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Table: 4, Figs : 7

Isolation of herbicidal compounds from *Melia azedarach* L. to control *Rumex dentatus* L. in wheat

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ABSTRACT

In field studies, we evaluated the herbicidal effects of *Melia azedarach* L. leaf extract against a weed *Rumex dentatus* L. and wheat (*Triticum aestivum* L.) crop. We isolated and identified a bioactive constituent in the methanolic extract of *M. azedarach* leaves with column chromatography and Gas Chromatography Mass Spectrometry (GC-MS). The leaf extract of *M. azedarach* at 10 % (w/v) concentration significantly reduced the *R. dentatus* weed biomass up to 65.24 % and subsequently increased the wheat yield by 41.4 %. While, the leaf extract of *M. azedarach* at 10 % concentration + Bromoxynil (70 g a.i.) + MCPA (70 g a.i) significantly reduced the *R. dentatus* weed biomass up to 96.77 %, with an increase of 67.6 % in wheat yield. In lab. study, 11 purified fractions (1, 2, 3, …, 11) from methanolic leaf extract of *M. azedarach* were tested for their herbicidal activity on leaf discs of *R. dentatus*. Fraction 7 showed the highest herbicidal activity and was identified as α-selinene through GC-MS analysis. It showed herbicidal activity against *R. dentatus* at 0.5 mg mL^{-1} concentration, but was not harmful to wheat. In lab bioassays, 2,4-D [2,4-dichlorophenoxyacetic acid] was used a reference compound that caused necrosis in *R. dentatus* at a concentration of 0.25 mg mL^{-1}. This study concluded that the α-selinene identified from *M. azedarach* may be developed as natural herbicide to avoid the harmful effects of synthetic herbicides. Besides, structure of α-selinene can be used as an analogue to synthesize commercial herbicides. To further improve the herbicidal activity of this compound, its derivatives need to be investigated.

Keywords: α-selinene, GC-MS, herbicidal compound, 2,4-D herbicide, *Melia azedarach*, *Rumex dentatus*, toothed dock, weed, wheat

INTRODUCTION

Toothed dock (*Rumex dentatus* L., Family: Polygonaceae), is a weed native of Eurasia and North Africa and has become major weed of wheat in Pakistan (Figure 1), hence, it was selected as target/recipient plant. Synthetic herbicides are widely used for its control but these cause numerous ill effects. So, there is urgent need for herbicidal compounds from natural resources. Weeds interfere in the normal growth of crops, hence, these are controlled by mechanical, biological, or chemical methods (17,31,32). Chemical methods i.e., herbicides are the most reliable to control weeds (25), but they cause harmful side effects viz., (i). health problems in human beings (12), (ii). toxicity in plants (9) and (iii). development of herbicide resistance in many weeds (8,33). So, there is need to replace the chemical method with eco-friendly weed control strategies (1,16,27).

M. azedarach (Meliaceae) is the prime source of medication since ancient times and has been used to treat different diseases (Figure 2). However, few studies have determined its effects on seed germination and plant growth. It's extracts decreased the seed germination and early growth of *Lactuca sativa* L. seedlings under laboratory conditions (21).

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Figure 1. (A): *Rumex dentatus* single plant and (B): *R. dentatus* population in wheat field

Figure 2. (A): *Melia azedarach* leaves and (B): tree.

Many plants, fungi and microbes are sources of herbicidal compounds (6,18). For example, phenolics and terpenoids from sunflower var. Suncross-42 are herbicidal to *Chenopodium album* L. and *R. dentatus* (4). This variety of sunflower contained herbicidal compound, annuionone, which inhibited the growth of *C. album*, *R. dentatus, Coronopis didymus* (L.) Sm., *Medicago polymorpha* L. and *Phalaris minor* Retz. (3). Moreover, extracts of certain plant species (*Bidens pilosa* L. and *Tephrosia candida* DC.) have been recommended for use as natural herbicides. These plant species controlled 80 % weeds and increased rice yield up to 20 % (13). Other studies reported herbicidal activity of extracts of *Carum carvi* L. seeds due to presence of cavone and limonene (24). According to Kordali *et al*. (20), *n*-hexane extract of *Nepeta meyeri* Benth. contained high concentration (83.7 %) of oxygenated monoterpene 4aα, 7α, 7aβ-nepetalactone, which was herbicidal to *Amaranthus retroflexus* L., *C. album*, *Cirsium arvense* (L.) Scop. and *Sinapis arvensis* L. weeds. The GC-MS analysis of *Syzygium aromaticum* L. extracts showed the presence of sesquiterpene hydrocarbons, phenolic compounds, oxygenated sesquiterpenes and oxygenated monoterpenes. These classes of compounds showed phytotoxic activity against

Mimosa pudica L. and *Senna obtusifolia* (L.) Irwin & Barneby (7). Under laboratory conditions lucerne (*Medicago sativa* L.) inhibited the growth of annual ryegrass (*Lolium rigidum* Gaud.) (40). However, assessment of herbicidal activity of *M. azedarach* under field conditions and isolation of herbicidal constituents of *M. azedarach* has not been reported. This study aimed (i). to determine the herbicidal activity of *M. azedarach* extracts against *R. dentatus* under field conditions, and (ii). to isolate and identify herbicidal compounds present in *M. azedarach* extracts using chromatographic and spectroscopic techniques.

MATERIALS AND METHODS

FIELD STUDY

Field experiments were conducted during 2012-2013 and 2013-2014 at Qasur, Pakistan (31.1179° N, 74.4408° E), altitude: 218 m, annual rainfall: 432 mm. It has semi-arid climate with mean maximum and minimum temperatures as 45 °C and 27 °C, respectively. All agronomical practices recommended by Agricultural Department were followed from field preparation till harvesting. Composite soil sample was taken from the experimental area before the start of experiment. Soil was sandy loam, pH 7.6, medium fertility [available potassium (44 mg kg^{-1}), EC (2.5 $mScm^{-1}$), organic matter (0.89 %) and available phosphorous (4.7 mg kg^{-1})]. Experiments were laid out in randomized complete block design with three replications. Each plot measured 1.54 m × 1.54 m. Nitrogen (N) at 160 kg ha^{-1}, P_2O_5 at 110 kg ha^{-1} and K_2O at 60 kg ha^{-1} were applied. Full doses of P_2O_5 and K_2O, and half-dose of N were applied as basal, while the remaining half N was top-dressed at wheat flowering. The details of experimental treatments are given below in Table 1.

Table 1. Details of treatments used in field experiments

Treatments	Treatments details
T_1	Weed free
T_2	Weedy check
T_3	*Melia azedarach* extract Conc. 10 %
T_4	*M. azedarach* extract Conc. 5 %
T_5	Bromoxynil + MCPA herbicide (Full dose @ 560 g a.i.)
T_6	Bromoxynil + MCPA herbicide (Half dose @ 280 g a.i.)
T_7	Bromoxynil + MCPA herbicide (Quarter dose @ 140 g a.i.)
T_8	Bromoxynil + MCPA herbicide (Quarter dose) + *M. azedarach* extract 10 %
T_9	Bromoxynil + MCPA herbicide (Quarter dose) + *M. azedarach* extract 5 %

a.i.: Active Ingredient

Test weed and wheat: The test plants were *R. dentatus* weed and wheat crop. The seeds of test weed *R. dentatus* were collected at maturity from wheat fields and sun-dried for one week. The test wheat variety was Sehar-2006. Wheat was sown in November in rows 22 cm apart by hand plough. After first irrigation, many weeds germinated in field. Only plants of *R. dentatus* were allowed to grow in experiment plots keeping the ratio of wheat plant to *R. dentatus* as 1:1. Weeds other than *R. dentatus* were removed by hand weeding.

Foliar sprays

For field assays, *M. azedarach* leaf extract (100 g L^{-1}) was prepared in distilled water. For preparation of this original concentration (10 %) of the leaf extract, healthy leaves of *M. azedarach* were collected, washed thoroughly and sun-dried. Thereafter, these leaves were completely dried in an electric oven at 35 °C. Dried leaves (1 kg) were soaked in sterilized distilled water (10 L) for 24 h and then passed through a cheese cloth. The extract was then passed through a filter paper to remove all the residues. It was further diluted with distilled water to get lower concentration of 5 %. First spray with leaf extract of *M. azedarach* was done on *R. dentatus* at 3-4 leaves stage and 2 subsequent sprays were done at 7-days interval. All sprays were done in the evening. The extract was sprayed at 100 L ha^{-1}. On the other hand, single spray of herbicide Bromoxynil + MCPA 200/200EC alone or a 1:1 mixture of herbicide and *M. azedarach* extract was done. Both wheat and weed (*R. dentatus*) were harvested at maturity (May). After drying of wheat and weed, various parameters were recorded viz., weed biomass, and wheat plants height, number of tillers, total dry biomass, grain yield and 1000 grains weight.

LABORATORY BIOASSAYS

Extract preparation: Leaves of *M. azedarach* were collected from healthy plants in March. The leaves were washed under tap water to remove impurities, sun-dried and ground to fine powder. One hundred g of leaves dry powder was extracted in 1 L *n*-hexane for 24 h at 25 °C. The contents were filtered using filter paper. This *n*-hexane filtrate was evaporated at 45 °C using the rotary evaporator (Model: Laborata 4000/Gl, Heildoph Germany) to get the concentrated extract of *n*-hexane (CE-*n*-hexane). We obtained 0.9 g CE-*n*-hexane 100 g^{-1} *M. azedarach* dry leaf powder. Residues left after extraction with *n*-hexane was air-dried at room temperature to remove traces of *n*-hexane. It was soaked in methanol in glass jar @ 100 g L^{-1}, stirred with glass rod and left overnight. It was first filtered through 4 layers of cheese cloth and then by filter paper (Whatman™ filter paper No. 1, pore size: 11 µm). The extract was centrifuged at 1000 rpm for 10 min and its precipitates were discarded. The extract obtained after filtration was dried under vacuum in a rotary evaporator at 45 °C. The extract obtained was regarded as concentrated methanolic extract (CME) and was 2.7 g 100 g^{-1} of *M. azedarach* dry leaf powder. All the solvents used in extraction were of analytical grade.

Bioassays: For laboratory bioassays, two types of solutions were used viz., (i). DMSO (dimethyl sulfoxide) alone and (ii). DMSO + concentrated extracts. To prepare stock solution of 4 mg mL^{-1} concentration, 4 mg of each concentrated extract (CE-*n*-haxane and CME) was dissolved separately in 100 µL DMSO. The final volume was made to 1 mL with autoclaved dH_2O. This stock solution was diluted to make lower concentrations of 0.0625, 0.125, 0.25, 0.5, 1 and 2 mg mL^{-1}. Young leaves of 25 days old *R. dentatus* plants grown in plastic pots were detached and used in these bioassays. Leaf discs of 1 cm dia were cut with the help of a cork borer and placed on the surface of glass slides. These glass slides were placed in Petri dishes lined with moistened filter papers. Droplets of 20 µL of each concentration were applied on the surface of *R. dentatus* leaf discs. Ten leaf discs of *R. dentatus* were used to determine the bioactivity for each concentration of the extract. Leaf discs were treated with 20 µL of DMSO as control solution at its highest concentration (100 µL mL^{-1} of distilled water), while its lower concentrations viz., 1.560,

3.125, 6.250, 12, 25 and 50 µL mL^{-1} were made with distilled water. These discs were incubated in incubator at 25 °C for 72 h, under fluorescent light and relative humidity of 60 %. Laboratory bioassays were done in completely randomized design. Ten discs of test plant were used to assess the bioactivity of each concentration. There were three replicates of each treatment. Symptoms of leaf colour and necrotic spots were observed by colour scale = 0-3 and necrotic spot scale = 4-10 (2).

Isolation and purification of compounds

Concentrated CME (5 mg) of *M. azedarach* was dissolved in 1 mL of HPLC grade methanol to prepare solution to isolate different fractions. Thin layer chromatography (TLC) (Kieselgel 60, F254, 0.25 and 0.5 mm, respectively) (Merck) was done to optimize the separation conditions. A three-solvent system (chloroform: isopropanol: acetonitrile in ratio of 10:30:60, respectively) was found the most suitable to separate maximum number of compounds. After optimization, column chromatography (Merck, Kieselgel 60, 0.063-0.200 mm) was done to isolate and purify the fractions. The solvent system was same as used in TLC. Fractions obtained were analyzed for their purity on TLC. Fractions having similar R_f values were pooled together, while impure fractions were purified again by column chromatography. This solvent system yielded 11 chromatographic fractions (CFrs): Retention factor (R_f) of each fraction (CFr) was calculated as under:

$$\mathrm{Rf} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Bioassays with chromatographic fractions

Bioassays with chromatographic fractions (CFrs) were done on leaves of *R. dentatus* and wheat. Two mg of each CFrs were dissolved in 50 µL of DMSO and the final volume was raised to 1 mL with autoclaved dH_2O to prepare stock solution of 2 mg mL^{-1} concentration. The lower concentrations (0.0312, 0.625, 0.125, 0.25, 0.5 and 1 mg mL^{-1}) were made by adding dH_2O. The commercial herbicide 2,4-D was used to compare its efficacy with isolated chromatographic fractions. Treatments for both 2,4-D and CFrs included 0.0312, 0.625, 0.125, 0.25, 0.5, 1 and 2 mg mL^{-1} concentrations. Laboratory bioassays were done in a completely randomized design and 10 discs were used for each concentration with three replications.

GC-MS of bioactive chromatographic fraction (CFr)

The biochemical constituents of bioactive fractions obtained by column chromatography of methanolic extract of *M. azedarach* were analysed by GC-MS. The GC-MS conditions were the same as previously published. Briefly, Helium gas used was 99.99 % pure, with constant flow rate at 1 mL min^{-1}. Injection volume was 2 µL. The injector temperature was 240 °C. Oven temperature was programmed first from 60 °C for 2 min with increase of 5 °C min^{-1} to 80 °C, then 10 °C min^{-1} to 310 °C for 5 min (39).

Statistical analyses

Symptoms regarding colour change as well as induction of necrotic spots were recorded following details mentioned in section describing bioassays with extracts. The field experiments data were analysed by ANOVA and Tukey's Test at 5 % significance level using computer software Minitab 17.

RESULTS AND DISCUSSION

FIELD STUDY

Weed growth: The *M. azedarach* extract at 5 and 10 % conc. significantly reduced the weed biomass of *R. dentatus* by 22.3 % and 65.24 % respectively. The commercial herbicide Bromoxynil + MCPA spray at full and half dose completely controlled the weed. The *M. azedarach* extract spray at 5 and 10 % conc. with quarter dose of Bromoxynil + MCPA (2-methyl-4-chlorophenoxyacetic acid) herbicide significantly reduced the weed biomass of *R. dentatus* by 82.7 % and 96.77 %, respectively. While quarter dose of Bromoxynil + MCPA spray decreased the weed biomass by 65.93 % (Figure 3). In previous research, culture filtrates of *Drechslera australiensis* and *Drechslera hawaiiensis* exhibited the herbicidal activity under field conditions and caused 58 % and 57 % reduction in weed biomass of *R. dentatus*, respectively (1). Ullah *et al.* (35) did field study to control weeds of wheat by aqueous extracts of allelopathic plants. Extracts of *Sorghum halepense*, *Parthenium hysterophorus* and *Helianthus annuus* significantly reduced weed density by 8 to 17 % and increased grain yield of wheat. Likewise when allelopathic crop, *Sorghum bicolor* was used as cover crop, it reduced weed density by 75 % in the subsequent barley crop that resulted in a significant increase in yield of barley (36).

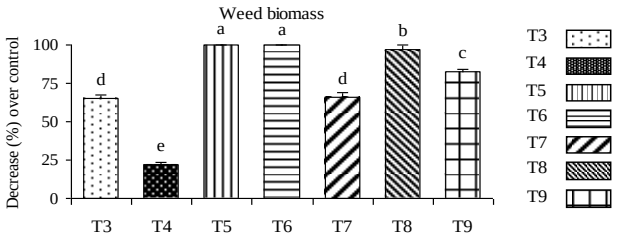

Figure 3. Inhibitory effects of *Melia azedarach* and Bromoxynil + MCPA herbicide on *Rumex dentatus* weed biomass in field study. T_3: *Melia azedarach* extract Conc. 10 %; T_4: *M. azedarach* extract Conc. 5 %; T_5: Bromoxynil + MCPA herbicide (Full dose @ 560 g a.i.); T_6: Bromoxynil + MCPA herbicide (Half dose @ 280 g a.i.); T_7: Bromoxynil + MCPA herbicide (Quarter dose @ 140 g a.i.); T_8: Bromoxynil + MCPA herbicide (Quarter dose) + *M. azedarach* extract 10 %; T_9: Bromoxynil + MCPA herbicide (Quarter dose) + *M. azedarach* extract 5 %.

Wheat growth: During the 1st and 2nd year of field experiments, *M. azedarach* extracts spray significantly increased the growth (plant height, tillers/plant, dry biomass) and yield attributes (grain yield and 1000 grains weight) of wheat, over weedy check. 10 % extract of *M. azedarach* significantly increased the wheat plant height, tillers/plant and dry biomass by 6.9 %, 11.1 % and 19.2 %, respectively. While the increase with 5 % extract of *M. azedarach* in plant height, tillers/plant, dry biomass was 1.78 %, 11.1 % and 5.5 %, respectively. Similarly, 10 % extract of *M. azedarach* significantly increased the wheat yield attributes (grain yield and 1000-grains weight) by 41.4 % and 27.6 %. On the other hand, 5 % extract of *M. azedarach* increased these yield attributes by 15 %, 7.8 %, respectively. 10% extract of *M. azedarach* + quarter dose of Bromoxynil + MCPA,

although did not completely control the *R. dentatus* but significantly decreased its growth. Consequently, there was 17.5 %, 22.2 %, 33.5 %, 67.6 % and 41.5 % increase in plant height, number of tillers, dry biomass, grain yield and 1000-grains weight of wheat than weedy check (Figure 4 and 5).

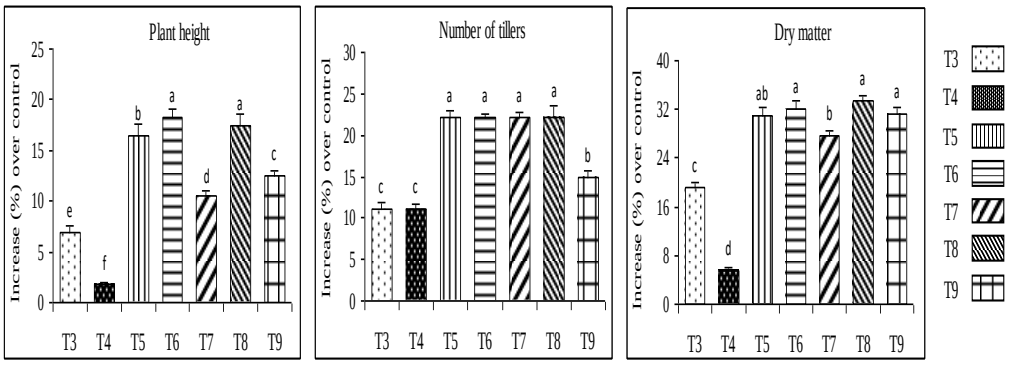

Figure 4. Effects of *Melia azedarach* and Bromoxynil + MCPA herbicide on wheat growth in field study. T_3: *Melia azedarach* extract Conc. 10 %; T_4: *M. azedarach* extract Conc. 5 %; T_5: Bromoxynil + MCPA herbicide (Full dose @ 560 g a.i.); T_6: Bromoxynil + MCPA herbicide (Half dose @ 280 g a.i.); T_7: Bromoxynil + MCPA herbicide (Quarter dose @ 140 g a.i.); T_8: Bromoxynil + MCPA herbicide (Quarter dose) + *M. azedarach* extract 10 %; T_9: Bromoxynil + MCPA herbicide (Quarter dose) + *M. azedarach* extract 5 %.

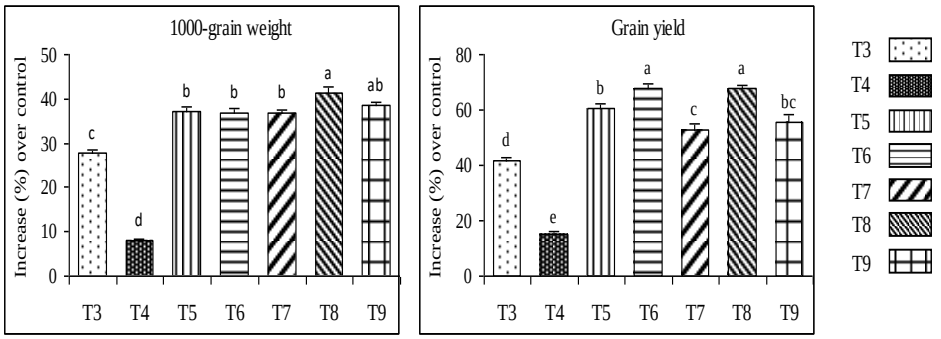

Figure 5. Effects of *Melia azedarach* and Bromoxynil + MCPA herbicide on yield attributes of wheat. T_3: *Melia azedarach* extract Conc. 10 %; T_4: *M. azedarach* extract Conc. 5 %; T_5: Bromoxynil + MCPA herbicide (Full dose @ 560 g a.i.); T_6: Bromoxynil + MCPA herbicide (Half dose @ 280 g a.i.); T_7: Bromoxynil + MCPA herbicide (Quarter dose @ 140 g a.i.); T_8: Bromoxynil + MCPA herbicide (Quarter dose) + *M. azedarach* extract 10 %; T_9: Bromoxynil + herbicide MCPA (Quarter dose) + *M. azedarach* extract 5 %.

LABORATORY BIOASSAYS

Effects of extracts on leaf discs of *R. dentatus*

The applied concentrated methanolic extract (CME) at 4 mg mL^{-1} concentration caused necrosis on leaf surface of *R. dentatus*. CME at 0.5 mg mL^{-1} extract concentration

also induced moderate discolouration of *R. dentatus* leaf discs, but the C-*n*-hexane extract was not necrotic even at the highest concentration (Table 2). In a similar research in paper disk assay, the extract of *M. azedarach* inhibited the seed germination and seedling growth of *Avena sativa*, *Brassica napus*, *Chenopodium album*, *Lactuca sativa* and *Sorghum halepense* (28). In the same experiment, 10 % (w/w) of *M. azedarach* crushed material used as soil amendment completely inhibited the germination of *A. sativa* and *S. halepense* (28). In another study, water extracts of *Galactia pendula*, *Leucaena glauca* and *M. azedarach* markedly suppressed the germination and seedling growth of radish (14).

Table 2. Effects of DMSO, concentrated *n*-hexane extract (C-*n*-hexane) and concentrated methanolic extract (CME) of *M. azedarach* on leaf surface colour and necrotic spots of *R. dentatus* in lab. bioassay.

DMSO conc. ($\mu L\ mL^{-1}$)	DMSO		Extract conc. ($mg\ mL^{-1}$)	C-*n*-hexane		CME	
	Colour	Necrotic Spot		Colour	Necrotic Spot	Colour	Necrotic Spot
Water	0	4	Water	0	4	0	4
1.560	0	4	0.0625	0-1	4	0-1	4
3.125	0	4	0.1250	0-1	4	1	4
6.250	0-1	4	0.2500	0-1	4	1	4
12.500	0-1	4	0.5000	0-1	4	1-2	4
25.000	0-1	4	1.0000	0-1	4	2	4
50.000	0-1	4	2.0000	0-1	4	2	4
100.000	0-1	4	4.0000	1	4	2	5

DMSO: Dimethyl sulfoxide, **CME:** Concentrated methanolic extract, Colour Scale: **0**= No change, **1**= Light change, **2**= Moderate change, **3**= Severe change. Necrotic spot (NS) Scale, **4**=No NS, **5** = NS≤ 1 mm, **6** = NS≤ 2 > 1 mm, **7** = NS≤ 3 > 2 mm, **8** = NS≤ 4 > 3 mm, **9** = NS≤ 5 > 4 mm

Chromatographic fractions

TLC revealed 11 fractions in the concentrated methanolic extract (CME) of *M. azedarach*. The retention factor (R_f) of each chromatographic fraction (CFr) followed the order: **1** (R_f 0.13) 33 mg >, **2** (R_f 0.15) 66 mg >, **3** (R_f 0.21) 77 mg >, **4** (R_f 0.25) 15 mg >, **5** (R_f 0.28) 30 mg >, **6** (R_f 0.33) 24.3 mg >, **7** (R_f 0.36) 14.6 mg >, **8** (R_f 0.43) 17 mg >, **9** (R_f 0.57) 13.5 mg >, **10** (R_f 0.78) 17.9 mg > and **11** (R_f 0.86) 10 mg.

(I). Effects of chromatographic fraction 7 from *M. azedarach* on *R. dentatus* and wheat

In laboratory bioassays, CME showed more pronounced herbicidal activity than C-*n*-hexane on leaf discs of *R. dentatus.* Therefore, CME was selected for further experimentation. Through TLC, its different fractions were purified and their different concentrations were applied on leaf discs of *R. dentatus* and wheat.

(i). *R. dentatus*: Bioassays with CFrs from CME of *M. azedarach* showed that CFr No. 7 at low concentration of 0.5 $mg\ mL^{-1}$ caused necrosis on *R. dentatus* leaf surface. The 2,4-D produced necrosis in *R. dentatus* leaves at lower conc. of 0.25 $mg\ mL^{-1}$. The chromatographic fraction 7 applied at 0.5, 1.0 and 2.0 $mg\ mL^{-1}$ concentration caused moderate to severe discolouration in leaf discs of *R. dentatus*, while 2,4-D at the highest concentration (2.0 $mg\ mL^{-1}$) caused slight discolouration (Table 3, Figure 6).

Table 3. Effects of chromatographic fractions (CFrs) from concentrated methanolic extract (CME) of *M. azedarach* on leaf colour and necrotic spots of *R. dentatus* in Lab. bioassay.

DMSO Conc. (µL 1000 µL^{-1})	DMSO effect	2,4-D/ Conc. (mg mL^{-1})	2,4-D effect	Effects of purified fractions										
				1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
				***R. dentatus* leaves colour**										
Water	0	Water	0	0	0	0	0	0	0	0	0	0	0	0
0.780	0	0.0312	0-1	0	0	0	0	0	0	0-1	0	0	0	0
1.560	0	0.0625	0-1	0	1	0	0	0	0	1	0-1	0	0	0
3.125	0	0.1250	1	0-1	0-1	0-1	1	0-1	0	1	1	1	0-1	0
6.250	0	0.2500	1	1	0-1	1	1	1	0-1	1	1	1	0-1	0-1
12.500	0-1	0.5000	1	1	1	1	1	1	1	1-2	1	1	1	1
25.000	0-1	1.0000	1	1	1	1	1	1	2	2	1	1	1	1
50.000	0-1	2.0000	1	1	1	1	1	1	3	3	1	1	1	1
				***R. dentatus* leaves necrotic spots**										
Water	4	Water	4	4	4	4	4	4	4	4	4	4	4	4
0.780	4	0.0312	4	4	4	4	4	4	4	4	4	4	4	4
1.560	4	0.0625	4	4	4	4	4	4	4	4	4	4	4	4
3.125	4	0.1250	4	4	4	4	4	4	4	4	4	4	4	4
6.250	4	0.2500	6	4	4	4	4	4	4	4	4	4	4	4
12.500	4	0.5000	7	4	4	4	4	4	4	5	4	4	4	4
25.000	4	1.0000	10	4	4	4	4	4	4	6	4	4	4	4
50.000	4	2.0000	10	4	4	4	4	4	4	8	4	4	4	4

DMSO: Dimethyl sulfoxide, Leaf colour scale: **0** = No change, **1** = Light change, **2** = Moderate change, **3** = Severe change. Necrotic spot scale: **4** = No NS, **5** = NS≤ 1 mm, **6** = NS≤ 2 > 1 mm, **7** = NS≤ 3 > 2 mm, **8** = NS≤ 4 > 3 mm, **9** = NS≤ 5 > 4 mm, **10** = NS≤ 6 > 5 mm.

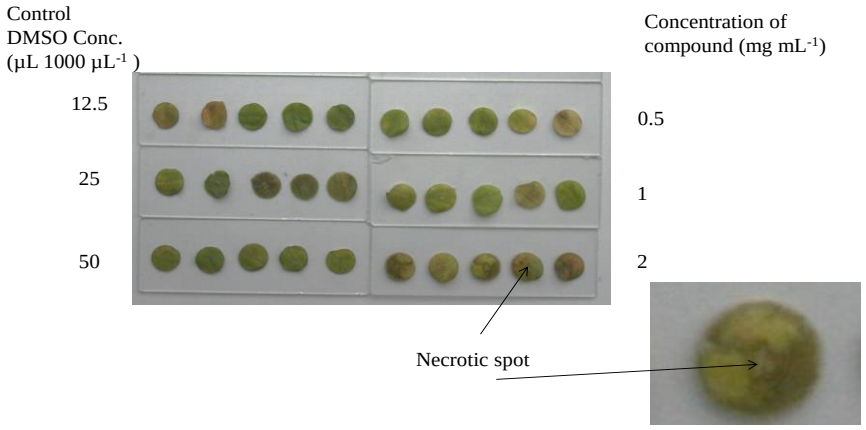

Figure 6. Herbicidal effect of fraction 7 from *M. azedarach* (identified as α-selinene) on leaf discs of *Rumex dentatus.*

(ii). Wheat: The application of chromatographic fraction 7 from *M. azedarach* at the highest concentration (2.0 mg mL^{-1}) did not cause necrotic spots on the leaf surface of wheat i.e., no harmful effects on wheat (Table 4). This is advantageous, for its use to control weeds in wheat crop. Numerous studies have reported the *M. azedarach* herbicidal activity against many weeds but the active herbicidal compound was not isolated and identified. Such research is needed to develop eco-friendly herbicide to reduce the negative impacts of synthetic herbicides on environment, human health and development of resistance to synthetic herbicides in many weed spp. Hence, new herbicides with novel mode of action should be explored (26).

Table 4. Effects of 2,4-D and identified compound, α-Selinene [fraction 7, from concentrated methanolic extract (CME) of *M. azedarach*] on leaf surface colour and necrotic spots in wheat

DMSO conc. (µL mL^{-1})	DMSO		Extract conc. (mg mL^{-1})	2,4-D		α-Selinene	
	Colour	Necrotic Spot		Colour	Necrotic Spot	Colour	Necrotic Spot
Water	0	4	Water	0	4	0	4
0.780	0	4	0.0312	0	4	0	4
1.560	1	4	0.0625	0-1	4	0	4
3.125	0	4	0.1250	0-1	4	0	4
6.250	0	4	0.2500	0-1	4	0	4
12.500	0	4	0.5000	1	4	0-1	4
25.000	0-1	4	1.0000	1	4	0-1	4
50.000	0-1	4	2.0000	1	4	0-1	4

DMSO**:** Dimethyl sulfoxide, Colour scale: **0** = No change, **1** = Light change, **2** = Moderate change, **3** = Severe change. Necrotic spot scale: NS: **4** = No NS, **5** = NS≤ 1 mm, **6** = NS≤ 2 > 1 mm, **7** = NS≤ 3 > 2 mm, **8** = NS≤ 4 > 3 mm, **9** = NS≤ 5 > 4 mm, **10** = NS≤ 6 > 5 mm.

II. GC-MS of purified compound

The GC-MS of purified chromatographic fraction 7 revealed the presence of single herbicidal compound, α-selinene [naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-,[2R(2.alpha.,4a.alpha.,8a.beta.)]-. Its chemical formula is $C_{15}H_{24}$ and molar mass is 204 g mol^{-1} (Figure 7).

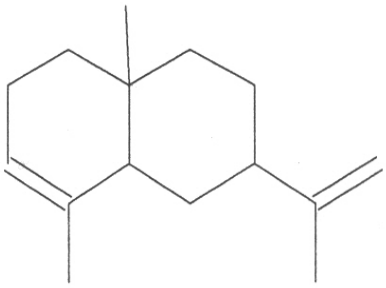

Figure 7. Structure of herbicidal compound, α-selinene.

α-Selinene is a sesquiterpenoid hydrocarbon and have herbicidal/phytotoxic activities. For example, β-caryophyllene inhibited seeds germination and seedling growth of *Brassica campestris*, *Raphanus sativus*, *Lactuca sativa* and *Mikania micrantha* (38). Moreover, δ-cadinene from root extract of *Chrysanthemoides monilifera* showed herbicidal activity on *Isolepis nodosa* (10). In another study, gas chromatographic analysis of extract of *Schinus lentiscifolius* leaves detected sesquiterpene hydrocarbons (41.52 %) and amongst these, δ-cadinene was detected in abundance (14.43 %). The *S. lentiscifolius* extract reduced the seed germination and growth in onion and lettuce and also reduced the mitotic index of onion and lettuce by 19 % and 25 %, respectively (29). Sesquiterpenic compounds present in *S. aromaticum* were herbicidal to two invasive weeds: *Mimosa pudica* and *Senna obtusifolia* (7). In another investigation, *Lantana camara* extract (rich in sesquiterpene hydrocarbons) was herbicidal against *Amaranthus hybridus*. These results suggested the possible use of these plant extracts as natural herbicides (37). In a previous study, sesquiterpenic compounds with silphinene and selinene type skeletons were reported from a fungus *Leptosphaeria maculans*. The herbicidal activity of *Helichrysum italicum* on *Brassica napus*, *Sinapis alba* and *Brassica juncea* was probably due to the presence of bioactive sesquiterpenes (23). Moreover, 4,5-dicaffeoylquinic acid isolated from the roots of *Echinacea purpurea* strongly inhibited the root growth of *Portulaca oleracea*, while, 1,3-dicaffeoylquinic acid inhibited the germination and shoot length of *Amaranthus viridis* and *P. oleracea* weeds (19). The biological activity of main components is regulated by minor components present in the extract of plant species (5,11,29). The herbicidal effects of extracts of *Salvia hierosolymitana* and *Salvia multicaulis* on the growth of *Raphanus sativus* and *Lepidium sativum* were due to the presence of α-selinene (22). The extract of *Callicarpa americana* also possesses the α-selinene, hence, its oil was mildly herbicidal to *Oscillatoria perornata*, *Oscillatoria agardhii* and *Selenastrum capricornutum* (34). A structurally related compound named as β-selinene was found in *Thymus mastichina* extract. It's extract at all test concentrations completely inhibited the seed germination and seedling growth of *Portulaca oleracea*, *Lolium multiflorum* and *Echinochloa crus-galli* (15).

Further research work is needed to investigate the mode of action of the identified pure compound α-selinene from *M. azedarach* and total synthesis of this compound. After total synthesis, it should be tested for herbicidal activity against many weeds under field conditions. The herbicidal activity of identified compound may be enhanced by preparing its derivatives. Besides, its toxicity to non-target fauna and flora needs to be investigated before its release into the market.

CONCLUSIONS

M. azedarach leaf extract at 10 % conc. significantly reduced the weed biomass of *R. dentatus* up to 65.24 %, with a subsequent increase in wheat yield (41.4 %). Whereas, the leaf extract of *M. azedarach* at 10 % concentration + quarter dose of Bromoxynil (70 g a.i.) + MCPA (70 g a.i) significantly reduced the *R. dentatus* weed biomass up to 96.77 %, with an increase of 67.6 % in wheat yield. The compound isolated and identified from *M. azedarach* was α-selinene. It showed herbicidal activity against *R. dentatus* leaves, at minimum conc. of 0.5 mg mL^{-1} but not harmful to wheat crop. The structure of compound we identified can be used as an analogue to synthesize commercial herbicides.

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DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who 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.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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