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ORIGINAL ARTICLE

Isolation of herbicidal compounds, quercetin and β-caryophyllene, from *Digera muricata*

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KEYWORDS

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Abstract Synthetic herbicides are available to control weeds but these herbicides have many concerns. Eco-friendly herbicides obtained from plants is a better alternative to synthetic ones. Despite that the herbicidal activity of *Digera muricata* extracts have been reported but there are no studies regarding the isolation and identification of herbicidal compounds from *D. muricata*. Herein, we are reporting the identification of two herbicidal compounds from chloroform extract obtained from *D. muricata*. The chloroform extract was initially tested on the germination and early growth of two weeds; *Avena fatua* and *Melilotus indicus* as well as wheat where a significant decline in % age germination and growth of both weeds was observed. Among the 8 different fractions obtained using different chromatographic techniques, fractions 2 and 7 were found to be phytotoxic to both test weeds. The herbicidal efficacy was tested at 200, 150, 100, 50, 25 µg/ml. These two fractions were further purified on Reversed Phase High Pressure Liquid Chromatography (RP-HPLC). Fraction 2 yielded 3 sub-fractions (2A, 2B & 2C), while fraction 7 yielded 2 sub-fractions (7A, 7B). Fraction 2B caused 43%, 53%, and 57% decline in seed germination, shoot dry weight, and root dry weight of *A. fatua,* while these values against *M. indicus* were 50%, 81% and 84%, respectively. Fraction 7A caused 25%, 36%, and 42% decline in seed germination, shoot dry weight, and root dry weight of *A. fatua,* while these values against *M. indicus* were 35%, 62% and 69%, respectively, at 200 µg/ml conc. Cyanazine caused 61% and 50% reduction in seed germination of *A. fatua* and *M. indicus,* respectively. The herbicidal effects of these two fractions were found nonsignificant against wheat. Fourier Transform Infrared Spectroscopy (FT-IR), elemental analysis (C,H) and Nuclear Magnetic

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Resonanace (NMR) analyses of these two fractions depicted the presence of quercetin (Fraction 2B) and β-caryophyllene (Fraction 7A). In post emergence bioassays, the isolated compounds caused significant decrease in the biomass of both weeds. Plasma membrane integrity assays revealed electrolyte leakage in treated leaf discs of both weeds. It was concluded that quercetin and β-caryophyllene isolated from *D. muricata* exhibited toxicity towards both test weeds without harming wheat.

1. Introduction

Weeds increase abiotic stresses like utilization of water, light, nutrients and unavailability of growth resources for crops (Soares et al., 2019; Silva et al., 2013). The roots of different plants excrete certain chemicals, produced during metabolism and are termed as allelochemicals (Broeckling et al., 2008). These allelochemicals are used by the plants for interactions with other plants and the environment (Aslani et al., 2014). These allelochemicals may interfere with the physiology of other plants and may enhance or retard the germination of the recipient ones (Majeed et al., 2017). The germination of Pirsabaq cultivar was totally failed due to aqueous extracts of *Melilotus indicus* (Siyar et al., 2017). Weeds e.g., *Avena fatua*, *M. indicus* and *Polypogon hissaricus* are the most dangerous weeds that decrease the wheat production in many regions of the world, including Pakistan (Siyar et al., 2019; Kong et al., 2019).

Despite the great success and widespread adoption of herbicides by farmers, the continued and heavy use of herbicides not only destroyed the ecosystem but also posed serious health risks and the evolution of herbicide resistant weeds (Iqbal et al., 2019). Owing to these negative effects of these weed killers, now there is demand of new groups of substances that are biodegradable, having plant based origin and may have the possibilities to be used as herbicides (Aryakia et al., 2015: Anwar et al., 2019). The allelochemicals disturb the growth of undesired plants by altering their cell structure, inhibit the cell multiplication, disruption in the plasma membrane or can create troubles in uptake of dissolved minerals and water. Utilization of these allelochemicals is environment-friendly and it helps to retard the applications of herbicides. So, allelopathy is an alternative method to control growth of undesired plants (Nawaz et al., 2020).

Plant-based natural herbicides can reduce the application of synthetic herbicides due to having the potential of producing those set of substances that are useful for weed killing mechanisms (Anwar et al., 2019; Ahmed et al., 2014). As an example, leaves aqueous extract of Jimson weed (*Datura stramonium*) at concentrations of 5, 15, 30, 40, 50 and 60% reduced the germination of sugar beet (*Beta vulgaris*) and *A. retroflexus* by 89% and 55%, at 5% concentration. However, at all other concentrations, 100% decline in germination was recorded (Yorulmaz et al., 2020). In another study, seed sprouting, seedling height, seedling vigor index and biomass of *Parthenium hysterophorus* were suppressed significantly due to the application of extracts of *Datura metel*, *Mangifera indica, Azadirachta indica* and *Tagetes erectus* at 50% and 75% concentration (Ramachandran et al., 2020). In another study, leaf extract of nerium (*Nerium oleander*), olives (*Olea europaea*) and castor oil (*Ricinus communis*) stopped the germination of purple nut sedge (*Cyperus rotundus*). Nerium extract caused 90% inhibitory effect at 5 and 10%, however, the 2.5% concentration showed the lowest inhibition effects on the germination of weeds, while in 2nd experiment, 10% nerium extract exhibited 100% inhibition (Al-Samarai et al., 2018). In another study, the isolated compounds viz., diterpenes, manool oxide and sesquiterpene hydrocarbons from *Pinus nigra* showed strong herbicidal efficacy against various weeds like *Phalaris canariensis*, *Trifolium campestre* and *Sinapis arvensis* (Amri et al., 2017).

There are few reports of phytotoxic activity of *Digera muricata* (Family Amaranthaceae). *D. muricata* aqueous extract decreased the germination and growth of *Pennisetum glaucum*. Major compounds detected by Gas Chromatography Mass Spectrometry (GC/MS) included quercetin, sinapic, ferluic acid, cystine, berbine and limonene from roots and shoots of *D. muricata* (Aziz & Shaukat, 2014). In another styudy, *D. muricata* extracts at 1, 3 and 5% concentrations significantly decreased the seed germination and seedling growth of *Pennisetum typhoideum* (Bindu & Jain, 2011). As the phytotoxic activity of *D. muricata* extracts have been reported by earlier workers so we hypothesized that *D. muricata* should possess herbicidal/phytotoxic compounds. To the best of our knowledge, previously there are no studies regarding the isolation and identification of herbicidal compounds from *D. muricata,* so herein we isolated the actual bioactive compounds from *D. muricata* and tested for their herbicidal activity. Identification of actual herbicidal compounds would act as structural analogues to synthesize of new herbicidal compounds (Natural herbicides) by herbicide companies to safeguard environment and human health.

2. Materials and methods

2.1. Collection of weeds

Two knotty weeds of wheat crop viz., *Avena fatua* and *Melilotus indicus* were chosen for study. The ripened/mature seeds of these weeds were collected in the month of May from wheat fields of District Gujrat, Pakistan at the end of the wheat growing season. These seeds were sun dried for 7 days and then packed in paper bags for further use.

2.2. Collection of test plant material

Test plant namely *Digera muricata* was harvested at flowering stage in the month of October, from the fields at Islam Nagar and Sabowal village, Gujrat, Punjab, Pakistan, to evaluate its herbicidal potential against the selected weeds namely *M. indicus* and *A. fatua*.

2.3. Preparation of extracts of test plant

For the preparation of plant extracts, procedure described by Akbar et al. (2020) was adopted with modifications. After collection, the plants were surface washed with water sprinkler to remove impurities like dust particles. Immediately after washing procedure, whole plants were air dried with the aid of fan to remove excessive moisture. These plants were cut with a fodder cutter into 2 cm pieces and the cut plant pieces were dried under shade to avoid leaching of metabolites by rainwater. The dried chopped plant material was ground to fine powder and incubated or soaked in autoclaved water @ 10 g: 100 ml (w/v) for 24 h at 50 °C in a water bath (H H-4). After the incubation period, the mixture was filtered through 4 layers of cheese cloth followed by filtration through Whatman filter paper no.1., using vacuum flask to get water extract of test plant. The water extract obtained in this way was regarded as original or 10% extract concentration. Extraction of metabolites was carried out using chloroform solvent in 1:1 ratio of 10% extract (400 ml) and chloroform (400 ml), in separating funnel (1L). The process was repeated thrice to maximize the extraction of compounds. These extracts were concentrated in a rotatry evaporator at 40 °C. Finally, the chloroform solvent was evaporated from the extract by continuous air flow on the extract contained in glass beaker.

2.4. Chromatographic procedures

Initailly, compounds present in chloroform extract of *D. muricata* were separated by Thin Layer Chromatography (TLC) for the optimization of maximum number of separated compounds and solvent system. The solvent system *n*-hexane and ethanol (1:3) was able to isolate 8 fractions. To get the fractions for bioassays, Preparative Thin Layer Chromatographic (PTLC) plates (20x20 cm) were used with the same solvent system. Fractions showing bioactivity (fractions 2 & 7) were further purified by Reversed Phase High Pressure Liquid Chromatography (RP-HPLC). Gradient elution was employed with solvent system dH_2O and acetonitrile. Fraction 2 yielded 3 sub-fractions (2A, 2B & 2C), while fraction 7 yielded 2 subfractions (7A, 7B). The herbicidal activity was tested at 200, 150, 100, 50, 25 µg/ml.

2.5. Pre emergence in vitro bioassays using chloroform extracts and fractions

Seeds of *Triticum aestivum*, *M. indicus* and *A. fatua* were surface sterilized with 1% sodium hypochlorite for 10 min (Akbar & Javaid, 2013) and then placed in Petri dishes (9 cm diameter) having sterilized filter paper moistened with 2.5 ml of plant extract. Two concentrations of chloroform extract (2 mg/ml) and diluted with dH_2O (1 mg/ml) were investigated. The control plant received the same amount of distilled water. Data regarding seed germination and early seedling growth were recorded after 14 days. Following parameters viz., seed germination, shoot dry weight, and root dry weight were noted to assess the herbicidal activity. The herbicidal activity of pure compounds was compared with broad-spectrum herbicide, cyanazine. Following treatments were investigated in *in vitro* assays. Each treatment was replicated thrice & there were 10 seeds per Petri plate.

Treatments in in vitro assays

Set 1- Melilotus indicus

T1 = Distilled water; T2 = 1 mg/ml chloroform extract; T3 = 2 mg/ml chloroform extract

Set 2- Avena fatua

T1 = Distilled water; T2 = 1 mg/ml chloroform extract; T3 = 2 mg/ml chloroform extract

Set 3- (1–8 Fractions) *Melilotus indicus*, *Avena fatua*

T1 = Distilled water; T2 = 25 µg/ml; T3 = 50 µg/ml; T4 = 100 µg/ml; T5 = 150 µg/ml; T6 = 200 µg/ml

Set 4- Subfractions/purified compounds/herbicide (Cyanazine) (2A, 2B & 2C) & (7A, 7B) (*Melilotus indicus*, *Avena fatua* & wheat); fraction concentrations as in set 3.

2.6. Spectroscopic analysis

Fractions showing herbicidal activity were analysed by spectroscopic methods to identify the active compounds.

The ^{1}H NMR and ^{13}C NMR spectra (Chloroform-*d*) were recorded using a Bruker 500 MHz spectrometer (Bruker, Freemont, CA, USA) and Fourier Transform Infrared Spectroscopy (FT-IR) analysis was carried out on Shimadzu FTIR–8400S spectrometer (Kyoto, Japan, υ, cm^{-1}). Melting point was determined by using a Digimelt MPA 160, USA apparatus and are reported uncorrected. Elemental analysis (C, H) was carried out on a Flash 2000 series elemental analyzer with thermal conductivity detector (TCD) system (Thermo Flash 2000 elemental analyzer, Thermo Fisher Scientific Inc., Waltham, MA, USA) and results are with ± 0.3%.

2.7. Post emergence herbicidal activity

2.7.1. Pot experiments

Pot experiments were conducted with *A. fatua*, *M. indicus* and wheat. Seeds were surface sterilized and sown in 500 g of soil contained in pots. After emergence, seedlings were thinned to keep 3 uniform seedlings in each pot. Sprays with desired concentrations of purified compounds as well as commercial herbicide (Cyanazine) were done at 3–4 leaves stage for *A. fatua* and wheat, while *M. indicus* seedlings were sprayed at 6–7 leaves stage. All mixtures contained 2% Tween 20 and sprays were done with manual spray bottle. Fresh weight measurements of shoot were done after 14 days of compounds/herbicide applications.

Treatments in post emergence pot assays

Following treatments were investigated in post emergence pot assays for purified compounds and cyanazine against *A. fatua*, *M. indicus* and wheat. Each treatment was replicated 5 times.

T1 = Distilled water; T2 = 25 mM; T3 = 50 mM; T4 = 100 mM; T5 = 150 mM; T6 = 200 mM T7 = 150 m M; T8 = 200 mM.

2.7.2. Plant plasma membrane integrity assays

For membrane integrity assays (an indicator of cellular damage), five leaves discs of 10 mm diameter were cut from plants after 2 h of application of the mixtures. These cut discs were transferred into 10 ml of dH_2O and shaken on a shaker for 3 h at 90 rpm. The mixtures were then transferred into test tubes for the measurements of electrolyte concentrations with

a conductivity meter. Five replications were done for membrane integrity assays (Bainard et al., 2006).

2.8. Experimental design

Completely Randomized Design (CRD) was followed in *in vitro* and pot assays.

2.9. Data analysis

Data were analyzed by analysis of variance (ANOVA) followed by Tukey's test to delimit treatment means using computer software Minitab 19.

3. Results and discussion

3.1. Herbicidal activity of chloroform extract of Digera muricata on the germination and growth of Petri plate grown Avena fatua and Melilotus indicus

The effect of chloroform extracts of *D. muricata* regarding germination of seeds and early seedling growth of two weeds viz., *A. fatua* and *M. indicus* was found significant. The chloroform extract showed reduction in germination of *A. fatua* and *M. indicus* by 46 and 62% at 1 mg/l concentration, respectively, whereas when the concentration was increased to 2 mg/l, there was 75 and 81% reduction in germination of seeds (Table 1). The chloroform extract of *D. muricata* showed reduction in shoot dry weight of *A. fatua* by 32% at 1 mg/l concentration, whereas when the concentration was increased to 2 mg/l, 73% reduction in shoot dry weight was observed. The dry weight of root was reduced to 35 and 60%, under the application of 1 mg/l and 2 mg/l concentrations of chloroform extract of *D. muricata*, respectively.

Table 1 Effect of different concentrations of chloroform extract of *Digera muricata* on the germination of two weeds, *Avena fatua* and *Melilotus indicus*.

Treatments	*Avena fatua*	*Melilotus indicus*
T1 = dH_2O	93 ± 3.3a	87 ± 3.3a
T2 = 1 mg/ml chloroform extract	50 ± 5.7b	33 ± 3.3b
T3 = 2 mg/ml chloroform extract	23 ± 3.3c	17 ± 3.3c

Data show means ± Standard error of 3 replications. Different letters show significance between the treatments at $P \leq 0.05$, as determined by ANOVA & Tukey's Test.

The susceptibility of *M. indicus* towards the chloroform extracts of *D. muricata*, was also found significant. The chloroform extract showed a reduced shoot dry weight by 60% at 1 mg/l concentration, whereas when the concentration was increased to 2 mg/l, 95% reduction in shoot dry weight was observed. The dry weight of root was reduced to 63 and 95%, under the application of 1 mg/l and 2 mg/l concentrations of chloroform extract of *D. muricata*, respectively (Figs. 1 & 2). In an investigation, the ethanol extract of *Sapindus mukorossi* was phytotoxic to *A. fatua* and *A. retroflexus*. Five phytotoxic compounds from *S. mukorossi* extract were identified. Of these five compounds, d-pinitol and oleanolic acid hindered the development of both test weeds (Ma et al., 2018). Two flavonoids; pectolaring and scutellarein 4′-methyl ether and two sesquiterpene lactones, elemanolide 11(13)-dehydromelitensin β-hydroxyisobutyrate and acanthiolide, isolated from leaves extract of *Onopordum acanthium*, showed phytotoxic activity against etiolated wheat coleoptiles. Elemanolide 11(13)-dehydromelitensin β-hydroxyisobutyrate arrested the development of wheat (Watanabe et al., 2014).

3.2. Herbicidal activity of fraction 2 & 7 from chloroform extract of Digera muricata on the germination and growth of Petri plate grown Avena fatua and Melilotus indicus

Fraction 2 from chloroform extract of *D. muricata* decreased the shoot dry biomass of *A. fatua* by 7, 18, 26, 38 and 45% at concentrations of 25, 50, 100, 150 and 200 µg/ml, respectively, while root dry biomass of *A. fatua* was inhibited by

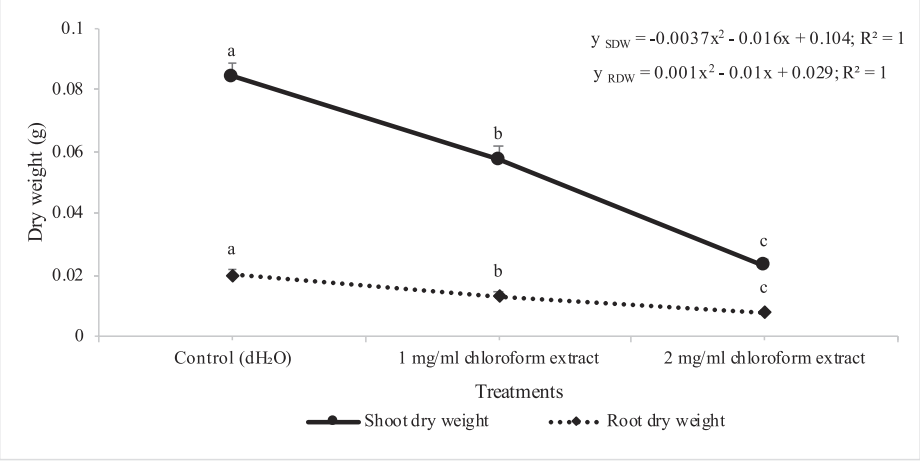

Fig. 1 Herbicidal activity of different concentrations of chloroform extract of *Digera muricata* on shoot and root dry weight of Petri plate grown *Avena fatua*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

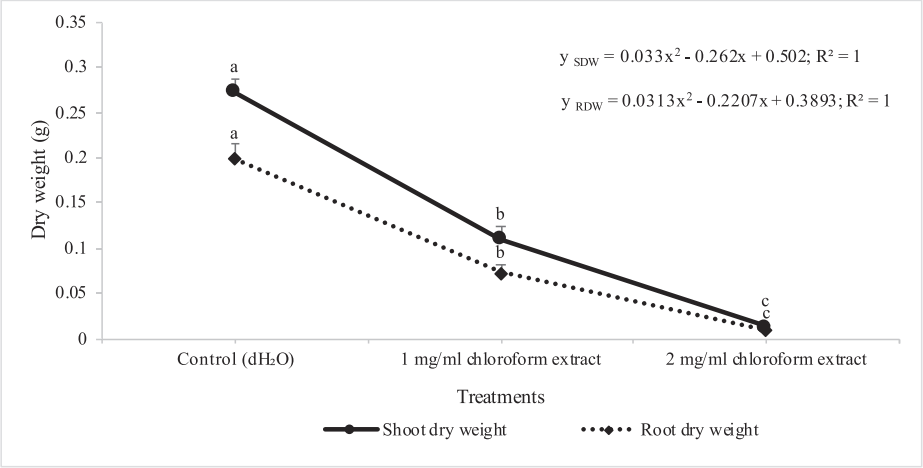

Fig. 2 Herbicidal activity of different concentrations of chloroform extract of *Digera muricata* on shoot and root dry weight of Petri plate grown *Melilotus indicus*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

11, 24, 27, 29 and 36%, respectively. On the other hand, fraction 7 from chloroform extract of *D. muricata* decreased the shoot and root dry biomass of *A. fatua* by 7, 16, 19, 20% and 24% and 5, 9, 12, 23 and 30%, at concentration of 25, 50, 100, 150 and 200 µg/ml, respectively (Figs. 3 & 4). Germination of seeds of *A. fatua* was also negatively affected by the applications of different employed concentrations of fraction 2 and fraction 7. There was 7, 11, 21, 25 and 36% decrease in the germination of seeds of *A. fatua* by the application of different concentrations of fraction 2, while these values for fraction 7 were 7, 11, 14, 14 and 18%, respectively.

Fraction 2 decreased the shoot dry biomass of *M. indicus* by 8, 22, 38, 54 and 62% at concentrations of 25, 50, 100, 150 and 200 µg/ml, respectively, while these concentations of fraction 2 decreased the root dry biomass of *A. fatua* by 10, 15, 20, 39 and 59%, respectively. On the other hand, fraction 7 decreased the shoot dry biomass of *M. indicus* by 6, 23, 30, 36 and 50% at fraction 7 concentrations of 25, 50, 100, 150 and 200 µg/ml, respectively, while root drybiomass of *M. indicus* was declined to 5, 16, 24, 34 and 48%, respectively (Figs. 5 & 6). There was 8, 12, 12, 23 and 35% decrease in the germination of seeds of *M. indicus* by the application of different concentrations of fraction 2, while these values for fraction 7 were 4, 8, 8, 15 and 23%, respectively (Table 2).

3.3. Herbicidal activity of fraction 2B & 7A from chloroform extract of Digera muricata on the germination and growth of Petri plate grown Avena fatua and Melilotus indicus

Fraction 2B from chloroform extract of *D. muricata* decreased the shoot and root dry biomass of *A. fatua* by 9, 28, 30, 49 and 53% and 13, 26, 40, 51 and 57% at concentrations of 25, 50,

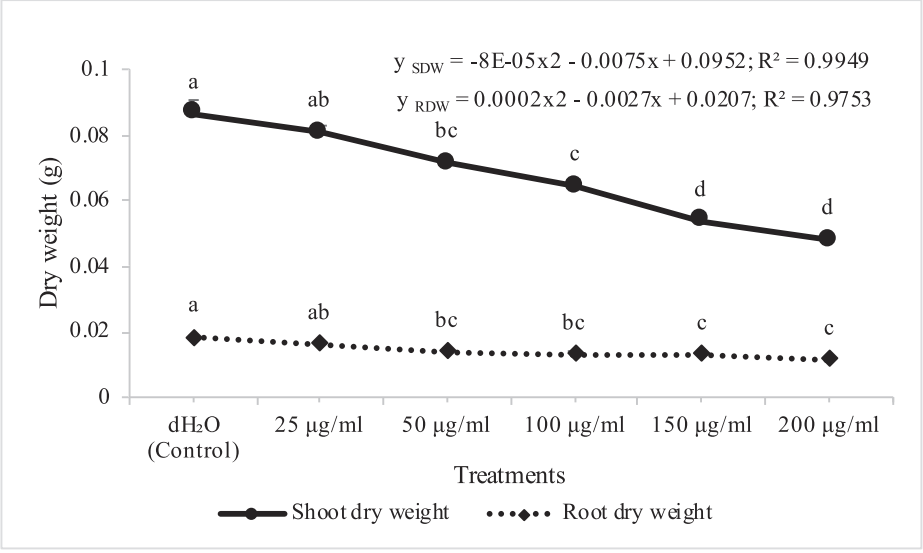

Fig. 3 Herbicidal activity of different concentrations of fraction 2 from chloroform extract of *Digera muricata* on shoot and root dry weight of Petri plate grown *Avena fatua*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

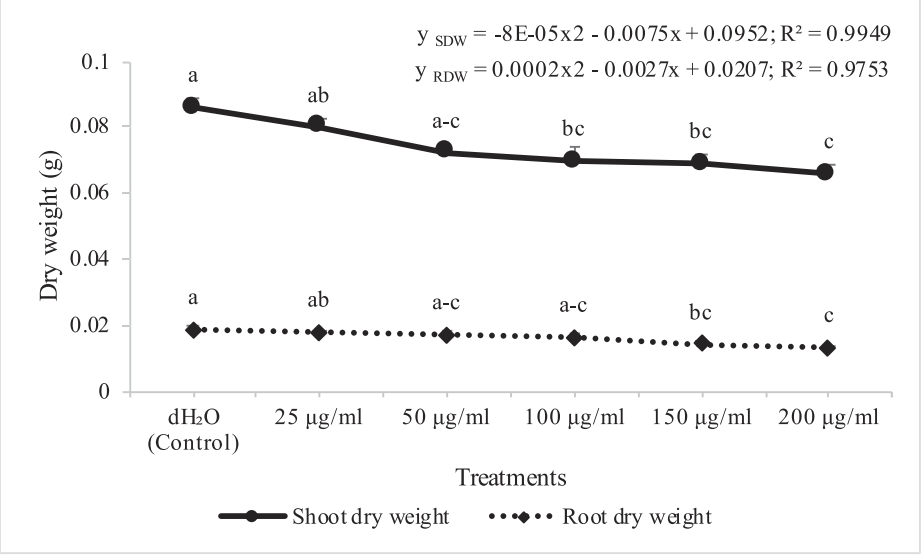

Fig. 4 Herbicidal activity of different concentrations of fraction 7 from chloroform extract of *Digera muricata* on shoot and root dry weight of Petri plate grown *Avena fatua*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard rrror of means and different letters show significance.

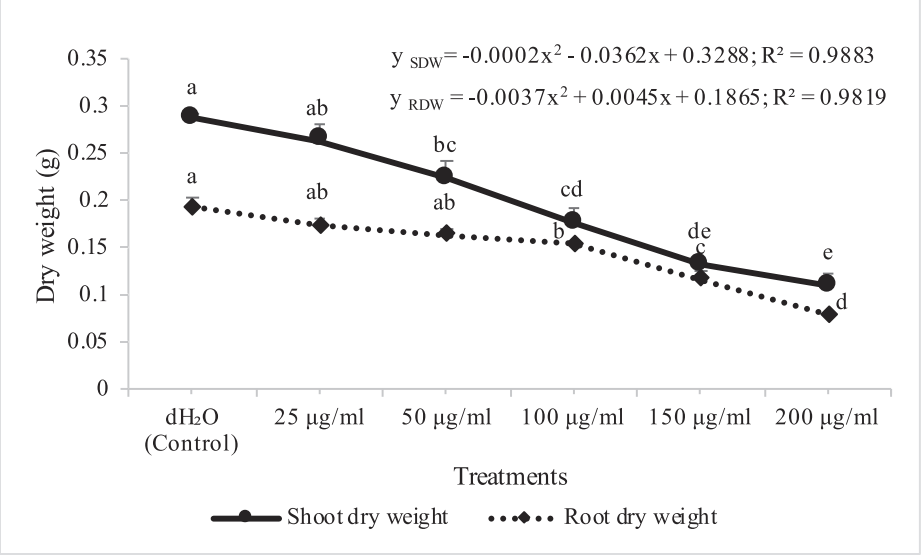

Fig. 5 Herbicidal activity of different concentrations of fraction 2 from chloroform extract of *Digera muricata* on shoot and root dry weight of Petri plate grown *Melilotus indicus*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

100, 150 and 200 µg/ml concentration, respectively. On the other hand, fraction 7A decreased the shoot and root dry biomass of *A. fatua* by 9, 18, 21, 31 and 36% and 7, 17, 20, 29 and 42% at concentrations of 25, 50, 100, 150 and 200 µg/ml, respectively (Figs. 7 & 8). Germination of seeds of *A. fatua* was reduced by the applications of different employed concentrations of fraction 2B and fraction 7A. There was 11, 14, 21, 32 and 43% decrease in the germination of seeds of *A. fatua* by the application of different concentrations of fraction 2B, while these values for fraction 7A were 14, 18, 21, 25 and 25%, respectively (Table 2). *Cynara cardunculus* extracts showed herbicidal/allelopathic potency against weeds that included *Trifolium incarnatum*, *Silybum marianum* and *Phalaris minor*. From the aerial parts of *C. cardunculus*, syringic acid, p-coumaric acid, myricitrin, quercetin and naringenin were detected (Kaab et al., 2020).

Fraction 2B from chloroform extract of *D. muricata* decreased the shoot dry biomass of *M. indicus* by 15, 31, 38, 53 and 81% at 25, 50, 100, 150 and 200 µg/ml concentration, respectively, while these concentations of fraction 2B decreased the root dry biomass of *M. indicus* by 16, 21, 25, 53 and 84%, respectively. On the other hand, fraction 7A from

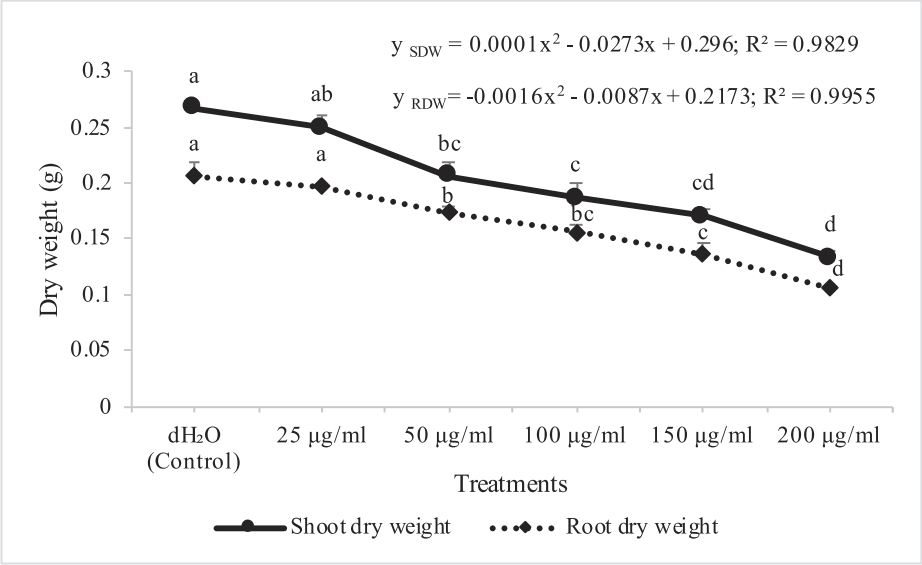

Fig. 6 Herbicidal activity of different concentrations of fraction 7 from chloroform extract of *Digera muricata* on shoot and root dry weight of Petri plate grown *Melilotus indicus*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show Ssignificance.

Table 2 Effect of different concentrations of fractions isolated from chloroform extract of *Digera muricata* on the germination of two weeds, *Avena fatua* and *Melilotus indicus*.

Treatments	*Avena fatua*		*Melilotus indicus*	
	Fraction 2	Fraction 7	Fraction 2	Fraction 7
T1 = dH_2O	93 ± 3.3a	93 ± 3.3a	87 ± 3.3a	87 ± 3.3a
T2 = 25 µg/ml	87 ± 3.3b	87 ± 3.3b	80 ± 5.8b	83 ± 6.7b
T3 = 50 µg/ml	83 ± 3.3c	83 ± 3.3c	77 ± 3.3bc	80 ± 5.8bc
T4 = 100 µg/ml	73 ± 3.3d	80 ± 5.8 cd	77 ± 5.8bc	80 ± 5.7bc
T4 = 150 µg/ml	70 ± 5.7e	80 ± 5.7 cd	67 ± 5.7d	73 ± 3.3c
T6 = 200 µg/ml	60 ± 5.7f	77 ± 3.3d	57 ± 3.3e	67 ± 3.3 cd

Data show means ± standard error of 3 replications. Different letters show significance between the treatments at $P \leq 0.05$, as determined by ANOVA & Tukey's range test.

chloroform extract of *D. muricata* decreased the shoot dry biomass of *M. indicus* by 11, 31, 37, 52 and 62% at fraction 7A concentration of 25, 50, 100, 150 and 200 µg/ml, respectively, while these concentations of fraction 7A from chloroform extract of *D. muricata* decreased the root dry biomass of *M. indicus* by 17, 21, 27, 44 and 62%, respectively (Figs. 9 & 10). Germination of seeds of *M. indicus* was also decreased by the application of different employed concentrations of fraction 2B and fraction 7A. There was 27, 31, 38, 50 and 50% decrease in the germination of seeds of *M. indicus* by the application of different concentrations of fraction 2B, while these values for fraction 7A were 8, 15, 23, 35 and 35%, respectively. The effect of synthetic herbicide, cyanazine was comparable with fraction 2B. This herbicide decreased the seed germination of *A. fatua* by 25, 32, 36, 43 and 61%, while, these values for *M. indicus* were 19, 23, 31, 46 and 50% at concentrations of 25, 50, 100, 150 and 200 µg/ml, respectively (Table 3).

The synthetic herbicide, cyanazine, used as reference compound, decreased the shoot dry biomass of *A. fatua* by 24, 39, 41, 56 and 65%, while root dry biomass of *A. fatua* was inhibited to 24, 46, 50, 61 and 68% at herbicide concentrations of 25, 50, 100, 150 and 200 µg/ml, respectively. On the other hand, cyanazine decreased the shoot dry biomass of *M. indicus* by 24, 34, 54, 65 and 83%, while, root dry biomass of *M. indicus* was inhibited to 16, 21, 25, 53 and 84% at herbicide concentrations of 25, 50, 100, 150 and 200 µg/ml, respectively (Figs. 11 & 12).

Parthenium hysterophorus is also known for phytotoxic behaviour against seedling growth of lettuce and some other plant seedlings (Shi et al., 2020). A novel kaurene-type compound, wedelienone was identified from aqueous methanol extract of *Wedelia chinensis*. Phytotoxic property of wedelienone was monitored by measuring inhibited growth of cress and timothy (*Phleum pratense*) at concentrations greater than 10–30 µM (Das et al., 2020). Brassicaceae seed meals (BSM) also showed herbicidal activity on the growth of wild oat, ryegrass, lettuce and pigweed. Among different BSM amendments, white mustard seed meals at 2000 kg ha^{-1} significantly inhibited the growth of tested weeds (Handiseni

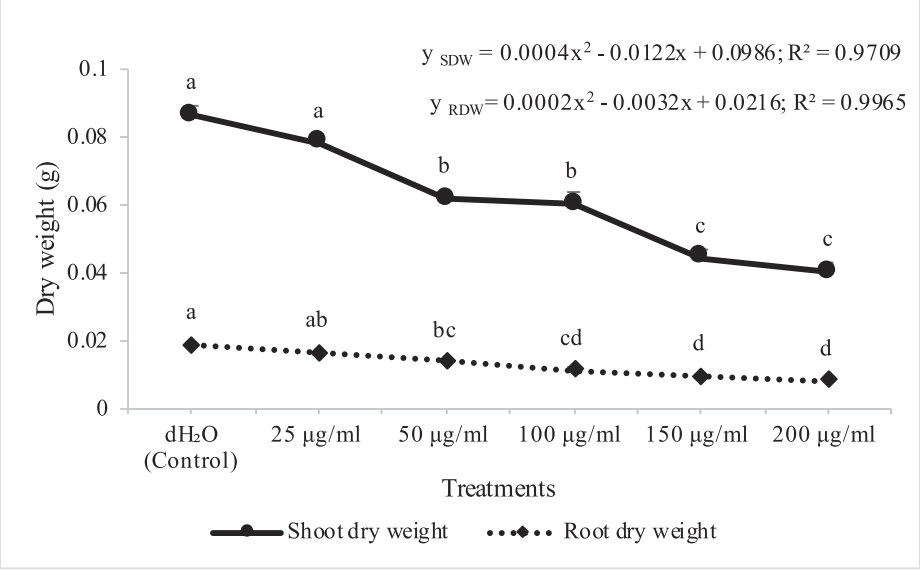

Fig. 7 Herbicidal activity of different concentrations of fraction 2B from chloroform extract of *Digera muricata* on **(A)** shoot dry weight and root dry weight of Petri plate grown *Avena fatua*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

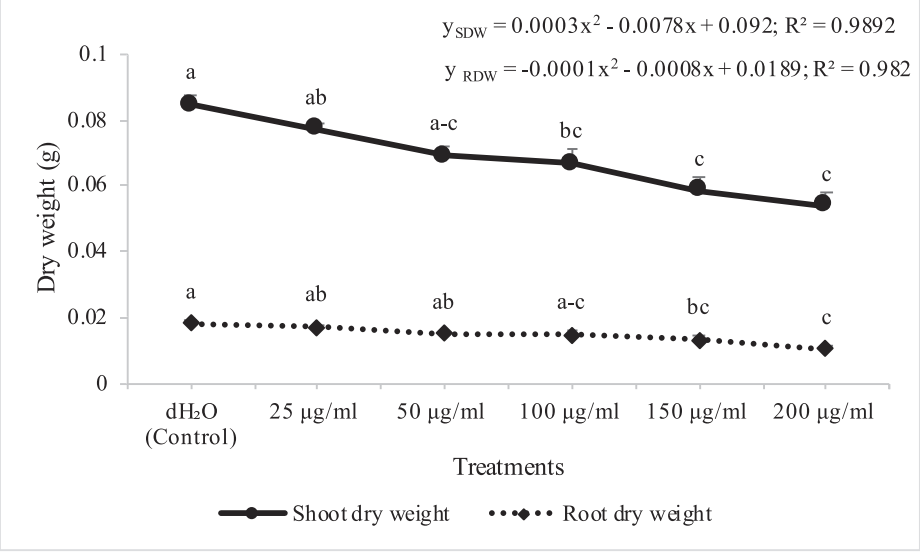

Fig. 8 Herbicidal activity of different concentrations of fraction 7A from chloroform extract of *Digera muricata* on shoot and root dry weight of Petri plate grown *Avena fatua*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

et al., 2011). Metabolites of *Aureobasidium pullulans* are known to possess herbicidal activity against *Galium aparine, Chenopodium album, Malva crispa, Polygonum lapathifolium* and *A. fatua*. *G. aparine* and *M. crispa* exhibited significant decline in fresh weight. Metabolites of *A. pullulans* exhibited little to non-toxic herbicidal effects on wheat, *Vicia faba, Hordeum vulgare*, *Brassica napus* and *Pisum sativum* (Guo et al., 2020). In the present study, the effect of extracts and fractions as well as synthetic herbicide against wheat was in general, non significant, so these data were not presented in this article.

3.4. Spectroscopic analysis

Spectroscopic analysis of active fractions revealed the presence of quercetin (Fraction 2B) and β-caryophyllene (Fraction 7A).

Quercetin

Solid; Mp: 316 °C; FT-IR (cm^{-1}): 3485 (OH), 1580 (C═O), 1480 (C═C), 1220 (C─O): ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.69 (s, 1H, H-11), 9.12 (s, 1H, H-20), 8.95 (s, 1H, H-21), 7.59 (dd, J = 7.5, 1.6 Hz, 1H, H-15), 7.32 (d, J = 1.5 Hz, 1H, H-19), 6.83 (d, J = 7.5 Hz, 1H, H-16), 6.29 (d, J = 1.4 Hz, 1H,

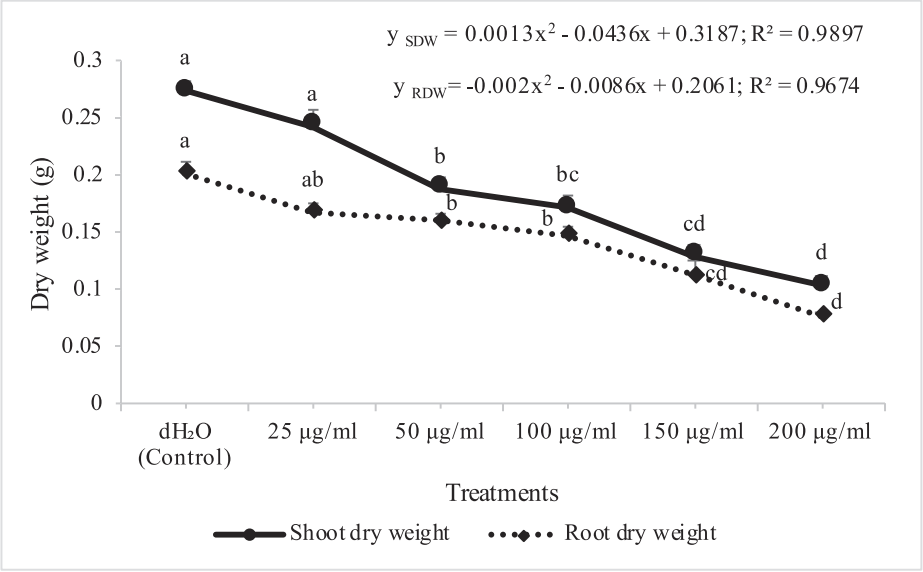

Fig. 9 Herbicidal activity of different concentrations of fraction 7A from chloroform extract of *Digera muricata* on shoot and root dry weight of Petri plate grown *Melilotus indicus*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

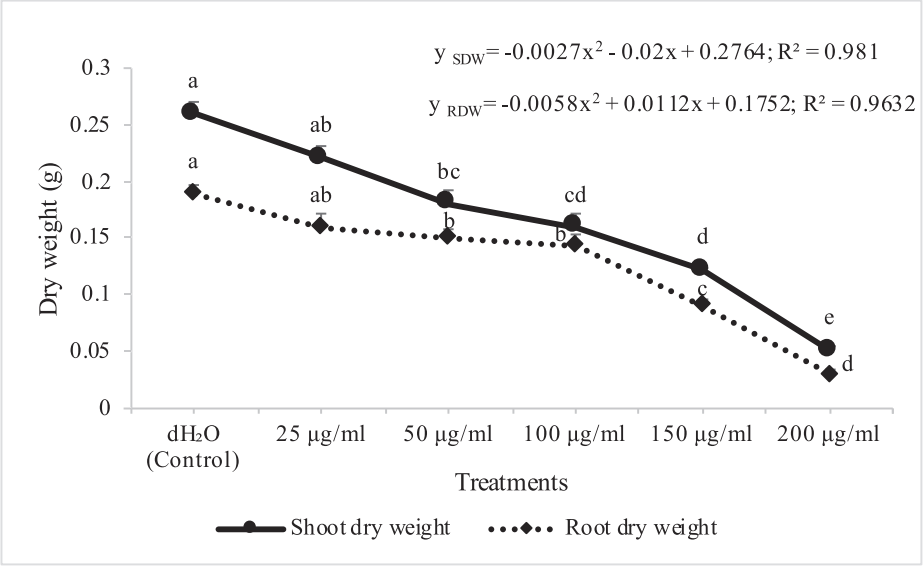

Fig. 10 Herbicidal activity of different concentrations of fraction 2B from chloroform extract of *Digera muricata* on shoot and root dry weight of Petri plate grown *Melilotus indicus*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

H-7), 6.15 (d, J = 1.4 Hz, 1H, H-9). ^{13}C NMR (125 MHz, Chloroform-*d*) δ 175.81 (C-3), 164.57 (C-8), 161.40 (C-10), 157.65 (C-1), 147.69 (C-17), 146.80 (C-5), 145.09 (C-18), 136.19 (C-4), 121.89 (C-14), 119.94 (C-15)), 115.60 (C-16), 115.17 (C-19), 103.23 (C-2), 99.54 (C-9), 94.34 (C-7). Chemical Formula: $C_{15}H_{10}O_7$; Molecular Weight: 302.24; Elemental Analysis: Calculated (C, H) 59.61, 3.34; Found; (C, H) 59.63, 3.36. The physical and spectroscopic data agreed with the literature for quercetin (Fernández-Aparicio et al., 2021; Grande, et al., 1985).

β-caryophyllene

Solid; FT-IR (cm^{-1}): 3395 (OH), 2904 (sp^2 CH), 1485 (C═C): ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.26 (dddd, J = 7.1, 4.8, 2.1, 0.9 Hz, 1H, H-4), 4.84 (ddq, J = 4.0, 2.0, 1.1 Hz, 2H, H-15), 2.44 (qt, J = 7.0, 1.1 Hz, 1H, H-16), 2.03 – 1.88 (m, 6H, H-1,8,9), 1.73 – 1.60 (m, 3H, H-12,17), 1.57 (s, 3H, H-10), 1.52 – 1.38 (m, 2H, H-2), 1.00 (d, J = 1.6 Hz, 3H,H-14), 0.95 (d, J = 1.5 Hz, 3H, H-13). ^{13}C NMR (125 MHz, Chloroform-*d*) δ 154.47 (C-7), 135.45 (C-5), 124.52 (C-4), 111.39 (C-15), 54.15 (C-3), 47.37 (C-6),

Table 3 Effect of different concentrations of isolated fractions from chloroform extract of *Digera muricata* and herbicide on the germination of two weeds, *Avena fatua* and *Melilotus indicus*.

Treatments	*Avena fatua*			*Melilotus indicus*		
	Fraction 2B	Fraction 7A	Herbicide	Fraction 2B	Fraction 7A	Herbicide
T1 = dH_2O	93 ± 3.3a	93 ± 3.3a	93 ± 3.3a	87 ± 3.3a	87 ± 3.3a	87 ± 3.3a
T2 = 25 µg/ml	83 ± 3.3b	80 ± 5.8b	70 ± 5.8b	63 ± 3.3b	80 ± 0b	70 ± 5.8b
T3 = 50 µg/ml	80 ± 5.8bc	77 ± 3.3bc	63 ± 5.7bc	60 ± 5.8bc	73 ± 3.3c	67 ± 3.3bc
T4 = 100 µg/ml	73 ± 3.3c	73 ± 3.3c	60 ± 5.7c	53 ± 5.7c	67 ± 3.3d	60 ± 5.7d
T4 = 150 µg/ml	63 ± 3.3d	70 ± 5.8c	53 ± 3.3c	43 ± 3.3d	57 ± 3.3e	47 ± 3.3e
T6 = 200 µg/ml	53 ± 3.3e	70 ± 5.8c	37 ± 3.3d	43 ± 3.3d	57 ± 3.3e	43 ± 3.3e

Data show means ± standard error of 3 replications. Different letters show significance between the treatments at $P \leq 0.05$, as determined by ANOVA & Tukey's range test.

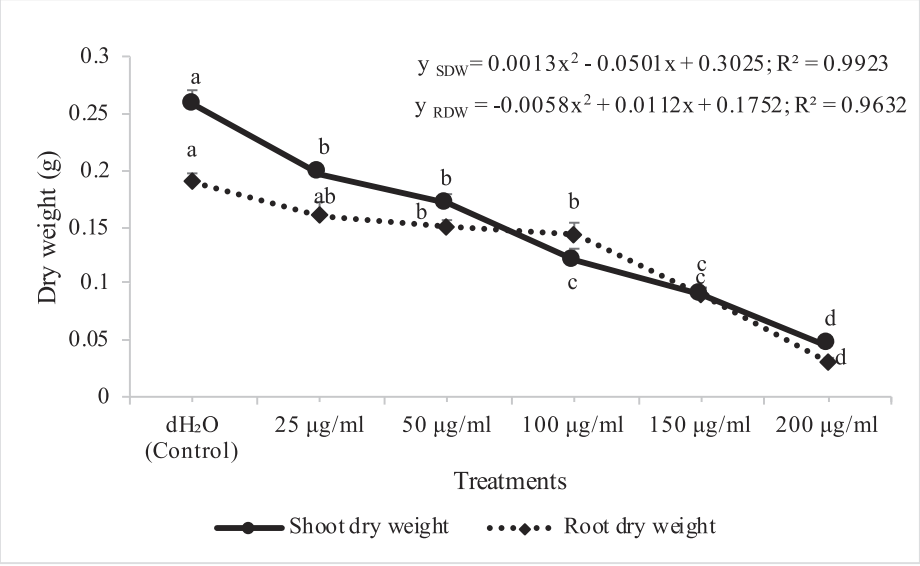

Fig. 11 Herbicidal activity of different concentrations of cyanazine herbicide on shoot and root dry weight of Petri plate grown *Melilotus indicus*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y Error bars represent standard error of means and different letters show significance.

39.79 (C-12), 39.20 (C-1), 34.53 (C-8), 33.33 (C-11), 28.26 (C-9), 27.50 (C-2), 26.37 (C-13,14), 16.56 (C-10). Chemical Formula: $C_{15}H_{24}$; Molecular Weight: 204.36; Elemental Analysis: Calculated (C, H) 88.16, 11.84; Found; (C, H) 88.14, 11.82. The physical and spectroscopic data agreed with the literature for β-caryophyllene (Dahham et al., 2015). The structures of these two compounds are presented in Fig. 13.

In an investigation, essential oil from *Tagetes erecta* exhibited herbicidal activity in terms of seed germination inhibition in weed, *Echinichloa crus-galli*. It's mode of action was described as blockage of α-amylase activity. Gas Chromatography Mass Spectrometry (GC/MS) analysis depicted the presence of piperitone, piperitenone, ocimine, sesquiterpenoids mainly neophytadiene and caryophyllene (Laosinwattana et al., 2018). Berberine, a plant secondary metabolite revealed more herbicidal effects than traditional herbicide glyphosate (Zhang et al., 2018). Extracted oils (EOs) from vegetative parts of *Copaifera* species and isolated active compounds (sesquiterpene hydrocarbons, including germacrene D, β-caryophyllene, α-humulene, δ-elemene, and δ-cadinene) by GC/MS depicted the phytotoxic activity against *Mimosa pudica* and *Senna obtusifolia*. *M. pudica* was more sensitive to these phytotoxic compounds in comparison with *S. obtusifolia* (Gurgel et al., 2019). EOs of various plant species showed phytotoxic effects against germination and early growth of *Cucumis sativus* and *Solanum lycopersicum*. Among oils extracted from different plants, Oregano was the most injurious as compared to rosemary EO (Ibáñez et al., 2020). EOs of *Vitex negundo*, showed herbicidal effect against *A. fatua* and *E. crus-galli*. GC/MS analysis depicted that *V. negundo* EO was rich in *β*-caryophyllene (Issa et al., (2020). EOs of *Piper dilatatum* and *Piper divaricatum* also exhibited phytotoxic effects against *Lolium perenne* and *Lactuca sativa*. Among isolated compounds, e.g., apiol (79%), eugenol (37%), methyl eugenol (36%), δ-3-carene (35%) and limonene (27%) at higher concentrations (Jaramillo-Colorado et al., 2019). Extracts from vegetative parts of *Inula graveolens* also revealed the phytotoxic activity against the growth of different monocotyledonous plant spp. In *in-vitro* experiments, plant extracts showed strong inhibitory effect, reduced the emergence rate

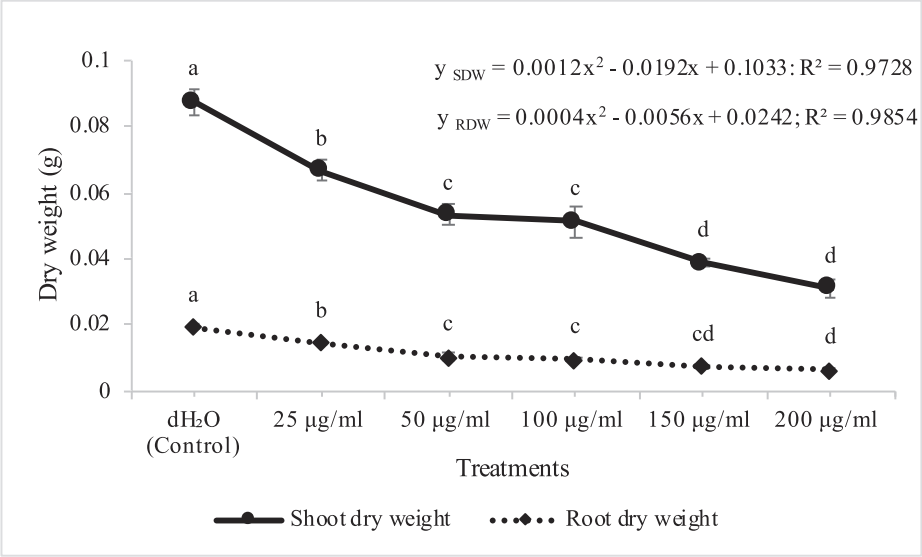

Fig. 12 Herbicidal activity of different concentrations of cyanazine herbicide on shoot and root dry weight of Petri plate grown *Avena fatua*. ANOVA and Tukey's range test were done on Minitab 19 with 3 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

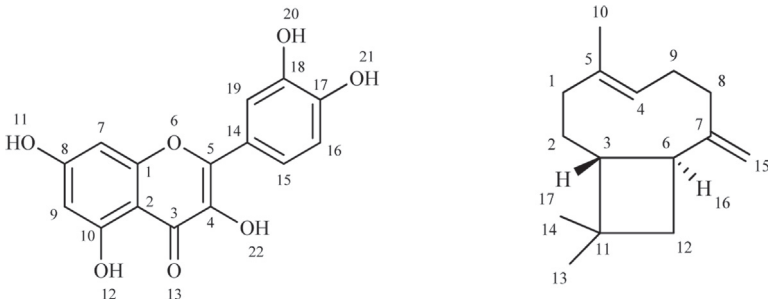

Fig. 13 Structures of identified compounds; quercetin β-caryophyllene.

and the growth of tested plant spp. Two phytotoxic compounds named as 2,3,11b,13-tetrahydroaromaticin (THA) and ilicic acid were isolated from plant extracts of *I. graveolens* (Abu Irmaileh et al., 2015). The compound royleanumioside, was separated from the chloroform extract of *Teucrium royleanum* which showed moderate phytotoxic activity against lettuce seedlings (Ahmad et al., 2015). Similarly, phenolic compounds isolated from water and methanol extracts of *Chrysanthemoides monilifera* exhibited phytotoxicity against *Isotoma axillaris* (Al Harun et al., 2015). Needles, stems and cones of *Pinus halepensis* also possess phytotoxic activities against weeds. Compounds named as a-pinene and and (Z)-caryophyllene were detected as major chemical constituents in various parts of *P. halepensis*. These extracts significantly inhibited seed germination of test weeds (Amri et al., 2012). A compound α-phellandrene, terpinen-4-ol, *trans*-pinocarveol and pinocarvone isolated from EOs of *Wedelia trilobata*, were responsible for the herbicidal activity against shoot and root growths of weedy rice and lettuce (Azizan et al., 2020).

Allelopathic interactions play very important role in the agro-ecosystems. The literature abounds with examples of plant extracts or extracts of other organisms showing allelopathic interactions in terms of modeling the growth of certain crops/plants. However, there are less examples showing actual compounds exhibiting phytotoxicity/herbicidal activity. Flavonoids are widely distributed in different plant parts. e.g., quercetin and kaempferol have already been described as allelopathic molecules. Moreover, the derivatives of quercetin like quercetin 3-methyl ether and its 4′-O-glucoside and 7-O-glucoside completely inhibited the growth of *Arabidopsis thaliana* at 100 ppm (Parvez et al., 2004). Quercetin, isolated from *Nerium oleander* declined germination of seeds, root/shoot growth of *Parthenium hysterophorus* in a dose dependent manner (Rajyalakshmi et al., 2011). Quercetin is a stronger inhibitor of auxin polar transport (Hernandez et al., 2009; Lewis et al., 2011). Flavonoids, such as quercetin, have been shown to inhibit polar auxin transport and to enhance consequent localized auxin accumulation in planta. Quercetin isolated

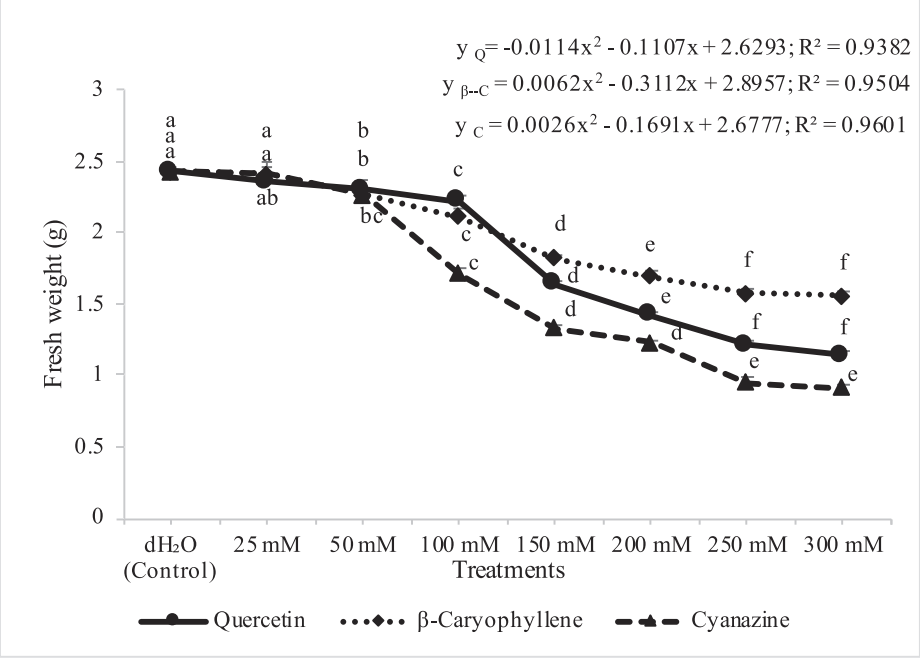

Fig. 14 Herbicidal activity of different concentrations of quercetin, β-caryophyllene and cyanazine herbicide on shoot fresh weight of pot grown *Avena fatua*. ANOVA and Tukey's range test were done on Minitab 19 with 5 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

from root of *Fagopyrum esculentum* showed inhibitory effect on *Phelipanche ramosa*. Besides reduction of radicle growth, haustorium induction was observed at the tip of *P. ramosa* radicles treated with quercetin at 0.1–0.05 mM (Fernández-Aparicio et al., 2021). The quercetin, apigenin and daucosterol isolated from *Shibataea chinensis* Nakai were more phytotoxic to germination (IC_{50} = 7.26–7.95 ppm), root (IC_{50} = 2.97–6.33 ppm) and shoot elongation (IC_{50} = 4.89–7.74 ppm) of lettuce than glyphosate (IC_{50} values greater than 20 ppm). Dihydroquercetin isolated from Egyptian plant *Jasonia montana* also showed the herbicidal activity against *Convolvulus arvensis* and *Calystegia inflata*, *Portulaca oleracea* and *Arabidopsis thaliana*. Dihydroquercetin isolated from *J. montana* decreased *C. arvensis* fresh biomass by 89.8%, when compared with control (Balah, 2011). In another investigation, dihydroquercetin isolated from *Centaurea maculosa* was shown to be phytotoxic in terms of inhibition of seed germination and growth of various plants, while quercetin was not phytotoxic to these plants (Bais et al., 2003).

β-caryophyllene, a sesquiterpene, has been described to be having allelopathic activities. β-caryophyllene affected seed germination and seedling growth of *Brassica campestris*, *Raphanus sativus, Lactuca sativa* and *Mikania micrantha*. Phytotoxicity of β-caryophyllene against these plants was dose-dependent and varied with the receptor plants (Wang et al., 2010). In another investigation, β-caryophyllene inhibited seedling growth of *B. campestris* and *R. sativus* (Wang et al., 2009). β-caryophyllene is also reported from *Artemisia lavandulaefolia*, and inhibited the seedling growth of *Achyranthes japonica* (Kil et al. 2000). β-caryophyllene isolated from *Senecio salignus* inhibited root length of *Physalis ixocarpa* by 42 and 53% at 50 and 150 µg/mL respectively. However, β-caryophyllene inhibited root length of *E. crus-galli* by 30% at 150 µg/ml concentration. β-caryophyllene also inhibited the dry biomass of *P. ixocarpa* by 37.5% in comparison with control at 100 µg/ml concentration.

3.5. Herbicidal activity of quercetin, β-caryophyllene and cyanazine on the fresh biomass of pot grown Avena fatua and Melilotus indicus

The toxic effects of quercetin, β-caryophyllene and cyanazine on the fresh shoot biomass were found significant against *A. fatua* and *M. indicus*. In *A. fatua,* the effect of 25–50 mM each of quercetin, β-caryophyllene and cyanazine was nonsiginificant when compared to control as well as when compared amonst themselves. However, the higher concentrations(100–300 mM) of quercetin, β-caryophyllene and cyanazine were found to cause significant decline in the fresh biomass of *A. fatua*. A dose dependant response was recorded from 25 to 200 mM concentrations, however, further increased concentrations did not depict significant effects. The highest inhibition in the fresh biomass of *A. fatua* was observed by the application of cyanazine followed by quercetin and β-caryophyllene was the least effective when compared with either quercetin or cyanazine.

In *M. indicus,* the effect of 25–50 mM of quercetin was found non significant however, the higher concentrations were found having significant effect on the fresh biomass of *M. indicus*. In case of β-caryophyllene and cyanazine, significant effects were observed at 50 mM and higher concentrations and these effects were obviously dose dependent up to 250 mM concentrations. Beyond 250 mM, nonsignificant effects of quercetin, β-caryophyllene and cyanazine were recorded (Figs. 14 and 15). The effects of quercetin, β-caryophyllene and cyanazine against wheat were in general non significant, so data were not presented in this paper. Quercetin was more active as compared to β-caryophyllene but the

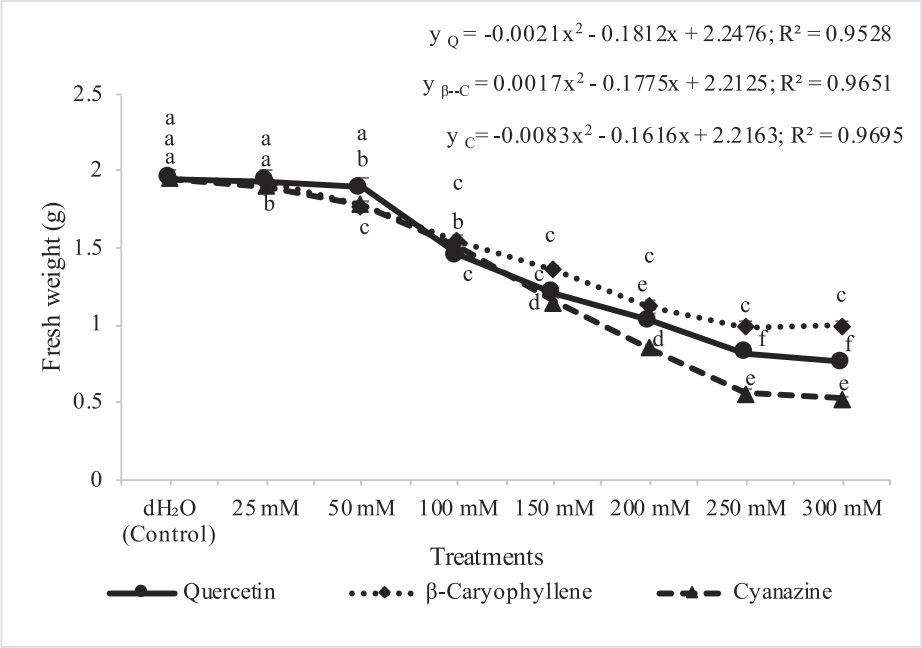

Fig. 15 Herbicidal activity of different concentrations of quercetin, β-caryophyllene and cyanazine herbicide on shoot fresh weight of pot grown *Melilotus indicus*. ANOVA and Tukey's range test were done on Minitab 19 with 5 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

cyanazine was even more effective when compared to quercetin. Application of phytotoxic compounds/essential oil is known to cause decrease in the biomass of test plants (Bainard et al., 2006; Ben Kaab et al., 2020).

3.6. Effect on plant plasma membrane integrity

The application of quercetin, β-caryophyllene and cyanazine significantly increased electrolyte leakage in both test weeds viz., *A. fatua* and *M. indicus*, and therefore increased conductivity of solutions. The highest conductivity was observed by the application of, cyanazine against both the test weeds. Quercetin was more effective in the increase of conductivity as compared to β-caryophyllene. *M. indicus* was more sensitive to the application of quercetin, β-caryophyllene and cyanazine. The effect of all these compounds on disintegration of membrane was in general dose dependent. The application of quercetin, β-caryophyllene and cyanazine caused significant loss of membrane integrity and this was evident by the increased electrolyte leakage in both test weeds viz., *A. fatua* and *M. indicus* (Figs. 16 & 17). Natural compounds like clove oil and its main constituents namely eugenol, β-caryophyllene, and α-humulene are known to cause membrane damage in planta and therefore caused electrolyte leakage leading to cell death (Stokłosa et al., 2012). In another study, clove oil and eugenol caused leaf cell membrane damage and electrolyte leakage in broccoli, lambsquarters and redroot pigweed (Bainard et al., 2006). Along with membrane damage, flavonoids like quercetin inhibited ATP production (Stenlid 1970). Flavonoids also influence auxin transport (Peer and Murphy, 2007) and promote the allelopathic effects (Bais et al., 2006), such as kaempferol, quercetin and naringenin (Macías et al., 2007). Moreover, these compounds are considered potent allelochemicals disturbing the mitochondrial O_2 regulation (Moreland and Novitski, 1988). Flavonoids destroy indoleacetic acid via IAA oxidase, resulting in the inhibition of ATP formation. Flavonoids may also interfere with the mode of actions of plant hormones such as auxins [Peer and Murphy 2007]. Quercetin can interact with components of both the ATP-generating and electron transport pathways. Interference with the phosphorylation pathway was evidenced by the higher sensitivity of the phosphorylation reaction than coupled whole-chain electron transport, inhibition of cyclic phosphorylation, inhibition of the light-activated Mg^{2+}-ATPase, and inhibition of the heat-activated Ca^{2+}-ATPase associated with CF_1 in chloroplasts of *Spinacea oleracea*. (Moreland and Novitzky, 1988). Any modification of the plant plasma membrane structure by allelopathic substances disturb its function and integrity (Lins et al., 2019). In that regard, the phytotoxic effects observed in the present study may be linked to the fact that plant plasma membrane is one of the targets of the test compounds, due to their lipophilicity and small size, as shown for some essential oil compounds (Lebecque et al., 2018). Mode of action of β-caryophyllene was described as inhibition of photosystem II and induction of chlorosis on treated leaves (Sánchez-Muñoz et al., 2012). Hence, it may be argued that the phytotoxic effects observed during the present study may be attributed to multiple effects of bioactive compounds as discussed by earlier workers because natural compounds may have more than one target site in the plants. In the present investigation, wheat was found resistant to the application of isolated compounds as well as commercial herbicide, cyanazine. The resistance of wheat to applied compounds as well as cyanazine could be explained due to the fact that the weeds are more sensitive to these compounds than the crops (Grichi et al., 2016), hence, these molecules can act as lead compounds for developing natural herbicides.

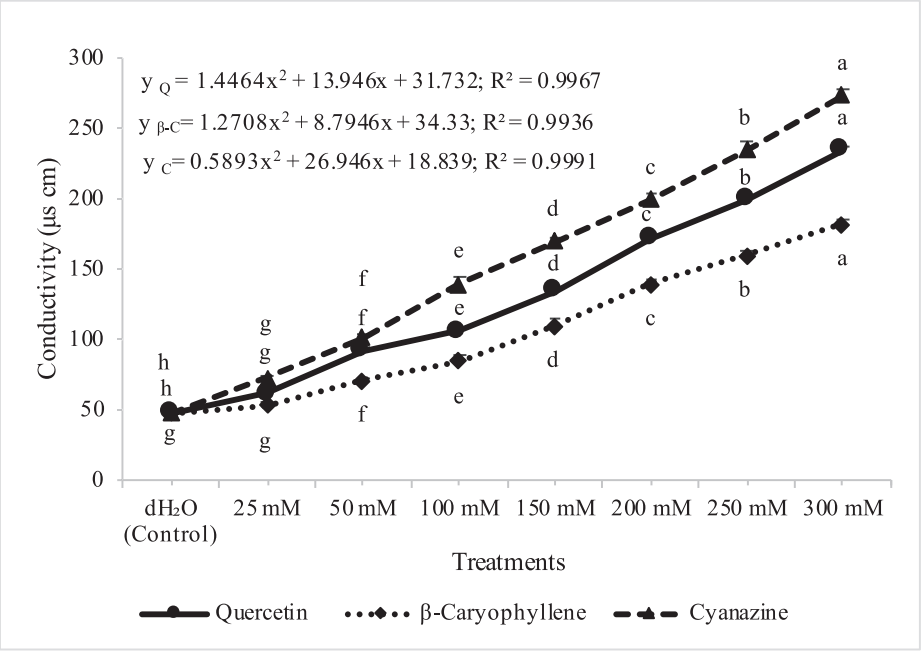

Fig. 16 Conducivity measurements at different concentrations of quercetin, β-caryophyllene and cyanazine herbicide on *Avena fatua*. ANOVA and Tukey's range test were done on Minitab 19 with 5 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

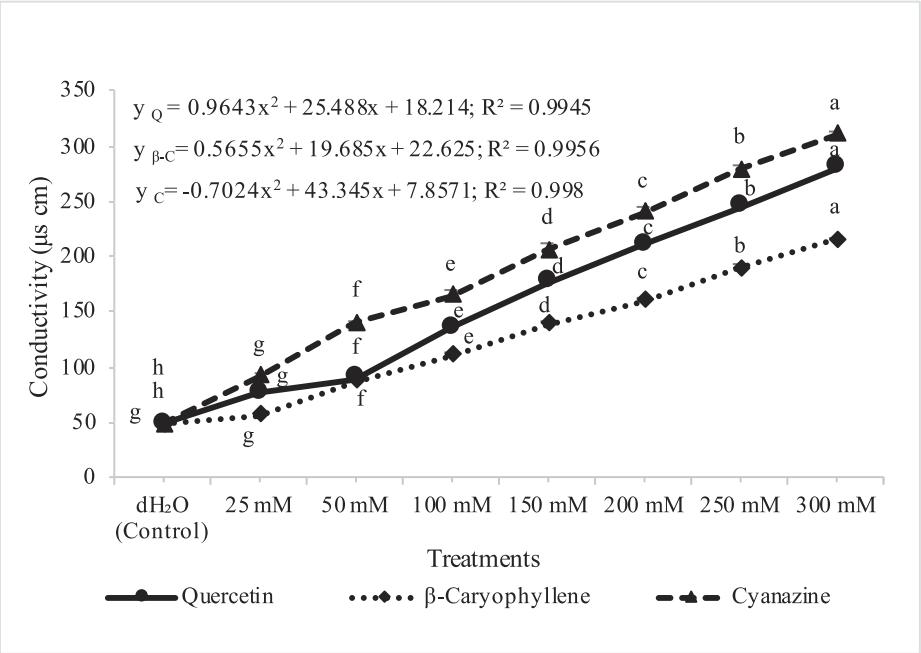

Fig. 17 Conducivity measurements at different concentrations of quercetin, β-caryophyllene and cyanazine herbicide on *Melilotus indicus*. ANOVA and Tukey's range test were done on Minitab 19 with 5 replicates at $P \leq 0.05$. Y error bars represent standard error of means and different letters show significance.

4. Conclusions and recommendations

The present study demostrated the herbicidal potential of *Digera muricata* against *Avena fatua* and *Melilotus indicus*. *In vitro* experiments depicted that chloroform extract of *D. muricata* as well as the purified compounds viz., quercetin and β-caryophyllene significantly decreased dry biomass of *A. fatua* and *M. indicus*. In post emergence bioassays, the isolated com-

pounds significantly decreased the biomass of both weeds. Membranne integrity assays depicted the electrolyte leakage in the both weeds by the application of quercetin, β-caryophyllene and cyanazine. As there were no bad effects of isolated compounds of *D. muricata* against wheat, so, total synthesis of these compounds can be made and used to manage weeds in wheat.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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