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Research Article

Identification of antifungal compounds from slender amaranth

Muhammad Akbar[a]*, Iram N. Sherazi[a], Tayyaba Khalil[a], Muhammad S. Iqbal[a], Shamim Akhtar[a], Salik N. Khan[b]

[a] Department of Botany, University of Gujrat, Gujrat, 50700, Pakistan; [b] Institute of Agricultural Sciences, University of the Punjab, Lahore-54590, Pakistan.

INFORMATION ARTICLE

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***Corresponding author:**
<makbarpu@gmail.com>

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Conflict of Interest:
The authors declare that there is no conflict of interest regarding the publication of this manuscript.

HIGHLIGHTS

- Antifungal activity of *Amaranthus viridis* leaves was evaluated against five phyto-pathogenic fungi.
- Ethyl acetate leaf fraction reduced the fungal growth up to 48%.
- GCMS analysis revealed 1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester) showing 58.5% peak area.

ABSTRACT

Background: Antifungal activity of slender amaranth (*Amaranthus viridis* L.) is well documented but such studies are scarce in Pakistan, especially against plant pathogens. It was hypothesized that *A. viridis* has antifungal activity against fungal phyto-pathogens also.
Objective: Identification of antifungal constituents from leaf extracts of *A. viridis*.
Methods: Different organic solvent extracts of *A. viridis* leaves were evaluated against 5 plant pathogenic fungal species viz. *Alternaria alternata, Aspergillus flavus, Drechslera australiensis, Fusarium oxysporum* and *Macrophomina phaseolina*. Antifungal activity of *A. viridis* was determined by serial dilution method. Six levels (0, 5, 10, 15, 20 and 25 mg mL^{-1}) of treatments of each *n*-hexane, chloroform and ethyl acetate were employed against all fungal species in a Completely Randomised Design (CRD).
Results: Generally, all organic solvent extracts reduced the fungal biomass significantly with the increase in extract concentration but ethyl acetate leaf fraction exhibited pronounced activity and reduced the fungal growth up to 44% in *A. alternata*, 39% in *A. flavus*, 48% in *D. australiensis,* 48% in *F. oxysporum* and 45% in *M. phaseolina.* Gas Chromatography Mass Spectrometry (GCMS) analysis of ethyl acetate leaf fraction revealed 09 compounds. Out of these 9 compounds, one compound identified as 1,2- Benzenedicarboxylic acid, mono (2-ethylhexyl) ester) showed 58.5% peak value.
Conclusions: It was concluded that 1,2- Benzenedicarboxylic acid, mono (2-ethylhexyl) ester) being in the highest concentration in the ethyl acetate leaf fraction of *A. viridis* may be responsible for antifungal activity. This compound can serve as structural analog to develop ecofriendly fungicides.

1 INTRODUCTION

Major threat being faced by the agri-based economies is the plant diseases (Strange and Scott, 2015). Studies revealed that major contributors for crop destruction are fungi (Wang and Jin, 2017). Some of the fungal pathogens that largely affect crops are *Alternaria alternata* that causes Alternaria black spot of rose, *Colletotrichum gloeosporioides* infects loquat and *Fusarium solani* causes strawberry fruit rot

(Abbas et al., 2017; Naz et al., 2017; Mehmood et al., 2017). Similarly, *Aspergillus flavus* produces maize ear rots and mycotoxin contamination (Shu et al., 2015). *Acacia nilotica* is affected by *Drechslera australiensis* (Ahmad et al., 2017). Fusarium head blight of wheat is caused by *Fusarium graminearum* (Li et al., 2015), *Macrophomina phaseolina* is a phytopathogen that causes stalk rot in soybean crop (Ramos et al., 2016). *M. phaseolina* has also been reported to infect about 500 plant species and no commercial fungicide has been recommended so far to combat this fungal pathogen (Javaid et al., 2017).

Agriculture sector has been using fungicides for fungal disease control that have substantially increased agriculture productivity. However, excessive use of these chemicals has resulted in food contamination (Wu et al., 2014), negative environmental impacts and disease resistance that have caused drastic impacts on food security and human health (Singh and Trivedi, 2017). Therefore, there is dire need to explore nature friendly methods of pathogen control (Kim et al., 2017).

At present, search for natural products, specifically linked to pathogen management in sustainable agriculture is very active (Droby et al., 2009; Sharma et al., 2009). Endeavors have been made to control plant infection by pathogens utilizing plant extracts (Chaudhary and Chaudhari, 2013; Elsharkawy and El-Sawy, 2015). Specific plant extracts having antimicrobial properties comprise numerous secondary metabolites such as alkaloids, flavonoids, glycosides quinones, saponins, tannins and terpenoids (Balakumar et al., 2011; Gillitzer et al., 2012; Sales et al., 2016). Weeds are well adapted in all types of agricultural lands because they have unique type of bioactive phytochemicals and these phytochemicals also have antimicrobial properties (Dhankhar et al., 2013; Chah et al., 2006). Extracts from plants viz. *Cinnamomum zeylanicum*, *Cymbopogon proximus*, *Laurus nobilis*, *Persea Americana* and *Zingiber officinale* showed significant antifungal activity against *Fusarium oxysporum* (Fawzi et al., 2009). *Phakopsora pachyrhiz* is a causal agent of soybean rust disease and it is the decisive foliar disease of soybean worldwide. Organic extracts and essential oil of *Nandina domestica* possess antifungal activity against *F. oxysporum* (Bajpai et al., 2009). Extracts of *Orobanche crenata* and *Sanguisorba minor* also represent as a natural mean for controlling *A. alternata* (Alternaria rot) and a number of other pathogens (Romanazzi et al., 2008; Gatto et al., 2016).

Amaranthus belongs to family Amaranthaceae and well known for having medicinal properties as well as its use as food (Bokaeian et al., 2013). Few studies have shown that extracts obtained from *Amaranthus* spp. have antimicrobial activities. According to the studies conducted so far, *Amaranthus hypochondriacus* extract possesses potent antifungal activities towards *A. alternata*, *F. solani, Candida albicans, F. oxysporum, Trichoderma* sp. and *Aspergillus ochraceus* (Rivillas-Acevedo et al., 2007; (Bahrami-Teimoori et al., 2017). Moreover, methanolic root extract of *Amaranthus spinosus* have possibility to be used as a bio fungicide to control spore propagation of *Phakopsora pachyrhizi* (Yusnawan, 2015). Past investigations have proved the antifungal potential of amaranth water-soluble extract, using agar diffusion, conidia germination and dry biomass tests, utilizing *Penicillium roqueforti* as the indicator fungus. The crude water-soluble extract had minimal inhibitory concentration (MIC) of 5 mg mL^{-1} and indicated hindrance towards countless fungal species isolated from bakeries (Rizzello et al., 2009).

As the natural products are attaining focus of scientists worldwide because they are safer and environment friendly (Martínez et al., 2017), therefore, weeds can be used as an inexpensive material for the management of microbial pests because of their wide availability and easy collection. Although there are number of reports regarding antifungal activities of *A. viridis* around the world but such studies are scarce in Pakistan especially against plant pathogens. Therefore, the purpose of present investigation was to explore antifungal activity of extracts of *A. viridis* against fungal plant pathogens that could be useful for the expansion of modern tools for the control of infectious phytopathogenic diseases.

2 Materials and Methods

2.1 Plant description and extraction of metabolites

The leaves of *Amaranthus viridis* were collected from healthy plants growing wild in suburbs of district Sialkot, Pakistan. Leaves were washed under running tap water to remove dust particles and other impurities. After washing, leaves were rinsed thrice in distilled autoclaved water for 1 minute and sun dried for 7 days. The dried plant leaves were converted to fine powder using pestle and mortar. Resulting powder was stored in glass containers and kept at 4 °C till further use.

Two hundred grams of leaf powder of *A. viridis* was soaked in methanol (1000 mL), in a glass container,

covered with glass lid for 7 days at 25 ºC. This mixture was stirred on periodically after every 12 hours, using glass rod. After this incubation period, the extract was first filtered through 4 layers of cotton cloth to remove larger particles. Final filtration was accomplished through Whatman filter paper No. 1. After filtration, metabolites were further purified by centrifugation. The metabolites thus obtained were concentrated at 45 ºC, using a rotary evaporator (Model: Laborata 4000/Gl, Heildoph Germany). This process was repeated three times to obtain adequate amount of methanol extracted metabolites. Extra solvent from the methanolic metabolites was evaporated under clean air currents to obtain methanol free extract. This methanol free extract comprising *A. viridis* leaf metabolites was reconstituted in sterilized dH_2O and further fractioned using different organic solvents viz. *n*-hexane, chloroform and ethyl acetate. All solvents used in the present study were of analytical grade. The resulting fractionated metabolites were evaporated under vacuum as in case of methanolic extract to get solvent free fractionated metabolites. This procedure was done in order to fractionate secondary metabolites in the methanolic extracts of *A. viridis* leaves (Akbar et al., 2014, 2017).

2.2 Fungal cultures

Fungal cultures were obtained from Fungal Culture Bank of Pakistan (FCBP), University of the Punjab, Lahore. Pakistan. All fungal cultures were maintained in the laboratory on potato agar medium, both as in slants as well as glass Petriplates. Following 5 fungal isolates/cultures were procured from FCBP, viz. *Alternaria alternata* (Accession No. 1285), *Aspergillus flavus* (Accession No. 1261), *Drechslera australiensis* (Accession No. 482), *Fusarium oxysporum* (Accession No. 1175) and *Macrophomina phaseolina* (Accession No. 1156).

2.3 Antifungal assays

For the preparation of stock solutions, *A. viridis* leaf extract (150 mg) each of *n*-hexane, chloroform and ethyl acetate were dissolved in DMSO (166 µL). Final volume (500 µL) was made by the addition of autoclaved distilled water. Control solution consisted of DMSO (166 µL) in distilled water (333 µL). Potato dextrose broth medium was autoclaved at 121 ºC for 20 minutes. When this growth medium was cooled down to 60 ºC, antibacterial (Amoxiline 250 mg) was added into the fungal growth medium to elude bacterial contamination. Six concentrations of each extract viz, 0, 5, 10, 15, 20 and 25 mg mL^{-1} were organized by pouring 0, 20, 40, 60, 80 and 100 µL stock solution and 100, 80, 60, 40, 20 and 0 µL of control solution respectively, to 1.1 mL of growth medium to make final volume of mixture (1.2 mL). All treatments in all experiments were replicated thrice. Spore suspension of each test fungus was freshly prepared and used with in 1 hour. The spore suspension (20 µL) of each test fungal isolate was separately added into growth mixture. This experiment was incubated for 3 days at 27 ºC for appreciable measurable fungal growth. After the completion of incubation at above mentioned specified conditions, filtration of fungal biomass was done on pre-weighted filter paper and this filtered material was dried in electric oven at 70 ºC to obtain a dry fungal biomass. Similar procedure was applied to all fungal species for recording biomass of selected plant pathogenic fungal species (Ali et al., 2017).

2.4 Identification of antifungal compounds through Gas Chromatography Mass Spectrometry (GCMS)

The biochemical constituents of ethyl acetate fraction of *A. viridis* extract were analyzed by using GC-MS with model number 7890A/5975C of Agilant Technologies in the Department of Chemistry, Forman Christian College University, Lahore, Pakistan. The GC-MS instrument was equipped with column # HP-5MS (30 m × 250 µm × 0.25 µm). Helium gas with 99.99% purity was used with constant flow rate at 1 mL $minute^{-1}$. Material was employed with injection of 2 µL volume. After injector the temperature was 240 ºC. Oven temperature was programmed first from 60 ºC for 2 min with increase of 5 ºC min^{-1} to 80 ºC for 0 minute, then 10 ºC min^{-1} to 310 ºC for 5 minutes and the total GC running time was 34 minutes. Relative quantities of all constituents were computed by using same method as described by Lakshmi et al. (2012) with slight modifications.

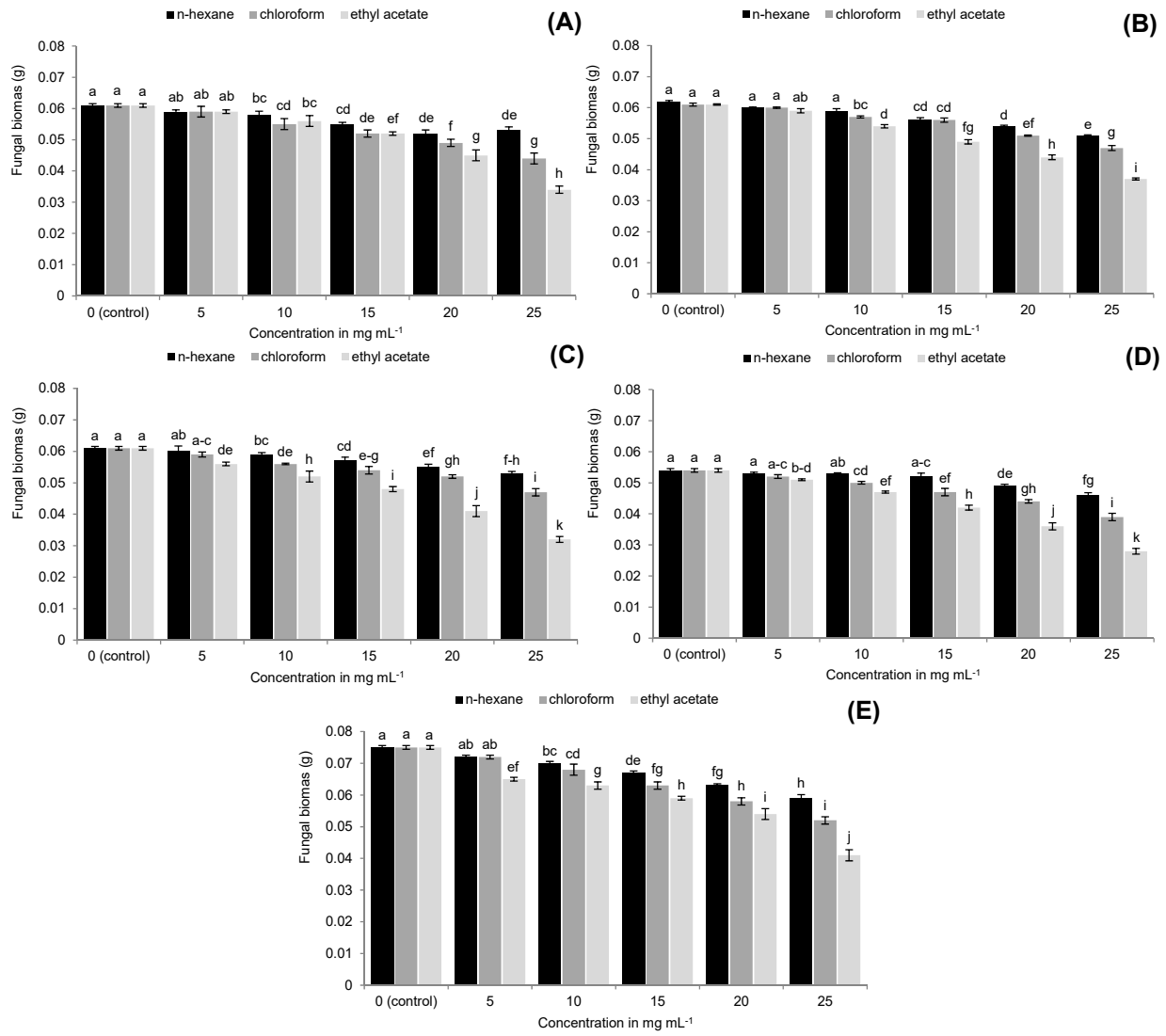
2.5 Statistical analysis

All the data were analyzed to demarcate treatment means through ANOVA followed by Fisher's LSD test using Minitab Statistical Software- Minitab 17.

3 Results and Discussion

3.1 Antifungal activity of *A. viridis* extracts against *A. alternata*

Data regarding inhibition effect of *n*-hexane, chloroform and ethyl acetate leaf metabolites of *A. viridis* against *A. alternata* are presented in Figure 1A. In general, *n*-hexane, chloroform and ethyl acetate extract of *A. viridis* leaves incurred significant inhibitory effects in different employed concentrations



Values topped with different letters show a significant difference ($P \leq 0.05$), as determined by Fisher's LSD test using statistical software Minitab 17. Vertical bars show standard errors of means of three replicates.

Figure 1 - Effect of different concentrations of *n*-hexane, chloroform and ethyl acetate extract of *Amaranthus viridis* leaf on biomass of (A) *Alternaria alternata,* (B) *Aspergillus flavus,* (C) *Drechslera australiensis,* (D) *Fusarium oxysporum* and (E) *Macrophomina phaseolina.*

viz. 10, 15, 20 and 25 mg mL^{-1} except the lowest employed concentration of 5 mg mL^{-1} where non significant effects were recorded. *n*-hexane extract of *A. viridis* leaves caused 3, 5, 10, 15 and 13% reduction in fungal biomass as compared to control at 5, 10, 15, 20 and 25 mg mL^{-1} concentration. In case of chloroform extract of *A. viridis* leaves, better antifungal activity was depicted by different employed concentrations. In this case, chloroform extract of *A. viridis* leaves caused 3, 10, 15, 16 and 28% reduction in fungal biomass as compared to control at 5, 10, 15, 20 and 25 mg mL^{-1} concentration, respectively. On the other hand, the highest antifungal activity was recorded in case of ethyl acetate extract of *A. viridis* leaves where different corresponding concentrations caused 3, 8, 15, 26 and 44% decline in biomass of test fungal species. This clearly shows that ethyl acetate extract of *A. viridis* leaves possess comparatively higher concentration of antifungal constituents as compared to *n*-hexane and chloroform extracts. In past, few studies have shown that extract of *Amaranthus* spp. possess antifungal activities e.g. *A. hypochondriacus* protein extract demonstrated antifungal activities against a number of fungal pathogens e.g. *A. alternata*, *Fusarium solani*, *Candida albicans*, *F. oxysporum*, *Aspergillus ochraceus* and *Trichoderma* sp. (Rivillas-Acevedo and Soriano-Garcia, 2007).

3.2 Antifungal activity of extract of *A. viridis* against *A. flavus*

Data regarding effect of different treatments of *n*-hexane, chloroform and ethyl acetate leaf extract of *A. viridis* against *A. flavus* are presented in

Figure 1B. In general, *n*-hexane, chloroform and ethyl acetate extract of *A. viridis* leaves caused significant inhibitory effects in different employed concentrations viz. 10, 15, 20 and 25 mg mL^{-1} except the lowest employed concentration of 5 mg mL^{-1} and *n-hexane* extract at 10 mg mL^{-1} where non significant effects in terms of reduction in fungal biomass were recorded. *n*-hexane extract of *A. viridis* leaves caused 2, 3, 8, 11 and 16% reduction in fungal biomass as compared to control at 5, 10, 15, 20 and 25 mg mL^{-1} concentration. In case of chloroform extract of *A. viridis* leaves, better antifungal activity was depicted by different employed concentrations. In this case, chloroform extract of *A. viridis* leaves caused 2, 6, 8, 16 and 23% reduction in fungal biomass as compared to control at 5, 10, 15, 20 and 25 mg mL^{-1} concentration, respectively. On the other hand, the highest fungicidal activity was recorded in case of ethyl acetate extract of *A. viridis* leaves where different corresponding concentrations caused 3, 11, 20, 28 and 39% reduction in test fungal species. This clearly showed that ethyl acetate extract of *A. viridis* leaves possess comparatively higher concentration of antifungal constituents as compared to *n*-hexane and chloroform extracts.

3.3 Antifungal activity of extract of *A. viridis* against *D. australiensis*

Data in Figure 1C show that different concentrations of *n*-hexane extract did not show potent antifungal activity against *D. australiensis*. Maximum 13% inhibition was seen against 25 mg mL^{-1} concentration as compared to control. While in case of lowest concentration of 5 mg mL^{-1}, biological activity was found statistically non significant as the fungal biomass did not reduce significantly at this level of *n*-hexane extract concentration. Data about the results of chloroform extract of *A. viridis* against plant pathogenic fungus *D. australiensis* are shown in Figure 1C. The fungal biomass was decreased from 3 to 23% in comparison with control. Very low non significant decrease was seen at 5 mg mL^{-1} concentration but this activity further increased significantly with the increased level of extract concentration where 8, 11, 15, 23% reduction in fungal biomass was recorded at 10, 15, 20 and 25 mg mL^{-1}, chloroform extract concentration. Effect of ethyl acetate extract on *D. australiensis* exhibited significant results at all concentrations tested as shown in Figure 1C. Maximum decline in fungal biomass was measured at the highest concentration of ethyl acetate and that was 47% as compared to control. It was clearly noted that ethyl acetate extract showed significant results against this fungus and the activity was increased with the increase in concentration of extract from 5-25 mg mL^{-1}. Moreover, ethyl acetate extract was found potent at minimum concentration of 5 mg mL^{-1} where other two extracts failed to exhibit any significant fungicidal effects. Furthermore, dose dependent effects were recorded as the fungicidal activity increased with increasing the extract concentration. There was a significant reduction of 15, 21, 33, 47% in fungal biomass as recorded at 10, 15, 20 and 25 mg mL^{-1} chloroform extract concentrations, respectively.

3.4 Antifungal activity of extract of *A. viridis* against *F. oxysporum*

Data in Figure 1D show that different concentrations of *n*-hexane extract did not show good antifungal activity against *F. oxysporum*. Maximum 15% inhibition was seen against 25 mg mL^{-1} concentration as compared to control. While, in case of concentrations ranging from 5-15 mg mL^{-1}, the fungal biomass did not reduce significantly. Data about the results of chloroform extract of *A. viridis* against plant pathogenic fungus *F. oxysporum* are shown in Figure 1D. The fungal biomass was decreased from 3.7 to 28% in comparison with control. Effects were found nonsignificant at 5 mg mL^{-1} concentration but this activity further increased with the increased level of extract concentration. In other tested chloroform extract concentrations of 10, 15, 20 and 25 mg mL^{-1}, there were significant inhibitory effects of 7, 13, 19 and 28%, respectively. Effect of ethyl acetate extract on *F. oxysporum* was found significant as shown in Figure 1D. Maximum decline in fungal biomass was measured at the highest concentration of ethyl acetate that was 48.15% as compared to control. It was clearly noted that ethyl acetate extract showed higher significant results against this fungus and the activity was increased with the increase in concentration of extract from 5-25 mg mL^{-1}. Different corresponding concentrations of ethyl acetate extract incurred 6, 13, 22, 33 and 48% decrease in fungal biomass at 5, 10, 15, 20 and 25 mg mL^{-1} ethyl acetate extract concentrations.

3.5 Antifungal activity of extract of *A. viridis* against *M. phaseolina*

Data about the effect of *n*-hexane extract of *A. viridis* leaves on the growth of *M. phaseolina* are shown in Figure 1E. Different *n*-hexane concentrations ranging from 5 to 25 mg mL^{-1} of *A. viridis* showed reduction in fungal biomass by 5 to 25% as compared to control but the inhibitory effect of *n*-hexane extract at 5 mg mL^{-1} concentration was non significant. Whereas, effect of chloroform extract of *A. viridis* on the biomass of

plant pathogenic fungi, *M. phaseolina* with same concentrations is shown in Figure 1E. According to data shown in figure, the growth of test fungus was gradually decreased from 3 to 31% with the increase in extract concentration as compared to control. Maximum, 45% decrease in biomass was noted against ethyl acetate extract at 25 mg mL^{-1} concentration. It was seen that ethyl acetate extract showed better response at all levels against the test plant pathogenic fungus, *M. phaseolina.*

In a previous investigation, *Amaranthus retroflexus* has proved to be having antifungal activity against a number of fungal pathogens. The 50% minimum inhibitory concentrations (MIC_{50}) against *M. phaseolina*, *A. alternata* and *F. oxysporum* were recorded to be 159.80 ± 14.49, 337.09 ± 19.72, and 328.05 ± 13.29 µg mL^{-1}, respectively. However, not all fungal species investigated were inhibited successfully by the application of *A. retroflexus* derived bioactive compounds like in case of *Trichoderma harzianum* and *Geotrichum candidum* (Bahrami-Teimoori et al., 2017). But in the present research work, leaf extract of *A. viridis* has shown fungicidal activity against all test plant pathogenic fungal species. This clearly shows that leaf extract of *A. viridis* has better chemical constituents that can be harnessed as natural antifungal compounds.

3.6 GC-MS analysis

A total 9 compounds were present and identified in the ethyl acetate fraction of *A. viridis*. The retention time (RT), concentration of component (%) molecular weight and their molecular formulas are presented in Table 1. Diphenylamine; 1-Hexadecanol, 2-methyl-; 1-Eicosene; 1-Docosene; 1-Eicosene; 1-Hexacosene; 2-Hexadecanol; 1,2-Benzenedicarboxylic acid, mono(2-ethylexyl) ester; 18, 19-Secoyohimban-19-oic acid, 16,17,20,21-tetradehydro-16-(hydroxymethyl)-, methyl ester, (15.beta., 16E)-. These compounds were also reported by (Mohamed, 2012; Alshammaa, 2017) in *A. viridis* during their research work. Amongst these compounds, 1,2-Benzenedicarboxylic acid, mono(2-ethylexyl) ester, was detected as having the highest concentration in the ethyl acetate extract from *A. viridis* leaves (Figure 2). A compound with slightly different structure from 1,2-Benzenedicarboxylic acid, mono(2-ethylexyl) ester reported in the present study was also reported in another study on *Senna occidentalis* extract that exhibited antifungal activity against *M. phaseolina* (Javaid et al., 2017). Moreover,

Table 1 - List of compounds identified in GC-MS analysis of ethyl acetate leaf extract of *Amaranthus viridis*

Sr.#	Retention time	Molecular formula	Name of Compound	Molecular weight	Peak value %
1	16.646	$C_{12}H_{11}N$	Diphenylamine	169	7.918
2	17.929	$C_{17}H_{36}O$	1-Hexadecanol, 2-methyl-	256	4.608
3	19.932	$C_{20}H_{40}$	1-Eicosene	280	8.871
4	21.751	$C_{22}H_{44}$	1-Docosene	308	5.154
5	21.934	$C_{20}H_{40}$	1-Eicosene	280	8.063
6	23.441	$C_{26}H_{52}$	1-Hexacosene	364	1.769
7	23.577	$C_{16}H_{34}O$	2-Hexadecanol	242	3.646
8	24.718	$C_{16}H_{22}O_4$	1,2-Benzenedicarboxylic acid, mono(2-ethylexyl) ester	278	58.521
9	24.966	$C_{21}H_{24}N_2O_3$	18, 19-Secoyohimban-19-oic acid, 16,17,20,21-tetradehydro-16-(hydroxymethyl)-, methyl ester, (15.beta., 16E)-	352	1.448

Note: Compounds highlighted in bold were detected in higher concentrations (>5%) during Gas Chromatography Mass Spectrometry (GCMS) analysis.

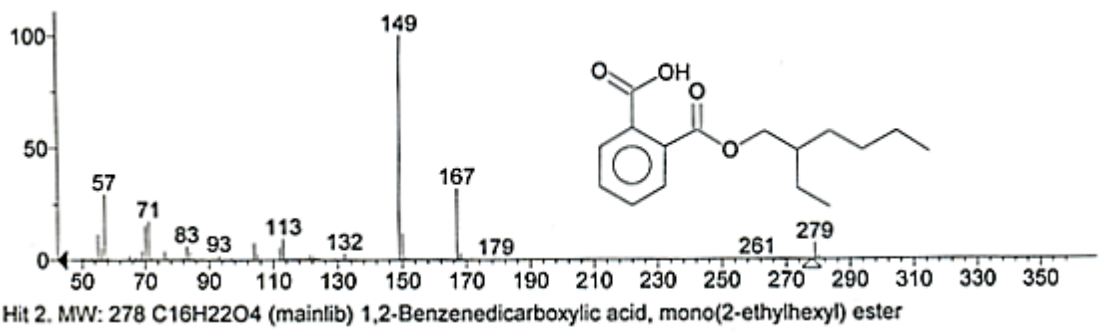


Figure 2 - 1,2-Benzenedicarboxylic acid, mono(2-ethylexyl) ester.

1- eicosene reported in the present study was also reported in another study describing antifungal activity of *Cenchrus pennisetiformis* against *Fusarium oxysporum* f. sp. *lycopersici* (Khurshid et al., 2018).

4 Conclusions

The present study concludes that ethyl acetate extract from *A. viridis* leaves contain potent antifungal constituents that can be elaborated further to elucidate their usefulness in designing commercial antifungal compounds. Especially, compound identified as 1,2-Benzenedicarboxylic acid, mono(2-ethylexyl) ester in our investigation being in the highest concentration in the ethyl acetate leaf fraction of *A. viridis* was responsible for antifungal activity observed against all test deadly pathogenic fungal species. These bioactive compounds from *A. viridis* can be exploited to develop ecofriendly fungicides against a number of plant pathogens.

5 Contributions

MA: conceived the main idea; INS: performed experiments; TK: did data analyses; MSI: did write up; SA: managed resources, and SNK: did formal analysis.

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