote for low, medium and high concentrations.

of H₂O₂, LPO and EL (compared with control) in *L. sativa* seedlings with increasing concentration of ash extracts might due to the OP as suggested by Uddin et al. (2013). The strong correlation between OP and H₂O₂ ($r = 0.94$), and between OP and LPO ($r = 0.94$) again suggest that the slight increase in H₂O₂ and LPO may be due to OP (Appendix A Fig. S2).

The litter ash (burnt both at high and low temperatures) mediated soil significantly reduced phytotoxicity to test species

compared with equivalent unburnt litter powder, and even had stimulatory impact compared with control. The stimulation of the growth of *L. sativa* exposed to litter ashes despite containing more OP compared with control (dH₂O), might indicate that OP had no inhibitory role in the soil system used in this experiment. As the unburnt litter powder and ashes have similar OP it is likely that the observed differences are due to the far greater allelochemical content of the unburnt litter. Although, Inderjit and Dakshini (1994) suggested that nutrients released from the decomposed plant litter may play an important role in overcoming allelopathic effects, we found significant phytotoxic impact of boneseed litter on test species. This contrast in our study may be due to the presence of high level of phenolics, in particular, ferulic acid in boneseed litter. The phytotoxicity of similar concentrations of ferulic acid has been established in an earlier study (Reigosa et al., 1999). The severe growth inhibition in response to boneseed litter powder may be due to the direct impact of allelochemicals on the test plant or, potentially, the degradation of soil NO₃, due to either enhancing denitrifying bacteria or suppressing nitrogen fixing bacteria by litter allelopathy as reported by Northup et al. (1998). One of the important mechanisms of allelopathic impact is inhibition of the activities of beneficial soil microorganisms by allelochemicals, which are known to vary with types of soil, inorganic nutrient status and types and concentration of allelochemicals (Blum and Shafer, 1988).

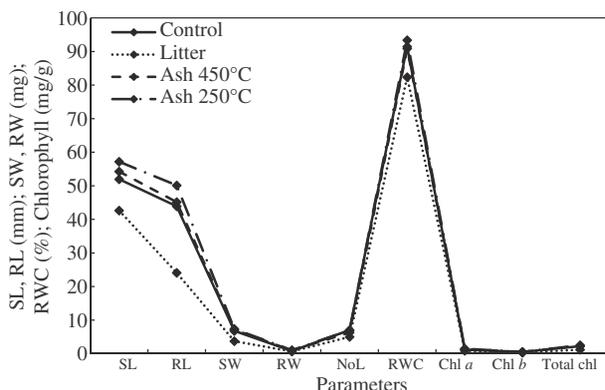


Fig. 5 – Comparative phytotoxic effects of boneseed unburnt litter and ash-mediated soil on biometric parameters of *L. sativa*. The X-axis denotes the parameters e.g., shoot and root length and weight (SL, RL, SW and RW), number of leaf (NoL), leaf relative water content (RWC), and leaf chlorophyll (chl a, chl b and total chl) content of *L. sativa* seedlings. Values on Y-axis denote their values (SL and RL in mm, SW and RW in mg, RWC in %, and chlorophyll in mg/g).

The reduction of chlorophyll production in *L. sativa* by boneseed litter compared with litter ash may suggest one of the mechanisms that led to the observed impacts on plant growth, similar to the effect reported by Gallardo-Williams et al. (2002). Although we didn't directly determine the allelopathic impact on photosynthesis, the decrease in chlorophyll could plausibly

suggest a reduction in photosynthesis, as suggested by Zhu et al. (2014). Furthermore, the very strong negative correlations between the chlorophyll content and hypocotyl ($r = -0.91$)/radicle ($r = -0.95$) length of *L. sativa* may play a role in explaining the observed growth stimulation/inhibition in test species (Appendix A Fig. S3). In addition, the significant increase of leaf RWC in test species exposed to ash compared with litter powder might cause substantial improvement in test species growth, a similar finding suggested by Hussain and Reigosa (2011). Although our findings indicate phytotoxicity reduction by burning, field evidence is imperative to demonstrate allelopathic impact in a more authentic way as edaphic and environmental factors work together in influencing allelopathic effects (Inderjit and Duke, 2003). There is a scope to extend this study in the future to demonstrate phytotoxicity on native species, as the current findings are limited to identification and demonstration of phytotoxicity of phenolic on model species only.

4. Conclusions

Burning of boneseed reduced the water soluble phenolic (WSP) compounds in ashes of boneseed organs (leaf, stem and root) by 99%–100% both at high and low temperatures showing non-significant variation with increasing burning temperatures. The four phenolic compounds that were detected in boneseed organs were either absent or negligibly found in ashes of boneseed organs, and the increase in temperature inversely related with their presence in ashes. Boneseed ash extracts significantly reduced phytotoxicity in the test species compared with unburnt powder extracts with greater reduction in ashes produced at lower temperatures. This reduction of phytotoxicity was due to the reduction/removal of allelochemicals in ashes compared with the unburnt boneseed materials. The stimulation of growth in *L. sativa* in response to the low and medium doses of ash extracts compared with control further demonstrate the significant impact of burning on reduction/removal of allelochemicals and consequent phytotoxicity in ashes. The inhibition in test species by highly concentrated ash extracts compared with control might due to osmotic potential as no impact should have happened from the negligible/zero level of WSP that was found in the ashes. The mechanism of inhibition by ash extracts (highly concentrated) to the test species does not involve excessive ROS production in contrast with unburnt powder extracts. The litter ash-mediated soil significantly increased the growth parameters including shoot and root, leaf RWC and leaf chlorophyll level of *L. sativa* compared with unburnt litter powder-mediated soil due to the substantial/complete removal of allelochemicals in ashes after burning. Further, the ash-mediated soil exhibited stimulation (significant for few parameters) in growth of *L. sativa* when compared with control. Our results suggest that burning is an appropriate method of boneseed control and that this approach will reduce the phytotoxicity of this species on native plants. However, further studies to identify more allelochemicals species (flavonoids, terpenoids, etc.) in boneseed ashes, and phytotoxicity studies in field condition with associated native species are recommended to be more conclusive. These findings may also be relevant to ecologists and land managers

aiming to control other allelopathic weeds through burning to facilitate plant growth.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jes.2015.09.020>.

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