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ARTICLE

Isolation and phytotoxicity of an active fraction and its pure compound (gallic acid) from sun spurge *(Euphorbia helioscopia L.)* against harmful weeds

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ABSTRACT An active fraction (F5) and its derived pure compound (gallic acid) were extracted from aerial parts of sun spurge (*Euphorbia helioscopia*). Gallic acid was chromatographically isolated and identified based on spectroscopic analysis ^{1}H and ^{13}C NMR. To investigate the phytotoxicity of F5 (0.1%) and gallic acid (0.001%), their effects were studied against 18 test weeds. In this study, we evaluated the alterations in germination percentage (G%) and speed (GS) as well as seedling length (SL), fresh weight (FW) and dry weight (DW). Also, the change in total phenolic content (TPC) and lipid peroxidation in response to treatment were measured. Our results showed variation in the phytotoxic effect of F5 and gallic acid depending upon weed species. Significant reduction of germination and seedling growth by F5 and gallic acid treatment was common for most of weeds and F5 was more toxic than gallic acid. All weed species accumulated polyphenols as a defence system, but it was not enough to prevent plant damage (lipid peroxidation). We concluded that using weed derived phytotoxic fractions (*e.g.*, F5) and pure compounds (*e.g.*, gallic acid) could play an effective role for weed control instead of using harmful chemical herbicides.
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KEY WORDS

Euphorbia helioscopia L.
gallic acid, phytotoxicity
weed control

Introduction

Losses in crop yield and production caused by weeds, are well documented in many studies (Swanton et al. 1993; Khedr and Hegazy 1998). *Euphorbia helioscopia* is a common herbaceous weed found in Egypt and it invades crops and vegetables, *viz.* wheat, chickpea, potato and lentil. *E. helioscopia* is stated to possess antioxidant, antifungal and antibacterial components (Uzair et al. 2009; Tanveer et al. 2010). Additionally, the phytotoxic potential of some *Euphorbia* species extracts on germination and growth of weeds and cultivated plants was studied (Steenhagen and Zimdahl 1979; Hussain 1980; Abu-Romman et al. 2010). Hegnauer (1989) recorded the major classes of secondary metabolites present in *Euphorbia* species *viz.* alkaloids, terpenes, cyanogenic glycosides, glucosinolates, lipids and tannins.

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Allelopathic effects and mechanisms underlying the activity of plant compounds (*e.g.*, phenols) have been reviewed in various papers (Li et al. 2010). Additionally, the phytotoxicity of some active compounds isolated from weeds at certain concentrations had been studied (Ghareib et al. 2010). Gallic acid is a phenolic acid, which known as an allelopathic agent (Rice 1984) and identified in some *Euphorbia* species *e.g.*, *Euphorbia lunulata* (Yang et al. 2011) and *Euphorbia hitra* (Yoshida and Chen 1988), also, in other plants *e.g.*, *Cornus officinalis* (Tian et al. 2000), *Juglans regia* L. (Zhang et al. 2008), *Leea indica* (Srinivasan et al. 2008) and exudates of *Phragmites australis* (Rudrappa et al. 2007). Many studies established the phytotoxicity and persistence of gallic acid in soil (Weidenhamer and Romeo 2004; Bains et al. 2009). Moreover, gallic acid showed an inhibitory effect on the germination and seedling growth of different weeds (Weidenhamer and Romeo 1989; Reigosa et al. 1999; Rudrappa et al. 2007; Rudrappa and Bains 2008). In the same way, Rudrappa and Bains (2008) reported the suppression effects of gallic acid on crop species and the model plant *Arabidopsis thaliana*. Studies of Rudrappa et al. (2007) conclusively established that, phytotoxicity of gallic acid on *A. thaliana*

and other species is because of ROS mediated destruction of the microtubule network of root cells.

The objective of this study was to isolate and identify the phytotoxicity of most active fraction and its isolated pure compound from *E. helioscopia* against germination and seedling growth of some harmful weeds.

Materials and Methods

Plant material

The aerial parts of *Euphorbia helioscopia* were collected from infected wheat fields in Beni-Suef, Egypt. The samples of *E. helioscopia* were then identified by Dr. Mohamed Fadl (Botany and Microbiology Department, Faculty of Science, Beni-Suef University) and authenticated by comparison with voucher specimens in the herbarium of Botany Department, Faculty of Science, University of Cairo, Cairo, Egypt, where voucher specimens were deposited (No. 11029). The leaves were washed five times with distilled water and were dried at room temperature. Grains or seeds of the test weeds (*Amaranthus hybridus* L., *Amaranthus lividus* L., *Avena sterilis* L., *Avena fatua* L.*, Cichorium endivia* L. *Convolvulus arvensis* L. *Corchorus olitorius* L. *Cynodon dactylon* (L.) Pers., *Cyperus rotundus* L., *Echinochloa colona* L., *Euphorbia helioscopia* L., *Euphorbia prostrate* Aiton, *Melilotus indicus* L., *Hibiscus trionum* L., *Panicum repens* L., *Paspalum distichum* L., *Phalaris paradoxa* L. and *Portulaca oleracea* L.), were used to investigate the phyotoxicity of active fractions and isolated compounds.

Extraction, fractionation and isolation of bioactive compounds

The air-dried plant materials (3.5 kg) were ground and extracted by soaking for 24 h with ethanol, then dried using rotary evaporator (SENCO, Shanghai Senco Technology Company, Shanghai, China) at 45 ºC and reduced pressure. Dried ethanol extract (272.5 g) was eluted by using gradients of heptane:ethanol (9:1 to 0:1) as a mobile phase to yield 7 fractions depending upon thin layer chromatography (TLC) analysis (Merck KGaA, Darmstadt, Germany). According to wheat coleoptiles bioassay, F5 (89.8 g) showed the highest bioactivity. The most active fraction (F5) was evaporated under reduced pressure at 45 °C to remove the solvent and then subjected to column chromatography (Merck, Germany) on a silica gel column (Sigma-Aldrich, USA). The mobile phase (dichloromethane:water) with gradient (10:0 to 3:10) was chosen for elution depending up on thin layer chromatography analyses (Merck KGaA, Darmstadt, Germany) to give 5 sub-fractions (F1-1 to F1-5). Sub-fraction 2 (F5-2) (3.14 g) showed the highest activity by using coleoptiles bioassay. Thin layer chromatography (TLC) analyses of F1-3 showed the presence of mixture of three compounds and one of them was very low, the major one was purified by subjecting to Sephadex LH-20 column (Sephadex LH-20, Merck, Germany) using n-butanol saturated with H_2O for elution, and 104.5 mg of the purified compound was yielded. The preliminary bioactivity test of the isolated pure compound indicated its phytotoxicity.

Nuclear magnetic resonance (NMR) identification

The compound in F1-3 was identified based on spectroscopic analyses and comparison of ^{1}H and ^{13}C NMR data with previous literature values.

According to the mass spectrometry analyses of the isolated compound [^{1}H NMR (DMSO-d6): δ 6.90 (s, 2H, ArH), 12.23 (1H, s, –COOH), 9.25 (s, 1H, OH), 8.87 (s, 1H, OH). During ^{1}H NMR analysis, peaks at d 8.87 and 9.25 ppm disappeared by the addition D_2O, which clearly indicated that both protons belong to the OH group and ^{13}C NMR (DMSO-d6): δ 169.8 (–COOH), 145.9 (C-3, 5), 137.8 (C-4), 122.3 (C-1), 110.6 (C-2, 6)]. Further comparison of our results was made with previous studies (Zhang et al. 2003; Wang et al. 2005), which helped us to identify our isolated compound as gallic acid.

Wheat coleoptile bioassay experiments

Wheat grains (*Triticum aestivum* L. cv. Sides) were sown in Petri dishes and incubated in the dark at 22±1 °C for 4 days (Hancock et al. 1964). The apical 2 mm of the shoots were cut off and discarded, then the next 4 mm of the coleoptiles were taken under a green safe light for bioassay. Four different concentrations for every fraction and sub-fractions were diluted in a phosphate-citrate buffer solution, containing 2% sucrose at pH 5.6 (Nitsch and Nitsch 1956) to prepare 0.001, 0.005, 0.01% solutions. Five wheat coleoptiles were placed in test tube, containing 4 ml of the experimental solution (three replicates of each solution) and control tubes were filled with 4 ml of the buffer solution. All test tubes rotated slowly for 24 h at 22±1 °C in the dark. The coleoptiles length was measured and the data expressed as percentage of differences as compared with the control in order to investigate whether the extracts had an additive or negative effect on the growth.

HPLC analyses of free phenolic compounds in aqueous methanol extract

After drying, the residue of active fraction (F5) was dissolved in *High Performance Liquid Chromatography (*HPLC*)* grade methanol to give 1000 ppm, then 20 µl of the methanol dis-

solved sample was injected into HPLC system (Shimadzu LC 10 AD chromatograph supplied with Shimadzu SPD-10 AUV-VIS). Phenomenex C18 column (25 cm x 4.6 mm, i.d, 5 mm particle size) was used as a stationary phase for HPLC determinations. The retention times of twenty-five highly purified phenolic compounds (Sigma-Aldrich, Germany) as well as our sample were detected at 254 nm.

Preparation of test solutions

The test solutions of active fraction (0.1%) and its pure compound (gallic acid) (0.001%) were prepared by dissolving in a very little amount of dimethyl sulfoxide (DMSO) (1%), and then completed with 10 mM MES (2-[N-morpholino] ethanesulfonic acid) to 100 ml. Selection of the appropriate concentrations were based on preliminary tests, that indicated the maximum inhibitory effects of the compounds on coleoptiles elongation and lettuce germination. MES and 1% DMSO were used as controls. The pH values were adjusted to 6.0 using 1M NaOH.

Bioassay experiments

Grains or seeds of our collected weeds were surface sterilized with 0.1% mercuric chloride for 5 min, and thoroughly washed with distilled water. Fifty, nearly uniform size and weight grains were placed on Whatman No.1 filter papers, wetted with 10 ml of aqueous solutions at controlled pH by using 100 mM MES and addition of 1M NaOH solution (as control, pH 6.0), or 10 ml of the 120, 60, 30 and 10 µM of phenolic acid (gallic acid) in sterile Petri-dishes (9 cm diameter). The Petri dishes were incubated in the dark for 10 days at 25/12 ºC ± 2 and about 97% relative humidity. Each treatment was replicated five times in a completely randomized experimental design. After 10 days, the germination percentage and the vigour value (V) were calculated. The vigour value (V) (germination speed) has been chosen to measure the germination speed. Vigour value may be calculated using the following formula (Bradbeer 1988).

V= (a/1+b/2+c/3+d/4+…….+ x/n) X 100/S.

Where a, b, c…x represent the number of seeds, which germinated after 1, 2, 3, n days of inhibition, respectively. S means the total number of germinated seeds.

Weed seedling's growth measurements; seedling length (SL), dry weight (DW) and fresh weight (FW) were recorded.

Total phenolic content (TPC)

The phenolic compounds were extracted from dried seedling tissues using 80% aqueous ethanol and their amounts were estimated by the Folin-Ciocalteau phenol reaction (AOAC, 1990). The phenolic content was obtained from a standard curve of gallic acid and expressed as mg phenolic g^{-1} dry weight. Subtraction of the phenolic aglycone content before and after hydrolysis gave the phenolic glycoside content.

Lipid peroxidation

Lipid peroxidation was determined by measuring malondialdehyde (MDA) (Heath and Packer 1968). The amount of MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} at 532 nm and expressed as nmol g^{-1} fresh weight.

Results and Discussion

Investigation of the bioactivity of fractions and subfractions

Among different fractions and subfractions, F5 and F5-2 showed the highest bioactivity by significant reduction of wheat coleoptile elongation (64.7 and 53.7% of length reduction at 0.1%, respectively) compared with respective controls at each stage. The active fraction (F5-2) was chromatographically separated on Sephadex LH-20 column to yield a pure compound (gallic acid) with an inhibitory effect on wheat coleoptiles elongation (35.9% of reduction at 0.001%).

F5 and gallic acid suppressed germination% and speed (vigour value)

In our study, we measured germination percentage (G%) and speed (GS) to evaluate the plant response to F5 and gallic acid applied at 0.1 and 0.001%, concentrations respectively (Fig. 1 and 2). Generally, the phytotoxic effect of both F5 and gallic depended upon the weed species investigated, i.e. the test species showed different responses to both CH_2Cl_2 fraction and tricin, where CH_2Cl_2 fraction had more reduction impact on measured parameters, indicating different tolerance of the weed species. Furthermore, F5 was more toxic than gallic acid, which may be attributed to the other phenolic compounds in F5 (some of these phenols were estimated by HPLC) that could overcome the phytotoxicity of gallic acid. F5 treatment resulted in a significant decrease in G% of all treated weed seeds and the % of reduction was more than 50% for all weeds except *A. sterilis, A. fatua, E. helioscopia* (26, 42 and 33%, respectively). Additionally, GS of most of weeds was significantly reduced, but the inhibition % was more than 50% only for few species (*M. indicus, P. repens*, *P. paradoxa*). The inhibitory potential of some *Euphorbia species* extracts on germination of weeds was studied (Steenhagen and Zimdahl 1979; Hussain 1980) with similar methods. Gallic acid significantly reduced the ger-

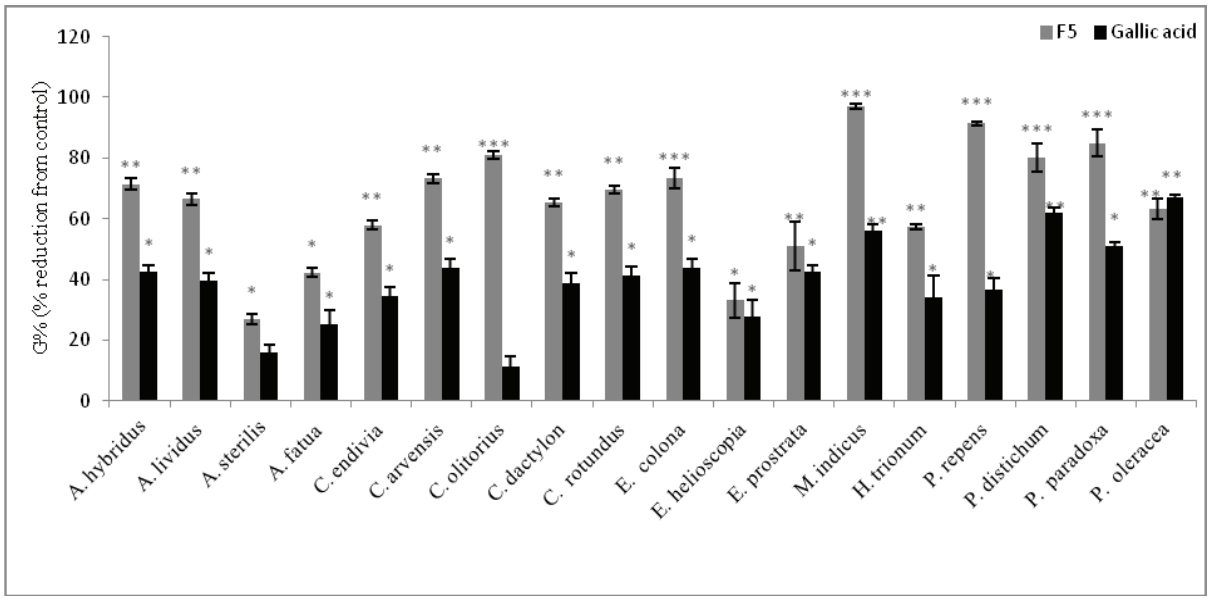

Figure 1. Effect of active fraction (F5) and gallic acid on the germination percentage of selected weed species (values are the mean of five replicates). Capped bars stand for standard errors. *, ** and *** significant difference at P< 0.05, 0.01 and 0.001.

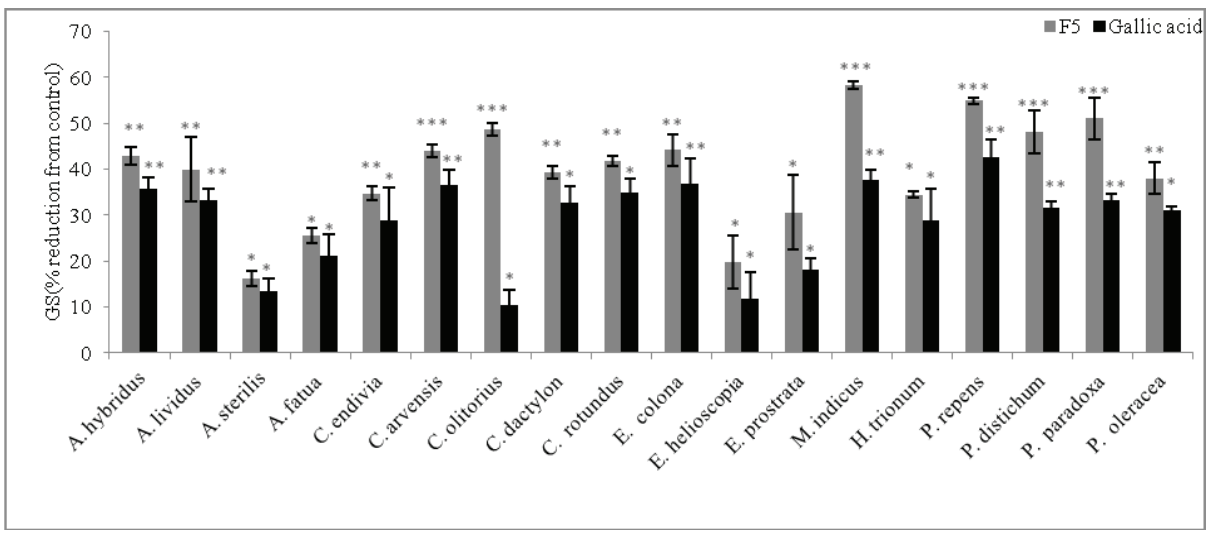

Figure 2. Effect of active fraction (F5) and gallic acid on the germination speed (GS) vigour value) of selected weed species as % reduction from control (values are the mean of five replicates). Capped bars stand for standard errors. *, ** and *** represent significant difference at P< 0.05, 0.01 and 0.001.

mination (G% and GS) of several weed species. The highest significant suppressions were recorded for G% in case of *M. indicus*, *P. distichum*, *P. paradoxa* and *P. oleracea* (56, 62, 51 and 67%, respectively). Ghareib et al. (2010) and Maighany et al. (2007) reported the inhibitory effect of some plant extract and isolated compounds at certain concentrations on germination. Hegab (2005) reported the suppression effect of gallic acid at high concentration, which could be a result of amylase activity inhibition. Also, Weidenhamer and Romeo (2004) recorded the inhibitory effects of higher concentrations of gallic acid and hydroquinone allelochemicals from *Polygonella myriophylla,* on bahiagrass. Similarly, Iqbal et al. (2003) showed a strong phytotoxic effect of gallic acid on several plant species. Furthermore, exuded gallic acid in the rhizosphere of *Phragmites australis* inhibited the growth of *A. thaliana* (Rudrappa et al. 2007).

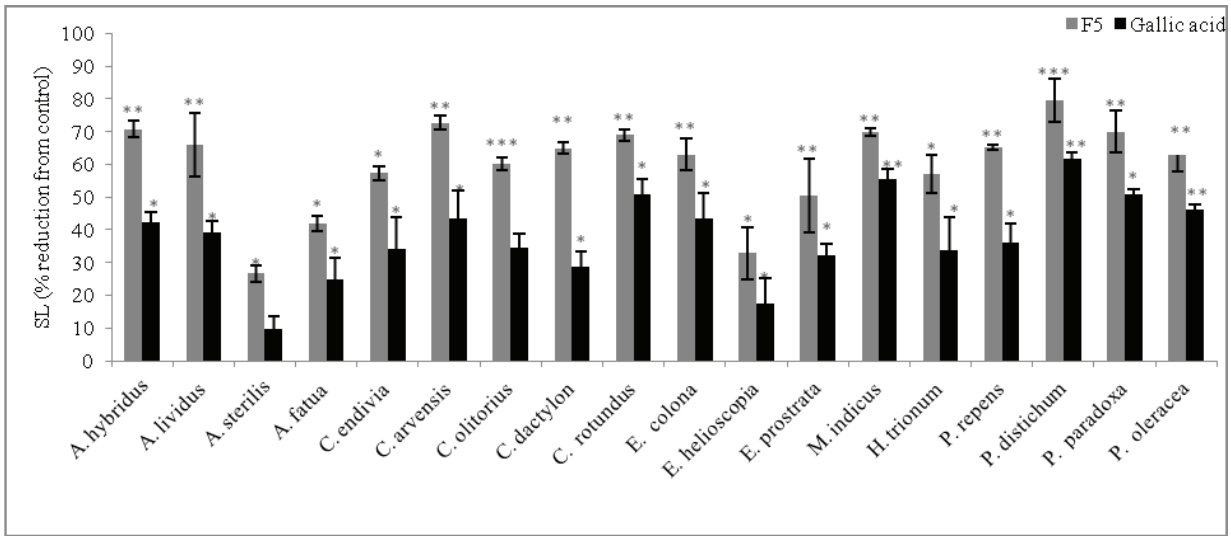

Figure 3. Effect of active fraction (F5) and gallic acid on seedling length (SL) of selected weeds species as % reduction from control (values are the mean of five replicates). Capped bars stand for standard errors. *, ** and *** represent significant difference at P< 0.05, 0.01 and 0.001.

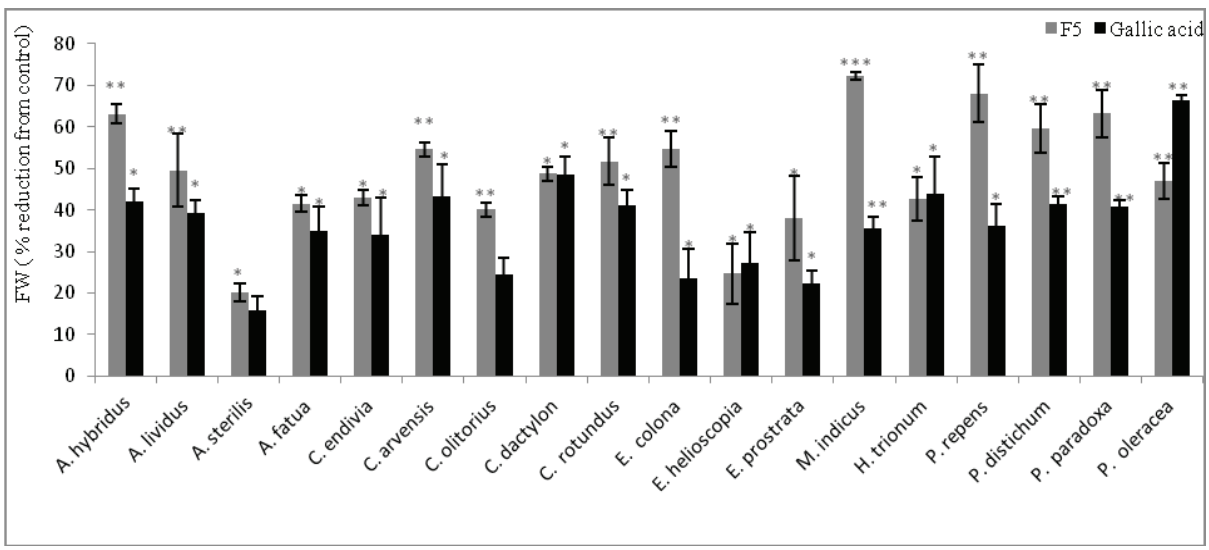

Figure 4. Effect of active fraction (F5) and gallic acid on fresh weight (FW) of selected weeds species (values are the mean of five replicates). Capped bars stand for standard errors. *, ** and *** represent significant difference at P< 0.05, 0.01 and 0.001.

F5 and gallic acid reduced seedling growth

We recorded the changes in seedling length (SL), fresh weight (FW) and dry weight (DW) as parameters for growth response to F5 and gallic acid treatments (Fig. 3, 4 and 5, respectively). F5 and gallic acid significantly inhibited all growth parameters and gallic acid was less phytotoxic. Percentage of reduction was species-specific. It was above 40% for most treated weed species and *A. hybridus, M. indicus, P. repens, E. colona, P. paradoxa* and *P. oleracea* showed the maximum inhibition (above 60%) for measured SL, FW and DW. These results agreed with Steenhagen and Zimdahl (1979), who indicated that extracts of stems, and leaves leafy spurge (*Euphorbia esula*) inhibited radicle elongation and germination of several test species. Additionally, gallic acid showed an inhibitory effect on the seedling growth of different weeds (Reigosa et al. 1999; Rudrappa and Bais 2008).

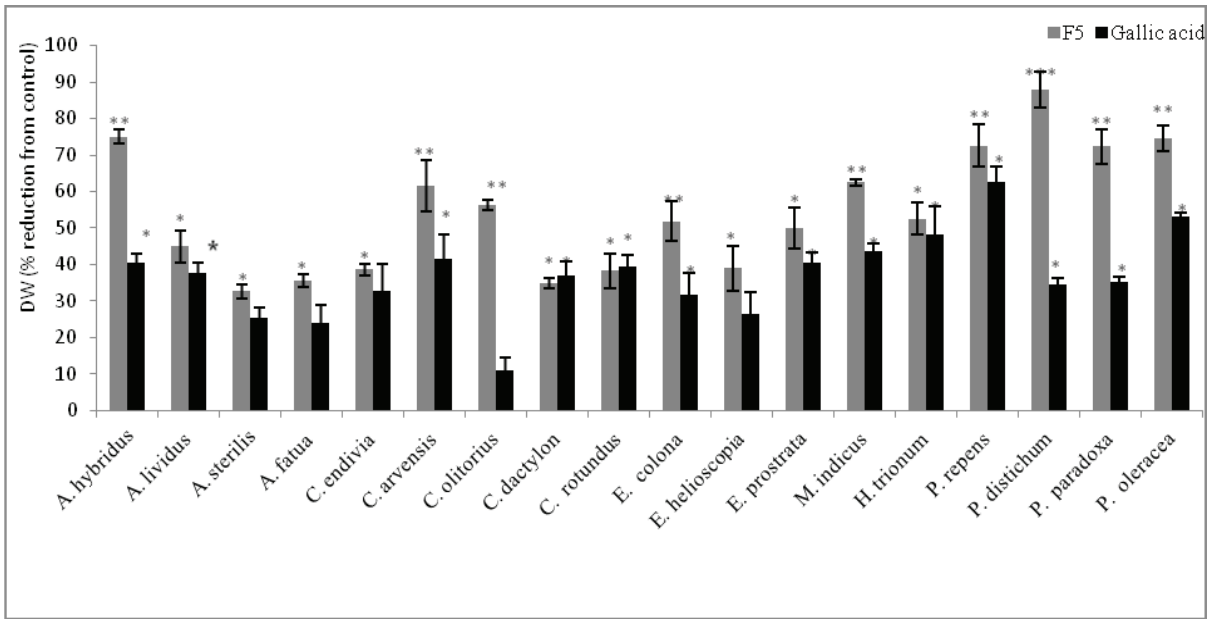

Figure 5. Effect of active fraction (F5) and gallic acid on dry weight (DW) of selected weeds species as % reduction from control (values are the mean of five replicates). Capped bars stand for standard errors. *, ** and *** represent significant difference at P< 0.05, 0.01 and 0.001.

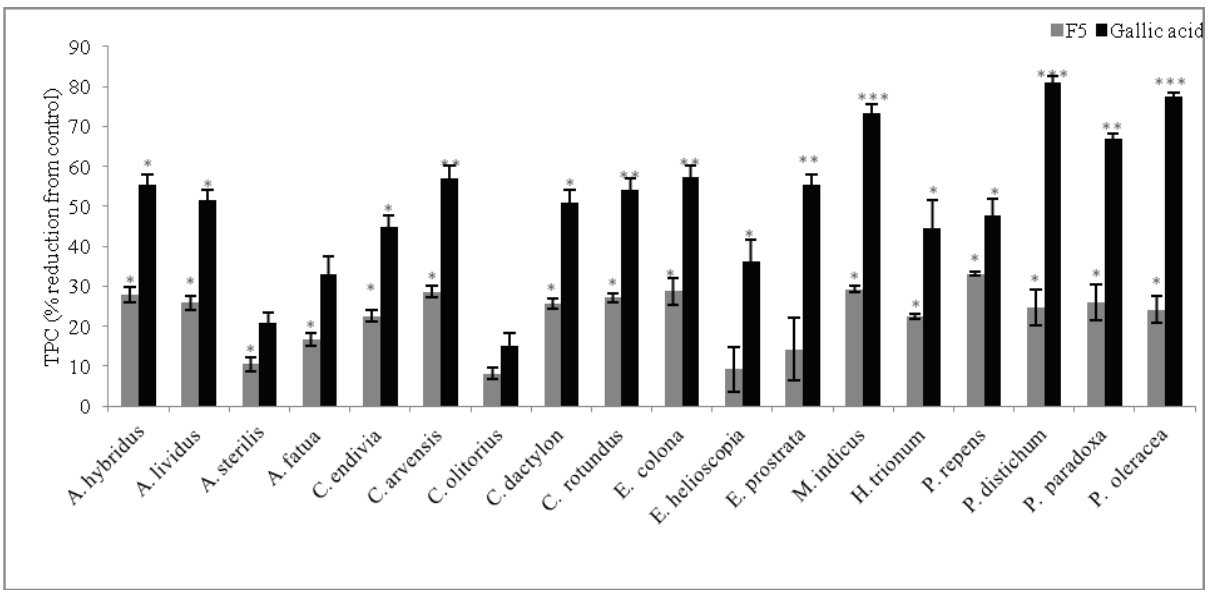

Figure 6. Effect of active fraction (F5) and gallic acid on total phenolic content (TPC) in shoot system of selected weeds (values are the mean of five replicates). Capped bars stand for standard errors. *, ** and *** represent significant difference at P< 0.05, 0.01 and 0.001.

F5 and gallic acid increased total phenolic content and induced lipid peroxidation

High accumulation of phenolic compounds was clearly recorded (Fig. 6), which could play a role as anti-oxidative defence mechanism. It is known, that phenolic compounds are related to antioxidant activity and play an important defense role in stabilizing lipid peroxidation (Hanasaki et al. 1994). Gallic acid treatment, which induced higher polyphenol levels than F5, decreased plant damage as shown by lower lipid

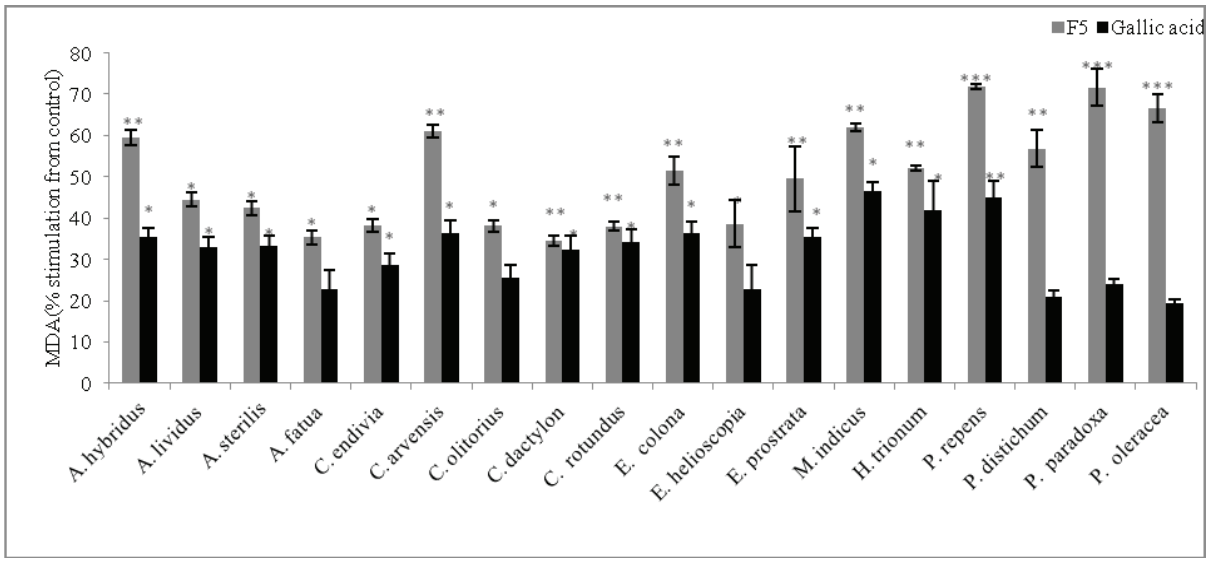

Figure 7. Effect of active fraction (F5) and gallic acid on lipid peroxidation (MDA) of selected weeds species (values are the mean of five replicates). Capped bars stand for standard errors. *, ** and *** represent significant difference at P< 0.05, 0.01 and 0.001.

peroxidation (MDA) (Fig. 7). It was clear that *P. distichum*, *P. paradoxa* and *P. oleracea* showed significant increases in polyphenols, which was accompanied by the lowest values of MDA (21, 24 and 19%, respectively) in comparison with respective controls. Although in most of the weeds investigated, the polyphenol content increased due to the treatment, but it was not enough to compensate the harmful effects of F5 and gallic acid that induced plant damage. However, phenolic acids (*e.g.,* gallic acid) at high levels suppressed the activity of antioxidant enzymes, which resulted in the stimulation of lipid peroxidation (Politycka 1996; Hegab 2005; Ghareib et al. 2010). Also, on applying high level of phenols they can convert to semiquinone radicals that donate electrons to molecular oxygen, forming superoxide anions (O^{2-}) and H_2O_2, which led to membrane system damage (Sakihama and Yamasaki 2002).

Table 1. HPLC analysis of free phenolic compounds in active fraction (F5) of *E. helioscopia.*

Standard phenolic compounds	Retention time [min.] Standard	Sample	Concentration [µg g^{-1} dry weight]
Resorcinol	13.731	13.970	28.47
Gallic acid	8.150	8.17	601.14
Protocatechuic acid	16.351	16.411	25.211
Caffeic acid	18.116	18.113	96.87
Vanillic acid	18.037	18.101	155.32
Coumarin	22.208	21.602	297.625
Kaempferol	24.853	24.984	79.645
Cinnamic acid	36.149	35.984	161.6
Apigenin	18.379	18.263	127.530
Total			1573.411

HPLC analyses

Twenty-five of highly purified phenolic compounds were used as standards and their retention times were detected at 254 nm. HPLC analyses identified eight phenolic compounds in our sample. By comparing their retention time with the standard compounds, they were found to belong different classes of phenolic compounds. In addition to gallic acid, we found some phenolic compounds *viz*, resorcinol, apigenin, kaempferol, coumarin, and protocatechuic, caffeic, vanillic, cinnamic acids (Table 1). Similarly, Yang et al. (2011) isolated and identified gallic, apigenin, kaempferol and protocatechuic acid in *Euphorbia lunulata.* Gallic acid concentration was markedly high representing 38.5% from the total content of the quantified free phenols. Also, vanillic and cinnamic acids, as well as coumarins and apigenin were found in high amounts as compared with the rest of identified phenols. Oppositely, protocatechuic acid and resorcinol had the lowest concentration (16 and 18%) from the total content of the quantified free phenols.

Conclusion

The results revealed that the phytotoxic fraction (F5) and its derived pure compound (gallic acid) at studied concentra-

tions might act as bio-herbicide, regarding to their activity to suppress the germination and growth of some target harmful weeds.

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