SHORT COMMUNICATION

Phytotoxicity, cytotoxicity and antioxidant activity of the invasive shrub *Austroeupatorium inulifolium* (Kunth) R.M. King & H. Rob

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ABSTRACT

Methanol and dichloromethane extracts of root, stem, leaves and flowers of invasive plant *Austroeupatorium inulifolium* were tested for cytotoxic, phytotoxic, antioxidant and antifungal activities. Significantly higher phytotoxicity was detected in methanol extracts of leaves and dichloromethane extracts of roots. This effect was most pronounced against amaranth seeds where the seed germination was reduced to 3.74 % with the addition of methanol extracts of leaves at 3000 mg/L. The dichloromethane extract of roots of *A. inulifolium* showed potent antifungal activity against *Cladosporium cladosporioides*. Cytotoxic activity was found in dichloromethane extract of roots (LD_{50} = 27.91 ± 8.55 mg/L), methanol extract of flowers (LD_{50} = 15.22±7.89 mg/L) and leaves (LD_{50} = 22.92±11.76 mg/L) against *Artemiasalina* (brine shrimp) larvae. The results also revealed significant antioxidant activity in methanol extract of leaves (IC_{50} =33.66± 0.03 mg/L) against the reference α-tocopherol (IC_{50} =10.02 ± 0.01 mg/L).

Keywords: invasive plant, cytotoxicity, phytotoxicity, antioxidant and antifungal activities.

INTRODUCTION

Plants have co-evolved with their respective environments for millions of years. During this process, secondary metabolites played a critical role in plants' adaptations to insect and microbial attacks and climatic conditions. Plant metabolites with various bioactivities including phytotoxic and antioxidant activities allow plants to live in a state of equilibrium with the environment. However, some plant species use these bioactivities to transform themselves into noxious weeds. The indigenous flora of Sri Lanka comprises about 7,500 species. Of the 3,154 flowering plants, about 894 (28 %) species are endemic to the island, some carrying varying levels of biological activity (Wijesundara *et al.,* 2012; Bandara *et al.,* 1989a; Bandara *et al.,* 1990a; Hewage *et al.,* 1997; Hewage *et al.,* 1998). Many bioactivities in plants have been used in medicinal applications (Banadara et al., 1989b; Bandara*et al.,* 1989c; Bandara*et al.,* 1990b; Williams *et al.,* 2011). Plant ecologists were always puzzled by the fact that exotic plant species with low densities in their native ranges eventually produce large populations in their introduced ranges. The 'novel weapons hypothesis' proposed that these exotic plants possess novel biochemical compounds with powerful bioactivities (Callaway and Ridenour, 2004). *Austroeupatorium inulifolium* (Kunth)

King and Robinson (Asteraceae) which is an aggressive invasive shrub native to South America has become a noxious invader in the up-country, wet zone of Sri Lanka, invading many natural and man-made ecosystems (Madawala *et al*., 2014). It is also listed as an 'agricultural and environmental weed in the Global Compendium of weeds (Randall, 2012). Even though it has been well identified as an invasive plant in many countries, it has been categorized as an invasive plant in Sri Lanka fairly recently. *A. inulifolium* has been invading the *Cymbopogon*-dominated grasslands in the Knuckles Conservation Area (KCA), displacing the grass. Once established, *A. inulifolium* can form mono-specific stands in their introduced range, influencing the growth and survival of the native flora (Haluwana and Madawala, 2013).

There have been many hypotheses to explain the mechanisms of exotic plant invasions into new landscapes and among them the 'novel weapon hypothesis' has attracted contrasting views since it was proposed in 2004 (Blair *et al.,* 2006; Duke *et al.,* 2009). According to this hypothesis, some exotic species credit their success of spread due to the production of bioactive compounds that native species never encountered before (Callaway and Ridenour, 2004; Thorpe *et al.,* 2009; Callaway and Aschehoug, 2000). Previous studies on other

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invasive species have confirmed the presence of bioactive compounds (Yan *et al.,* 2010; Shao *et al.,* 2010; Ens *et al.,* 2009; Xie *et al.,* 2010). These bioactive compounds can reach the soil environment through leaching, root exudation or litter decomposition. In the present study, we attempted to investigate the potential role of bioactive compounds in *A. inulifolium* by using bioassays with extracts of different parts of the shrub including stems, roots, flowers and leaves and to relate them to its invasive success. Apart from the isolation of nine new norlabdane derivatives with antimicrobial activity in *A. inulifolium* (Saito *et al*., 2011)**,** no studies have been carried out to assess the bioactive profile of *A. inulifolium* with an aim to explain its invasive nature of the plant.   We report herein, the bioactivities of A. *inulifolium* including cytotoxic, phytotoxic, antioxidant and antifungal properties.

MATERIALS AND METHODS

Austroeupatorium inulifolium, belongs to the family Asteraceae*,* is a shrub that can grow up to 1-5 m in height. It bears a creamy white fragrant inflorescence (Figure 1). Leaves are opposite, spear-shaped and pubescent, abruptly narrowing to a wedge-shaped petiole. It is well known as an aggressive invader in many countries.

Figure 1. The creamy white inflorescence of *A. inulifolium.* (Photo Credit: InokaPiyasinghe).

Extraction of potential bioactive compounds
Plant samples were collected from Riverston area in the Knuckles Forest Reserve (KFR) in the central of Sri Lanka. Plant parts (roots, stems, leaves and flowers) were separated and air-dried before grinding into a powder. The resulting powdered plant material ($\approx$600 g) was subjected to

sequential extraction with 5 L of CH_2Cl_2 and CH_3OH separately after maceration for 24 hours (in cleaned and dried glass bottles) with constant shaking. The extract was then filtered and concentrated using a rotary evaporator at 30 °C to obtain the final yield of extract in paste form. The crude extract was then stored at 4 °C until further use. The CH_2Cl_2 and CH_3OH crude extracts were subjected to cytotoxic, phytotoxic, antioxidant and antifungal bioassays.

Cytotoxic Activity
Cytotoxic activity was determined using a brine shrimp lethality assay (McLaughlin, 1982). Brine shrimp eggs were added to fresh artificial sea water and allowed to hatch in a beaker which was aerated and illuminated using a 20 W bulb. The temperature was maintained at 27 °C. A series of concentrations (0.75, 2, 7.5, 20, 75, 200, 750, 2000 mg/L) of the dichloromethane and methanol extracts of different plant parts were tested with 1-day old brine shrimps (three replicates and 10 shrimps per experiment). After 24 hours, the number of surviving nauplii of brine shrimps was counted and LD_{50} value (concentration at 50 % survival) was calculated using probit analysis of MINITAB version 16. The known cyctotoxic lactone,   (4   S)-4-methyl-2-(11-dodecynyl)-2 butenolide, from the genus *Hortonia* was used as the positive control (Ratnayake *et al.,* 2001).

Phytotoxic activity
The phytotoxic activity was determined using a seed assay with two monocot species, maize (*Zea mays*) and bluegrass (*Poa sp.*), and two dicot species, amaranth (*Amaranthus sp.*) and radish (*Raphanus sativus*). Ten seeds (surface sterilized with 0.5 % $HgCl_2$) from each species were treated with 5 ml of 250, 750 and 3000 mg/L of the CH_2Cl_2 and CH_3OH extracts of different plant parts in petri dishes (9 cm diameter) lined with Whatman No. 3 filter paper. Distilled water mixed with 1 % DMSO (v/v %) was used as the negative control and [(4S)-4-methyl-2-(11-dodecynyl)-2-butenolide] as the positive control. Petri dishes were sealed with Parafilm to prevent any water losses and the petri plates were stored in the dark at room temperature. Tests were carried out in triplicate for each concentration. Cumulative germination was determined by counting the number of germinated seeds daily over a period of 6 days and the Germination Index was calculated using the formula (Chiapuso *et al*.,1997),

$$GI = (N_1) \times 1 + (N_2\text{-}N_1) \times 1/2 + (N_3\text{-}N_2) \times 1/3 + \ldots + (N_n\text{-}N_{n\text{-}1}) \times 1/n$$

where N_1, N_2, $N_3 \ldots N_n$: proportion of germinated seeds observed afterwards 1, 2, 3… n-1 and n is the number of days. On the 7th day, shoot and root lengths of the seedlings were measured. The inhibitory or stimulatory percentage was calculated using the

equation (Chung *et al.*, 2001), Inhibition (-) or Stimulation (+) % = (Extract – Control)/(Control) × 100.

Antioxidant activity

Antioxidant activity was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Budzianowski and Budzianowska, 2006). The reduction of DPPH in the extract was measured using a concentration series ranging from 1 – 500 mg/L at 517 nm using a UV-visible spectrophotometer (Shimadzu UV-1800) after 30 minutes against the CH_3OH blank. The initial absorbance of DPPH and each test solution (without DPPH) were measured at 517 nm separately. α- Tocopherol (Vitamin E), concentration series ranging from 1 – 500 mg/L was used as the positive control. Tests were carried out in triplicate for each concentration. Percentage antioxidant activity was calculated using absorbance readings using the equation, $[A_0 - A_t/A_t]$ x 100 where A_0 is the initial absorbance value and A_t is the absorbance of the test sample after 30 min of adding DPPH. The % antioxidant activity was plotted against the concentration gradient for all three trials separately and trend lines were constructed for the linear range of the plot. The IC_{50} value was calculated for each trend line from which the average was taken.

Antifungal activity

Quantitative analysis of antifungal activity using the TLC bioassay method with the fungus, *Cladosporium cladosporioides* was carried out. A concentration series of the extracts (250, 750 and 2500 mg/L) of different plant parts was prepared and spotted on a TLC plate. A conidial suspension of *C. cladosporioides* was carefully sprayed using an atomizer onto the TLC plates. After 2 days of incubation, the presence of antifungal compounds was detected using inhibition zones. Antifungal active spots appeared white against the background of grey-green mycelia.

RESULTS AND DISCUSSION

Yields of potential bioactive extracts

After extraction and concentration, crude yields of methanol and dichloromethane extracts of different plant parts of *A. inulifolium were* obtained (Table 1). In both extracts, the highest percentage yield was obtained from the leaves.

Cytotoxic effects

Moderate cytotoxic activity was detected in the CH_2Cl_2 extract of roots (27.91 ± 8.55 mg/L), and CH_3OH extracts of leaves (22.92 ± 11.76) and flowers (15.22 ± 7.89). The LD_{50} value of the positive control, (4 S)-4-methyl-2-(11-dodecynyl)-2 butenolide, was 0.80 mg/L (± 0.37).

Phytotoxic activity

Germination index (GI) generally decreased with increasing concentrations of CH_3OH and CH_2Cl_2 extracts of *A. inulifolium* (Table 2). However, CH_3OH-leaf, CH_2Cl_2-root and CH_2Cl_2-stem extracts showed the most significant reductions in germination index with increasing concentrations (from 250 to 3000 mg/L). As an example, CH_3OH-leaf extracts decreased the GI from about 68 % to 4 % in amaranth seeds, while CH_2Cl_2-root extracts reduced the GI of same seeds from 61 % to 8 %, when increase the concentration from 250 mg/L to 3000 mg/L. In contrast, all concentrations of CH_2Cl_2-flower extracts showed no influence on the germination of seeds tested in the assay. CH_2Cl_2-leaf extracts too showed no impact on the germination of maize seeds.

Both CH_3OH and CH_2Cl_2 extracts of *A. inulifolium* roots and shoots negatively influenced the growth of both monocot and dicot seedlings tested, and this inhibition gradually increased with increasing concentrations (Figure 2). CH_3OH extracts of *A. inulifolium* leaves negatively affect the growth of seedlings while CH_2Cl_2 extracts did not show clear inhibition apart from *amaranth* seedlings.

Table 1. Mass and the yield (as a percentage) of methanol and dichloromethane crude extracts of different plant parts of *A. inulifolium* collected from Riverston area in the Knuckles Forest Reserve, Sri Lanka.

Plant part	Dry mass (g)	Mass of CH_3OH crude extract (±0.001g)	% yield of CH_3OH crude extract	Mass of CH_2Cl_2 crude extract (± 0.001g)	% yield of CH_2Cl_2 crude extract
Flower	77	4.14	5.37	0.67	0.86
Leaf	283	32.07	11.33	20.49	7.24
Stem	410	4.65	1.13	1.02	0.25
Root	173	3.41	1.97	2.15	1.24

Table 2. Germination Index (GI, %) of monocot (maize and bluegrass) and dicot (amaranth and radish) seeds under different concentrations (250, 750 and 3000 mg/L) of plant extracts of *A. inulifolium.*

Extract	Conc. (mg/L)	Monocots		Dicots	
		Maize	Bluegrass	Amaranth	Radish
CH_2Cl_2-leaf	250	98.1[a]	88.1[a]	83.6[a]	95.0[a]
	750	90.6[a]	77.2[ab]	71.7[b]	86.2[b]
	3000	89.9[a]	69.4[b]	43.2[c]	71.5[c]
CH_3OH-leaf	250	98.9[a]	89.5[a]	67.6[a]	79.4[a]
	750	91.3[a]	68.8[b]	46.8[b]	68.3[b]
	3000	74.8[b]	60.8[b]	3.7[c]	44.1[c]
CH_2Cl_2-flower	250	101.8[a]	98.7[a]	92.5[a]	92.2[a]
	750	97.0[a]	93.3[a]	90.3[a]	88.7[a]
	3000	88.6[a]	91.9[a]	89.5[a]	86.1[a]
CH_3OH-flower	250	95.1[a]	92.7[a]	89.1[a]	90.0[a]
	750	87.6[ab]	88.3[a]	72.5[b]	72.1[b]
	3000	80.9[b]	77.4[b]	48.3[c]	66.8[c]
CH_2Cl_2-root	250	99.9[a]	87.4[a]	61.4[b]	98.3[a]
	750	69.9[b]	57.8[b]	42.0[c]	91.7[a]
	3000	61.1[b]	35.3[c]	8.2[d]	75.0[b]
CH_3OH-root	250	89.2[ab]	61.5[b]	68.3[b]	100.0[a]
	750	63.8[c]	63.3[b]	58.0[bc]	92.8[b]
	3000	74.1[bc]	30.5[c]	51.1[c]	93.9[b]
CH_2Cl_2-stem	250	80.3[ab]	72.8[b]	98.3[a]	61.3[b]
	750	65.2[b]	52.4[c]	52.9[c]	89.2[ab]
	3000	40.9[c]	8.8[d]	7.8[d]	76.4[b]
CH_3OH-stem	250	90.2[ab]	75.3[b]	58.2[b]	98.3[ab]
	750	85.1[b]	59.9[b]	56.3[b]	92.2[ab]
	3000	49.7[c]	25.9[c]	35.0[c]	92.8[b]

Means with the same letters in a column (between different concentrations) indicate no significant differences at $P < 0.05$.

Interestingly, CH_3OH-leaf and CH_3OH-flower extracts showed higher inhibition on the growth of dicot seedlings compared to that of monocots. In contrast to the effects of other extracts, CH_2Cl_2-flower extracts stimulated the growth of dicot and monocot seedlings, showing highest stimulatory effect at 250 mg/L concentration, though the differences are not significant. Furthermore, the monocot seedlings were more stimulated than that of dicot seedlings (Figure 2).

Antioxidant activity of A. inulifolium
When comparing the antioxidant activity of

CH_3OH and CH_2Cl_2 extracts of *A. inulifolium* with α-tocopherol (IC_{50} =10.02 ± 0.01 mg/L), the CH_3OH extract of leaves (IC_{50}=33.66± 0.03 mg/L) showed a significant antioxidant activity among the extracts at $P<0.05$.

Antifungal activity of A. inulifolium
Interestingly, the antifungal activity was only observed in CH_2Cl_2-root extracts. According to the results obtained, the average diameters of inhibition zones shown in the presence of the CH_2Cl_2 extract of *Austroeupatorium* roots were 16.0 and 24.6 mm at concentrations 750 and 2500
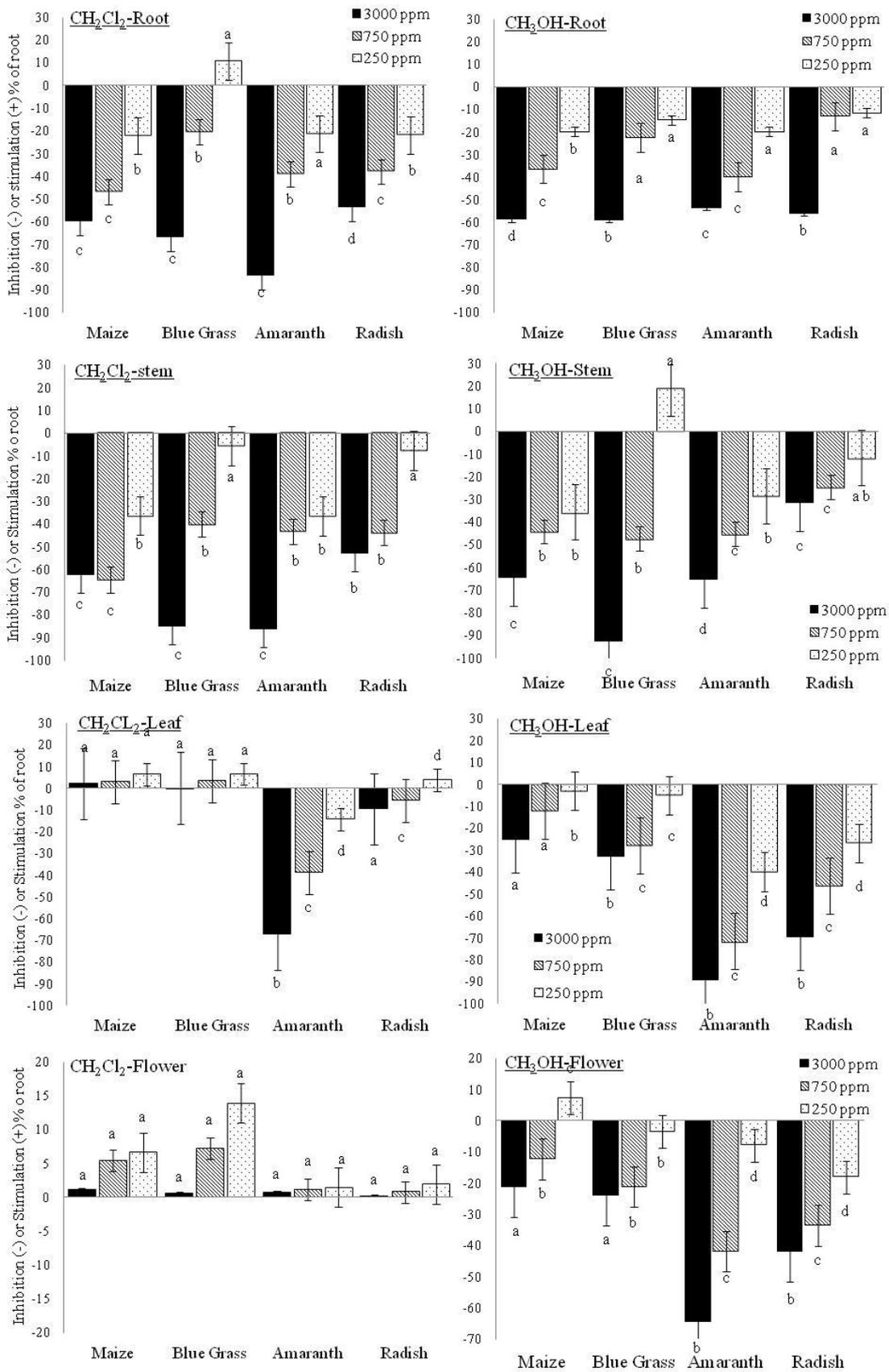
mg/L, respectively (Table 3).

Figure 2. Inhibitory or Stimulatory effects (%) of different concentrations (250, 750 and 3000 mg/L) of *A. inulifolium* extracts on the growth of monocot (maize and bluegrass) and dicot (amaranth and radish) seedlings.

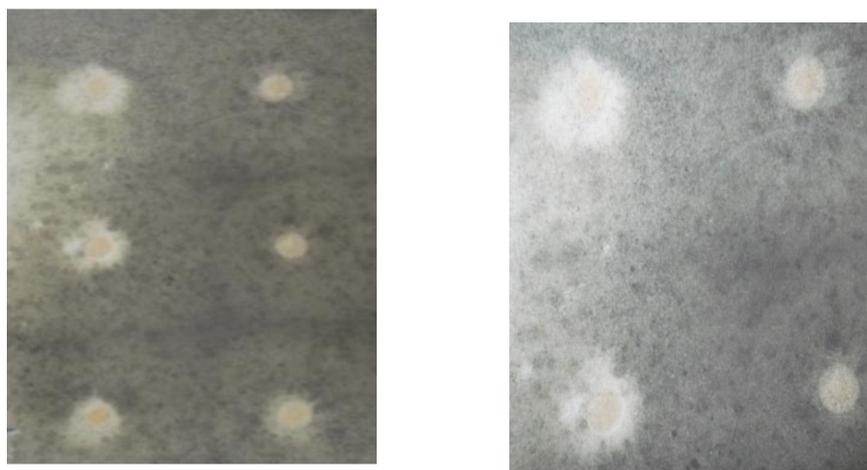Different letters indicate significant differences among concentrations at P<0.05.

Figure 3. Inhibition zones given by CH₂Cl₂ extract of *A. inulifolium* roots (a) 750 mg/L (b) 2500 mg/L.

Table 3. Diameters of the inhibition zones given by different concentrations of root dichloromethane extract of *A. inulifolium* (2500 mg/L and 750 mg/L).

Concentration/(mg/L)	Diameter of the inhibition zone ($\pm$ 0.5/mm)		
	Trial 1	Trial 2	Trial 3
2500	24.0	27.0	23.0
750	16.0	14.0	18.0

DISCUSSION

When consider the extraction of potentials of bioactive compounds in *A. inulifolium,* the CH_3OH extracts recorded a higher yield than that of CH_2Cl_2 extracts. This may be due to the higher content of polar compounds in *A. inulifolium* compared to that of non-polar compounds. Cytotoxic results suggested that *A. inulifolium* roots, flowers and leaves contain cytotoxic compounds which can be useful as antiproliferative and antitumor activities, as well as pesticidal and other bioactive agents. There is evidence to support that natural cytotoxic compounds are good contenders for anticancer drugs (Rahman *et al.,* 2001). In addition, these cytotoxic compounds also play an important role in these plants to minimize pest attacks compared to co-existing natives, determining their successful establishment in their introduced ranges (McGraw and Eloff, 2008).

The effects of bioactive compounds on seed germination have been tested using germination index (GI) in previous studies (Chiapuso *et al*., 1997). GI is considered as a sensitive indicator of phytotoxic effects (Ahmed and Wardle, 1994). Results showed that germination of tested species were affected by all extracts which either delayed or reduced germination of all test seeds. However, a significantly higher phytotoxic effect was detected in CH_3OH-leaf and CH_2Cl_2–root extracts of *A. inulifolium* (P<0.001). The CH_3OH-leaf extract at 3000 mg/L showed the most pronounced impact against amaranth seeds with GI of 3.74 %. The analysis showed that CH_2Cl_2-flower extract had no significant effect on the GI (P < 0.05) of both monocot and dicots, although it has declined the seed germination. The degree of effect on germination increased with the increasing concentrations of extracts and this was evident in extracts of all plant parts of *A. inulifolium*. The delayed seed germination can have important biological implications, as this can negatively affect the establishment of seedlings of co-occurring natives (Chaves *et al.,* 2001), and thereby increasing the chances of competing for resources with neighbouring species (Xingxinag *et al.,* 2009).

However, the effect was more pronounced in CH_3OH extracts of *A. inulifolium*, where polar compounds are commonly found. Methanolic extracts of some plant species were known to contain phenolics and other toxic substances (Belicova *et al.,* 2001; Al Harun *et al.,* 2015). These phenolics may inhibit the germination process through their interference in the indole acetic acid metabolism, or synthesis of protein and

iron uptake by plants (Blum, 1998; Castro *et al.,* 1984).

Phytotoxic results showed that the root and shoot development of germinating test seeds are highly inhibited by the CH_2Cl_2 extracts of root and stem, and by all extracts of CH_3OH. In contrast, slight stimulatory effects on the root and shoot growth of monocot seedlings was shown by CH_2Cl_2 extracts of leaves and flowers. The shoot growth appeared less sensitive to *A. inulifolium* extracts than the radicle, which eventually grows into roots. The phytotoxic compounds are highly active against meristmatic tissue in growing roots. This perhaps is the reason for differing responses of root and shoot to *A. inulifolium* extracts. Phytotoxicity has been suggested as the key strategy for the impressive success of many invasive plants that has been dominated in their invaded plant communities (Ridenour and Callaway, 2001).

In some instnaces, there were slight stimulatory effects on root and/or shoot by different extracts of *A. inulifolium*. According to results, inhibition or stimulation of root and shoot and the germination index varied with plant extract, concentration and seed type. The stimulatory bioactive compounds can be used to develop eco-friendly, cheap and effective Green Growth Promoters (Oudhia *et al.,* 1998). Phytotoxic compounds inhibit germination and seedling growth probably by affecting cell division and elongation, processes that are very important at early stages, or by interfering with enzymes involved in the mobilization of nutrients necessary for germination (Batlang and Shush, 2007).

Antioxidant results revealed that the MeOH extract of leaves contains compounds with antioxidant activity. Generally, plant polyphenolic compounds are responsible for antioxidant activity. In addition, they also act as iron chelators. The latter property may be important in an invasive plant like *A. inulifolium* where it contributes to altering the soil bacterial flora in their growing habitats (Morel *et al.*, 1993).

In the antifungal assay, larger inhibition zones were observed with CH_2Cl_2 extracts, suggesting that the solvent has the potential to extract many active antifungal compounds. Chromene, an antifungal compound, found in roots of the invasive plant *Eupatorium riparium* which also belongs to the same genus as *A. inulifolium* (Bandara *et al.,* 1992). Furthermore, the antifungal, phytotoxic and antioxidant activities were also tested in essential oils extracted from roots and inflorescence of *Eupatorium adenophorum* (Ahluwalia *et al.,* 2014), further supporting this genus's worldwide distribution.

CONCLUSIONS

The results suggest that *A. inulifolium* contained phytotoxic, cytotoxic and antifungal activities. The presence of phytochemicals in *A. inulifolium* may suppress the growth of other plants in the habitat they invade. Cytotoxic compounds can kill pests thereby minimizing pest attacks. Antifungal compounds can reduce fungal attacks on the plant too. These characteristics can favour invasive species to invade and establish in their introduced ranges successfully. Therefore, the survival and spread of *A. inulifolium* may have been favored by the presence of these phytotoxic compounds where they can inhibit the growth of co-habiting plants. These factors may have contributed to the aggressive nature of *A. inulifolium.*

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