

## Herbicidal potential of *Alternaria citri* Ellis and Pierce metabolites against *Parthenium hysterophorus* L.

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### ABSTRACT

We tested the metabolites of fungal species *Alternaria citri* Ellis and Pierce for management of invasive weed parthenium (*Parthenium hysterophorus* L.). Fungal metabolites were prepared in two growth media viz., Malt extract broth (MEB) and Potato dextrose broth (PDB). In laboratory bioassays, metabolites from PDB proved more inhibitory to parthenium germination (92 %), shoot biomass (98 %) and root biomass (97 %) than the metabolites from MEB (43 %, 32 % and 78 % reduction, respectively). Therefore, the metabolites from PDB were selected for pot experiment. In pot culture, 2 concentrations of metabolites, original (X) and concentrated (2X) were sprayed thrice at 4 days intervals on 1-week, 2-week and 3-week old parthenium plants. One-week old plants were highly susceptible to metabolites spray and herbicidal activity of metabolites decreased with increase in age of parthenium plants. There was 66 %, 49 % and 31 % reduction in shoot dry biomass of parthenium due to concentrated metabolites spray on 1-week, 2-week and 3-week old plants, respectively. Fungal metabolites from PDB were fractionated using chloroform and analyzed by GC-MS and 13 compounds were identified. Predominant compounds were hexadecane (20.27 %), stearic acid hydrazine (12.9 %), phenol, 2,6-bis(1,1-dimethylethyl)- (11.98 %) and 10-methylnonadecane (11.05 %).

**Keywords:** *Alternaria citri*, fungal metabolites, GC-MS, germination, lab. bioassays, Malt extract broth (MEB), natural herbicides, parthenium, *Parthenium hysterophorus*, pot culture, Potato dextrose broth (PDB), seedling growth, weed.

### INTRODUCTION

*Parthenium hysterophorus* is an invasive weed plant native to Mexico or South America and now widely spread to different parts of Pakistan (7,8). It is found in cultivated fields, open woodlands, grasslands, river banks and pure patches with no other plant in its vicinity, thereby reducing the number of indigenous plants (22). It adversely affects the plants biodiversity, crop production, human health, animal husbandry and even degrades natural ecosystem (4). It also causes agricultural and ecological losses (15). People exposed to this plant develop severe allergic reactions, asthma, dermatitis, diarrhea, breathlessness, choking and hay fever (19). Its success is attributed to its fast growth, efficient use of resources, allelopathic effects and high reproduction potential (16).

The mechanical, chemical and biological measures are used to control it, but each has its own limitations (6). Amongst all these options, the chemical weed control is widely used (8,18), but the shoot dies and new sprouts grow from roots. Due to ill effects of synthetic agro-chemicals, there is need to develop eco-friendly methods of its control (2). Use of natural compounds from plants (10) and fungi (3,9) are ecofriendly methods of

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weed control. Numerous fungal species from *Aspergillus* Micheli, *Drechslera* S. Ito, *Fusarium* Link and *Trichoderma* Pers. possess herbicidal activity against parthenium weed (11,12).

*Alternaria citri* is a fungal pathogen that causes black rot disease in citrus during storage. It attacks on fruits of wide range of citrus varieties, causing inner black discoloration and maceration of fruit during storage (14). The brown to black spots on fruits are formed due to production of *A. citri* toxin (ACT). This toxin is active in tangerines, grapefruits and oranges (24). Metabolites of some species of *Alternaria* namely *Alternaria alternata* (Fr.) Keissl. and *Alternaria japonica* Yoshii also exhibit herbicidal potential against parthenium weed (13). However, reports regarding herbicidal activity of metabolites of *A. citri* against parthenium weed are lacking. Therefore, this study aimed to investigate the herbicidal activity of metabolites of *A. citri* against parthenium and identification of the compounds present in the fungal metabolites.

## MATERIALS AND METHODS

The studies were conducted in Punjab University, Lahore, Pakistan [31°15' - 31°45' N and 74°01' - 74°39' E, 217 m altitude]. Minimum and maximum temperatures during the study period ranged from 9-12 °C and 20-26 °C, respectively.

### Laboratory bioassay

An isolate of *A. citri* was obtained from Fungal Culture Bank of Pakistan (FCBP), University of Punjab, Lahore Pakistan. The fungal isolate was re-cultured on malt extract agar medium (MEA). To produce metabolites of *A. citri*, the fungus was incubated in malt extract and potato dextrose broths (2 % each). These media were poured separately into 250 mL glass flasks @ 100 mL in each flask and autoclaved. After autoclaving, these flasks were cooled down to room temperature and seeded with bits of cultures of *A. citri* under aseptic conditions using laminar air flow cabinet. The seeded flasks were kept in an incubator at 25 °C for 15 days. Thereafter, flasks were removed from incubator and metabolites were separated from fungal mycelia by filtration through double-layered muslin cloth and filter paper and centrifuged at 600 rpm for 5 min. After centrifugation, these metabolites were passed through Millipore filter for final purification. These metabolites were designated as original fungal metabolites (X). Lower concentration (½X) was prepared by adding distilled autoclaved water (12).

Twenty-five seeds of parthenium were sown on filter papers in sterilized glass Petri plates (9-cm dia) moistened with 5.0 mL of each concentration of metabolites. Each treatment was replicated four times in a completely randomized design. Plates were incubated at 12 h light and dark with 70 % relative humidity in an incubator. After 2 weeks, data of seed germination (%), shoot and root length and dry biomass were recorded.

### Pot culture

Pot assays were done in plastic pots (10-cm dia and 15 cm deep) and filled with 450 g sandy loam soil during February-March 2017. Seeds of parthenium were surface sterilized with 1 % sodium hypochlorite solution and 10 seeds were sown per pot on February 14, 2017 at 0.5 cm depth. After germination, pots were divided into 3 sets based on time of original (X) and concentrated (2X) metabolites spray (prepared in potato dextrose broth) as under:

**Set I** : First spray was done on 7 days old plants

**Set II** : Second spray was done on 14 days old plants.

**Set III**: Third spray was done on 21 days old plants.

Control pots were sprayed with distilled water. Each treatment was replicated five times in a completely randomized design. In each set of pots, 4 sprays were done at an interval of 4 days. Pots were watered as per requirement and harvested after 45 days of growth. To study the efficacy of fungal metabolites, data of plant height and dry biomass of root and shoot were recorded 45 days after sowing.

#### **GC-MS analysis**

The selected fungal metabolites were partitioned with chloroform fraction and then chloroform fraction was subjected to GC-MS analysis. It was passed through a nylon membrane filter of 0.22  $\mu\text{m}$  pore size with 47 mm in diameter, with the help of filtration assembly. The sample was analyzed on a GC-MS-QP 2010 chromatograph. The ionization voltage was 70eV, m/z scan range 55-950 Da, fitted out with a capillary column DB-5 (30 m  $\times$  0.25  $\mu\text{m}$ , film thickness 0.25  $\mu\text{m}$ ). The oven temperature was held at 45  $^{\circ}\text{C}$  for 1 min, then automated from 45–100  $^{\circ}\text{C}$  at a rate of 5  $^{\circ}\text{C min}^{-1}$ , held for 1 min, increased up to 200  $^{\circ}\text{C}$  at the rate of 10  $^{\circ}\text{C min}^{-1}$  and was kept at the final temperature for 5 min. Helium was used as a carrier gas. The injector and detector temperatures were 200  $^{\circ}\text{C}$  and 250  $^{\circ}\text{C}$ , respectively. The percentage composition of volatile compounds was computed from GC peak areas. Qualitative analysis was based on comparison of retention time, indices and mass spectra with the corresponding data given in literature (NIST Library 2010 ).

#### **Statistical analysis**

All the data were analyzed by analysis of variance followed by application of LSD test at 5 % level of significance for separation of treatment means, using Statistix 8.1 software.

## **RESULTS AND DISCUSSION**

#### **Laboratory bioassays**

The fungal metabolites prepared in either of two-growth media significantly reduced the germination, shoot and root growth of parthenium (Figure 1 and 2). However, the fungal metabolites from potato dextrose broth (PDB) were more inhibitory to parthenium germination and growth than the metabolites from malt extract broth (MEB). There was 92 %, 98 % and 97 % reduction in germination, shoot biomass and root biomass of parthenium due to metabolites from PDB as compared to 43 %, 32 % and 78 % reduction, respectively, from metabolites from MEB. Metabolites from PDB were selected for pot experiment owing to their better herbicidal activity against germination as well as shoot and root growth of parthenium. In a similar study, Javaid *et al.* (13) also reported better herbicidal activity of metabolites of *A. japonica* in PDB than in MEB against parthenium weed. Likewise, there are reports that metabolites of other fungal species namely *Drechslera australiensis* and *Trichoderma* spp. reduced germination and arrested seedling growth of parthenium and other weeds such as *Rumex dentatus* in laboratory bioassays (2,3,12).

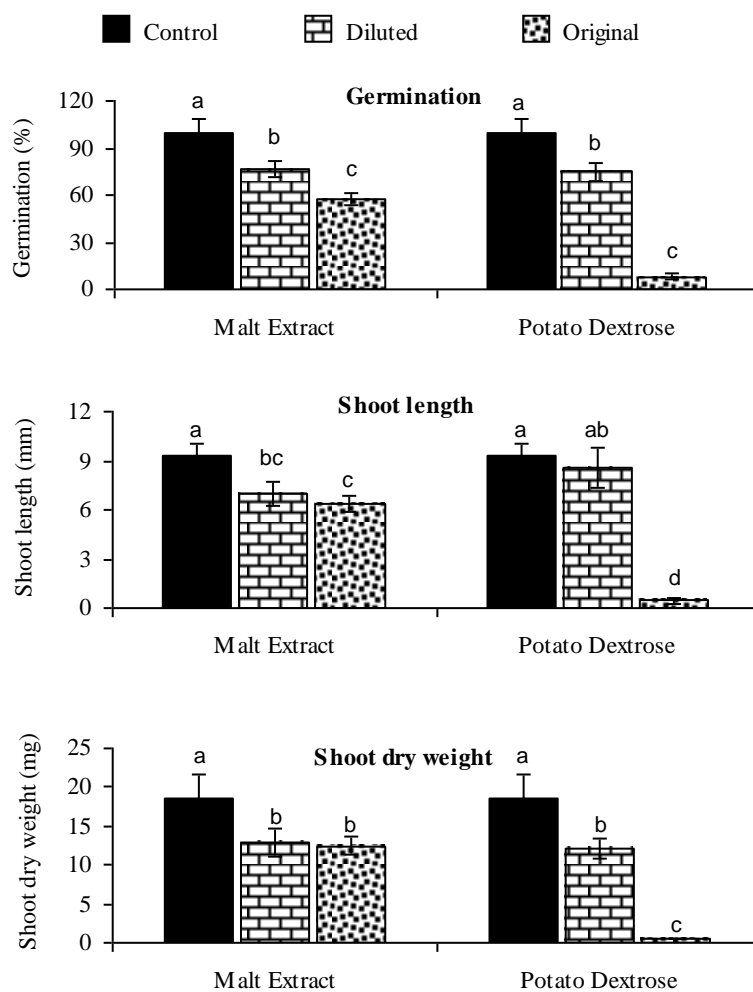


Figure 1. Effects of original (X) and diluted (1/2X) metabolites of *Alternaria citri*, prepared in malt extract broth and potato dextrose broth, on germination and shoot growth of parthenium seedlings in laboratory bioassays. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ( $P \leq 0.05$ ) as determined by LSD Test.

### Pot culture

Metabolites from PDB were selected for pot experiment due to their better herbicidal activity against germination as well as against shoot and root growth of parthenium. In pot culture, parthenium plants of different ages (1-, 2- and 3-weeks old plants) were sprayed with original and concentrated fungal metabolites. There were marked variations in the effects of fungal metabolites on plants of different ages. One-week old plants were most susceptible to fungal metabolites spray than older plants. The

concentrated metabolites sprayed on 1-week, 2-weeks and 3-weeks old parthenium plants caused 66 %, 49 % and 31 % reduction in shoot dry biomass, respectively. Likewise, there was 75 %, 49 % and 24 % suppression in root dry biomass due to spray with concentrated metabolites on 1-week, 2-weeks and 3-weeks old plants, respectively (Figure 3). These results are in agreement with findings of earlier studies, where herbicidal activity of fungal metabolites was reduced with age of parthenium weed. Original metabolites of *Alternaria japonica* reduced the shoot biomass of 1-, 2- and 3-weeks old parthenium plants by 40 %, 31 % and 17 %, respectively (13). It clearly indicated that as the parthenium plants mature, they become resistant to fungal metabolites because these metabolites contain very low concentrations of active herbicidal constituents. It is likely, if concentrations of these active herbicidal constituents is increased, their efficacy against the older parthenium plants may be increased.

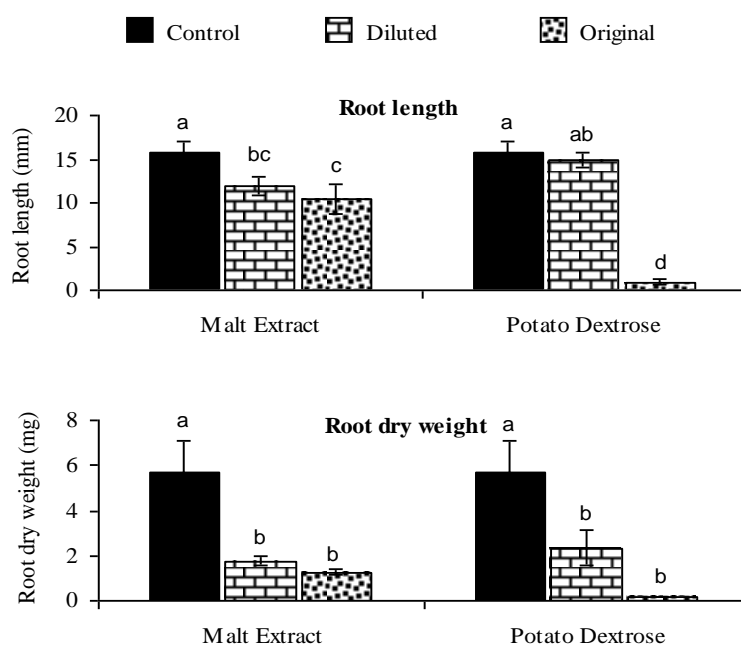


Figure 2. Effects of original (X) and diluted ( $\frac{1}{2}X$ ) metabolites of *Alternaria citri*, prepared in malt extract broth and potato dextrose broth, on root growth of parthenium seedlings in laboratory bioassays. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ( $P \leq 0.05$ ) as determined by LSD Test.

### GC-MS analysis

GC-MS analysis showed that there were 13 compounds in chloroform fraction of the fungal metabolites prepared in PDB. Among these, hexadecane was the most abundant compound followed by stearic acid hydrazine; phenol, 2,6-bis(1,1-dimethylethyl)- and 10-methylnonadecane with peak areas of 20.27 %, 12.9 %, 11.98 % and 11.05 %, respectively.

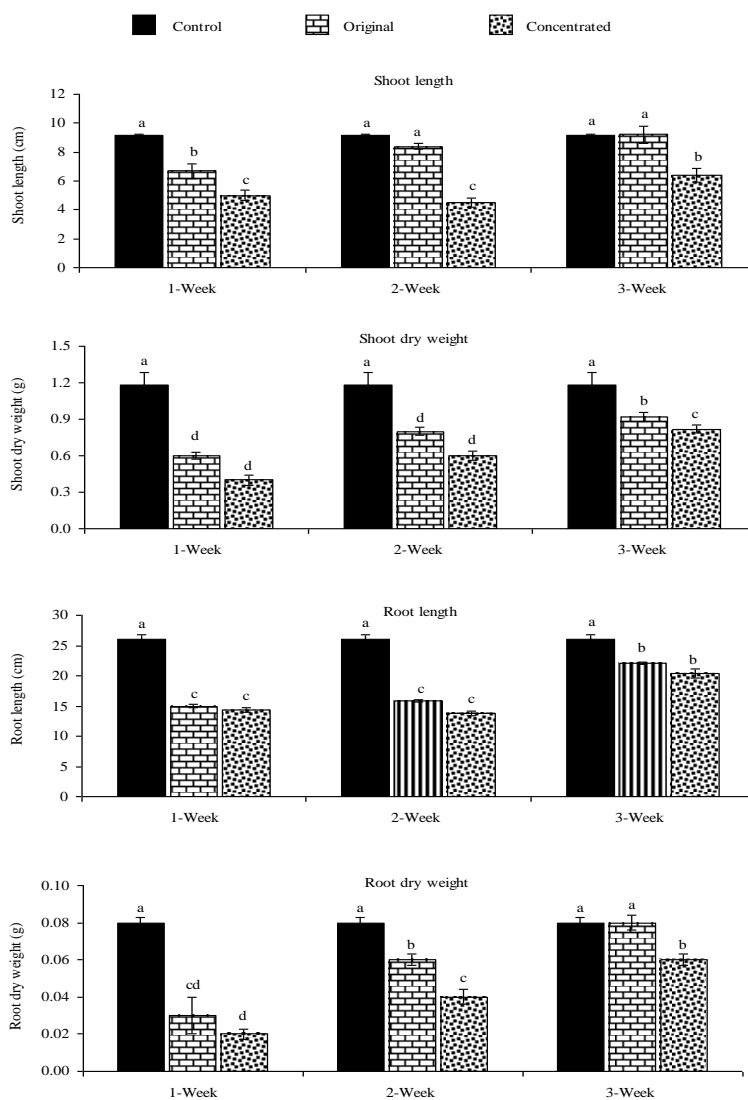


Figure 3. Effects of original and concentrated metabolites of *Alternaria citri* prepared in potato dextrose broth on shoot and root growth of parthenium in pot trial. Vertical bars show standard errors of means of five replicates. Values with different letters at their top show significant difference ( $P \leq 0.05$ ) as determined by LSD Test.

Other important identified compounds namely octadecane (7.37 %); 16-hexadecanoyl hydrazide (5.52 %); heptacosane (5.06 %); 1,2-hydrazinecarboxamide (5.45 %); octane, 2,4,6-trimethyl- (4.60 %); *p*-xylene (4.60 %); 1,2- hydrazinedicarboxamide (4.14 %); undecane, 3,8-dimethyl- (3.68 %) and hydrazinecarboxamide, N-phenyl (2.30 %) were present in moderate concentrations (Table 1).

Table 1. GC-MS characterization of chloroform fraction of *Alternaria citri*.

Comp. No.	Retention time (min)	Names of compounds	Molecular formula	Molecular weight	Peak area (%)
1	5.317	1,2- Hydrazinedicarboxamide	C <sub>2</sub> H <sub>6</sub> N <sub>4</sub> O <sub>2</sub>	118	4.14
2	5.608	<i>p</i> -Xylene	C <sub>8</sub> H <sub>10</sub>	106	4.60
3	6.422	Hydrazinecarboxamide, N-phenyl	C <sub>7</sub> H <sub>9</sub> N <sub>3</sub> O	151	2.30
4	40.167	Octane, 2,4,6-trimethyl-	C <sub>11</sub> H <sub>24</sub>	156	4.60
5	45.300	1,2-Hydrazinecarboxamide	C <sub>2</sub> H <sub>6</sub> N <sub>4</sub> O <sub>2</sub>	118	5.06
6	46.983	Undecane, 3,8-dimethyl-	C <sub>13</sub> H <sub>28</sub>	184	3.68
7	47.575	Stearic acid hydrazine	C <sub>18</sub> H <sub>38</sub> N <sub>2</sub> O	298	12.9
8	47.875	Octadecane	C <sub>18</sub> H <sub>38</sub>	254	7.37
9	48.500	16-Hexadecanoyl hydrazide	C <sub>16</sub> H <sub>34</sub> N <sub>2</sub> O	270	5.52
10	49.433	Heptacosane	C <sub>27</sub> H <sub>56</sub>	380	6.45
11	50.483	10-Methylnonadecane	C <sub>20</sub> H <sub>42</sub>	282	11.05
12	51.067	Hexadecane	C <sub>16</sub> H <sub>34</sub>	226	20.27
13	41.042	Phenol, 2,6-bis(1,1-dimethylethyl)-	C <sub>14</sub> H <sub>22</sub> O	206	11.98

Literature showed that compounds namely hexadecane; octadecane; heptacosane and undecane, 3,8-dimethyl- possess herbicidal and phytotoxic activities and the herbicidal activity of metabolites of *A. citri* prepared in PDB might be due to these compounds. Hexadecane present in the highest concentration has also been reported in *Peumus boldus* and *Trianthema portulacastrum* weeds with potent herbicidal and phytotoxic properties against the annual weed *Portulaca oleracea* (5). This compound was present in *T. portulacastrum* aerial parts extracts that were herbicidal against *Cenchrus echinatus*, *Brassica tournefortii* and *Lactuca sativa* (1). Similarly, octadecane was previously isolated from *Pereskia grandifolia*, which exhibited the phytotoxic activity against *Phalaris canariensis*, *Sinapis arvensis* and *Raphanus sativus* (23). Heptacosane was identified in *Geranium koreanum* and *Achillea vermicularis* and was tested against two different weed species namely *Portulaca oleracea* and *Amaranthus viridis* (20,21). The tested compound significantly inhibited the seed germination and seedling growth of both these species. Likewise, undecane, 3,8-dimethyl- was isolated from aqueous extract of *Nicotiana plumbaginifolia* and showed herbicidal potential against *Cassia tora* (17).

## CONCLUSIONS

Metabolites of *A. citri* from the potato dextrose broth exhibited herbicidal potential against germination and growth of parthenium weed. The herbicidal activity might be due to the presence of undecane, 3,8-dimethyl-; octadecane; heptacosane and hexadecane in the metabolites as these compounds possess phytotoxic and herbicidal activities. Further studies are needed to isolate the active herbicidal constituents for preparation of natural product based herbicides to control parthenium weed.

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## DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We have not excluded any author that substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

## CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

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