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Full Length Article

Potential Antifungal Constituents of *Sonchus oleraceous* against *Macrophomina phaseolina*

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Abstract

This study was done to identify possible antifungal compounds in *Sonchus oleraceous* L. (family Asteraceae) against *Macrophomina phaseolina* (Tassi.) Goid., a highly destructive fungal pathogen. Initially, methanolic extracts (10 to 50 mg mL^{-1}) of stem, leaf, inflorescence and root of this Asteraceous weed were assayed against *M. phaseolina* which reduced biomass of the fungal pathogen by 54–84%, 7–73%, 51–87%, and 49–82%, respectively, over control. The most effective stem extract was fractionated with four solvents of variable polarities. Laboratory trials with 3.125 to 200 mg mL^{-1} concentrations revealed that chloroform fraction was the most antifungal followed by *n*-hexane fraction resulting in 60–90% and 15–66% control in fungal biomass, respectively. GC-MS examination of chloroform fraction resulted in identification of 14 compounds. Hexadecanoic acid, methyl ester (13.26%), 11-octadecanoic acid, methyl ester (13.12%), 9, 12-octadecadienoyl chloride, (Z,Z)- (12.96%), 1-docosanol (8.62%), and 1, 2-benzenedicarboxylic acid, diisooctyl ester (8.28%) were the pre-dominant compounds in this fraction that might be the cause of *M. phaseolina* management.

Keywords: Antifungal; Asteraceous weed; Charcoal rot; *Sonchus oleraceous*

Introduction

The fungus *Macrophomina phaseolina* (Tassi.) Goid. is a devastating necrotrophic phytopathogen causing root rot, stem blight and charcoal rot in over 500 plant species (Khan *et al.* 2017). The most important host crops include sorghum, soybean, linseed, mungbean, alfalfa, maize, cotton and sunflower (Wang *et al.* 2019). The pathogen has a wide geographical distribution and has been reported in subtropical and tropical regions of the world (Zivanov *et al.* 2019). It attacks at any plant growth stage from seed germination to harvest thus causing average yield losses of 45–60% in severe cases (Farnaz *et al.* 2018). It is an important phytopathogen which survives as microsclerotia in plant debris or in soil for up to 12 years serving as a primary source of inoculum (Islam *et al.* 2012). The pathogen ultimately disrupts the root vascular system and leads to premature plant death (Hemmati *et al.* 2018). For decades, many fungicides have been tested to control the spread of charcoal rot pathogen but due to the persistent nature of *M. phaseolina* these are not effective in lower concentrations (Aravind and Brahmbhatt 2018; Iqbal and Mukhtar 2020). The intensive use of synthetic products has accompanied serious health concerns to humans and animals which clearly indicate the need to search for alternative effective disease management strategies (Kalsoom *et al.* 2019). Research during the last few years has led to the possibility of natural plant-based products as a realistic option against the fungal pathogens with potential stability and environmentally safe alternate to the chemical control (Shuping and Eloff 2017; Khan and Javaid 2020).

Sonchus oleraceous L. belongs to family Asteraceae, commonly known as sowthistle or milk thistle, is predominantly a winter-active annual weed plant growing in Europe, Asia, North Africa, America and Australia (Manalil *et al.* 2020). It is commonly used in the form of decoction or infusion for the procurement of diarrhea, rheumatism, inflammation, cancer, iceterohepatitis, snake venom poisoning as well as to alleviate the hypoalimentation associated problems (Saxena and Kumar 2020). It is a dicotyledonous broad-leaf plant which grows rapidly on moist, saline and fertile soil with plentiful sunlight (Peerzada *et al.* 2019). This weed inhibits growth and reproduction of pathogenic microorganisms through the production of bioactive constituents such as flavonoids, phenolic acid, luteolin 7-glucoside, apigenin 7-glucuronide, alkaloids, phenyl propanoides, taraxasterol, saponins, coumarins, steroids, terpenes, ionone glycosides and lignans (Juhaimi *et al.* 2017; Alrekabi and Hamad 2018). It has attracted the attention of many weed biologists globally due

to the presence of natural phytotoxins (Chen *et al.* 2019). However, literature about its antifungal activity against *M. phaseolina* is missing. Thus, this study was carried out to assess *in vitro* antifungal potential of *S. oleraceous* against the *M. phaseolina* and the detection of potent antifungal phytoconstituents.

Materials and Methods

Bioassays with methanolic extracts

Plants of *S. oleraceous* were collected from the waste lands of Lahore, Pakistan and washed with tap water to remove adhesive soil particles and kept under shade for drying of excessive water. Thereafter, inflorescence, stem, leaf and roots were separated and dried completely in an electric oven at 40ºC. Each dried plant part (200 g) was flooded separately in 2 L methanol for 15 days followed by a filtration process and *in vacuo* evaporation at 45ºC in order to obtain methanolic extracts in the form of gummy masses.

Screening bioassays were carried out with 9 g of each of inflorescence, stem, leaf and roots methanolic extract and dissolved in 5 mL of dimethyl sulfoxide (DMSO) in order to prepare a stock solution and raised the volume to 15 mL by adding distilled water. Likewise, a control solution was prepared simultaneously without the addition of plant extract. Different extract concentrations *viz.* 5, 4, 3, 2, 1, 0% (w/v) were prepared in sterilized beakers by adding the measured quantities of control (0, 1, 2, 3, 4, 5 mL) and stock solution (5, 4, 3, 2, 1, 0 mL) to 55 mL autoclaved malt extract (ME) broth contained in conical flasks and divided into four equal aliquots under aseptic conditions each serving as a replicate. Next, mycelial agar plugs (5 mm) of *M. phaseolina* were prepared from 7-day-old culture and placed in each flask incubated at 27°C for 7 days. Afterwards, the fungal mats were harvested on filter papers and weighed after drying at 60°C (Akhtar and Javaid 2018).

Bioassays with fractions of methanolic stem extract

Three kilograms dried stem of *S. oleraceous* was dipped in methanol for two weeks followed by filtration in order to remove suspended particles. The filtrate was evaporated at 45°C and the resultant 140 g viscous stem extract was mixed in distilled water (250 mL) and partitioned with four organic solvents on the basis of increase in their polarities. Firstly, it was partitioned with 500 mL of *n*-hexane in a separating glass funnel and the procedure was repeated five times. Subsequently, the left over phase was progressively extracted with chloroform (500 mL), ethyl acetate (400 mL) and *n*-butanol (400 mL). The obtained solvents were evaporated and kept separately in air tight jars for further experimental studies (Javaid *et al*. 2017).

Each extract (1.2 g) was dissolved in DMSO (1 mL) and volume was made up to 6 mL by the addition of ME broth to prepare a stock solution of 200 mg mL^{-1}. Through serial double dilution of this solution, 100, 50, 25, 12.5, 6.25 and 3.125 mg mL^{-1} solutions were prepared. Similarly, a control set of treatments was also obtained by adding DMSO (1 mL) in ME broth (5 mL) and serially double diluted for comparison with the experimental set. The conidial and mycelial suspension was prepared from 7-day-old culture of *M. phaseolina*, 20 µL of it was transferred to each test tube, and incubated for 7 days at 27ºC. The experimental design was a completely randomized with 3 replications. Finally, the fungal mats were filtered, dried at 60ºC and weighed (Akhtar *et al*. 2020).

GC-MS analysis

Chloroform fraction showed the best antifungal potential therefore, it was chosen for GC-MS study. For this, the selected fraction was dissolved in chloroform and filtered through a Millipore filter paper to separate unwanted particles. Thereafter, the sample was run on GC-7890A Agilant Technologies attached with MS 5975C mass spectrometer. The capillary column was 30 × 0.25 µm ID × 0.25 µm df. The electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas of 99.999% purity was used as a carrier gas at a constant flow rate of 1 mL min^{-1}. The injector temperature was maintained at 260°C, the ion-source temperature was 200°C, the oven temperature was programmed from 50°C (isothermal for 2 min), with an increase of 10°C min^{-1} to 310°C, ending with a 4 min isothermal at 310°C.

Mass spectra were obtained at 70 eV; with source temperature 250°C and MS Quad temperature 150°C. The solvent delay was 4 min, and the total GC-MS running time was 36 min. Retention indices were used for the identification of extract components and also by comparing their mass spectral fragmentation patterns with those reported in the literature and stored on the MS library (NIST database). The concentrations of identified compounds were calculated from total area of GC peaks without applying any correction factor.

Statistical analysis

Data were analyzed by ANOVA followed by application of Tukey's HSD test to delineate treatment means at $P \leq 0.05$ using Statistix 8.1.

Results

Antifungal activity of methanolic extracts

A significant difference ($P \leq 0.001$) was recorded in antifungal activity among the four parts (P) of *S. oleraceous.* Likewise, the effect of concentrations (C) and P × C was also significant. Overall, every concentration of the four parts of extracts had significant effect in controlling fungal growth. Among these, stem and root extracts were the best against *M. phaseolina* causing 56–84% and 51–87% decline

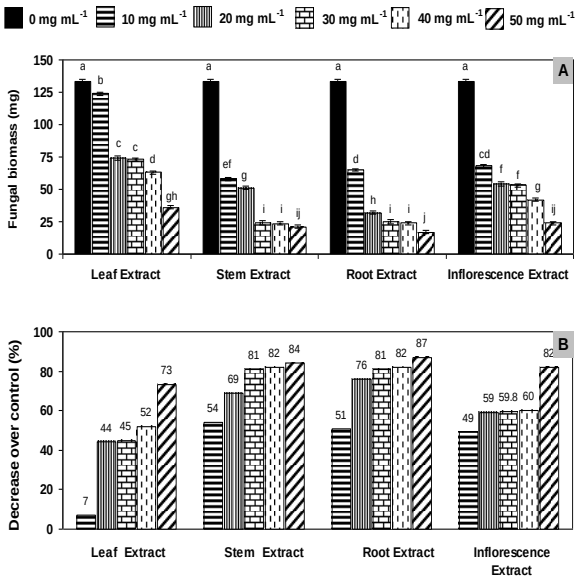

Fig. 1: Effect of methanolic extracts of different parts of *S. oleraceous* on biomass of *M. phaseolina*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Tukey's HSD Test

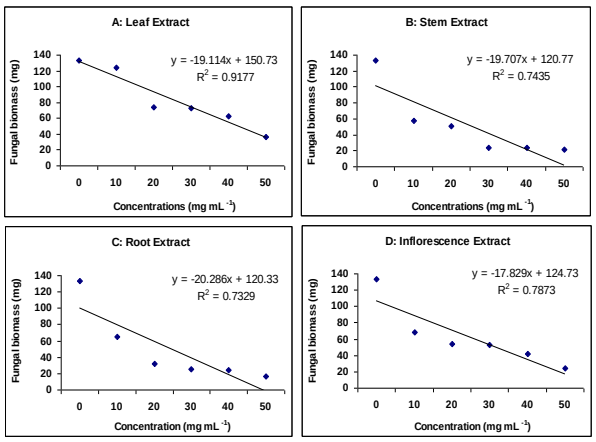

Fig. 2: Regression analysis for the effect of different concentrations of methanolic leaf, stem, root and inflorescence extracts of *S. oleraceous* on biomass of *M. phaseolina*

in biomass, respectively over control. Inflorescence extract also showed a marked antifungal activity but less pronounced than that of stem and root extracts. This extract caused a decline of 49–82% in fungal biomass. Leaf extract indicated the least antifungal activity and decreased fungal growth by 7–73% (Fig. 1A–B). The relationship between concentrations of various extracts and *M. phaseolina* biomass was linear (Fig. 2).

Antifungal activity of fractions of methanolic stem extract

Data concerning the antifungal activity of the five sub-fractions of stem extract on *M. phaseolina* growth demonstrated a noticeable difference in antifungal potential among the sub-fractions. The most effective among these was chloroform sub-fraction, which caused 60–90% reduction in *M. phaseolina* biomass over control. Likewise, *n*-hexane sub-fraction also showed noticeably high antifungal activity. Its different concentrations caused 15–68% control in fungal biomass. All the other three sub-fractions showed an insignificant effect. Other sub-fractions reduced fungal biomass just by 2–21% (Fig. 3, 4).

GC-MS analysis

The GC-MS chromatogram is shown in Fig. 5 that indicates 14 compounds. Details of compounds regarding their molecular formulae and weights, retention time and peak area percentages are shown in Table 1. The identified compounds of this sub-fraction generally belonged to alkenes, fatty acid methyl esters, fatty alcohols, phenolics and aliphatic aldehyde etc. Among the identified compounds, four were ranked as major compounds because they constitute 47.97% of the total compounds. The most abundant among these was hexadecanoic acid, methyl ester **(5)** followed by 11-octadecenoic acid, methyl ester **(8)** with very close peak areas of 13.26 and 13.12%, respectively. The other two major compounds were 9,12-octadecadienoic acid, methyl ester, (E,E)- **(7)** and 1-docosanol **(10)** with 12.95 and 8.62% peak areas, respectively. Compounds such as 1,2-benzenedicarboxylic acid, diisooctyl ester **(13)** (8.28%), 1-docosene **(4)** (6.56%) and 1-elcosene **(6)** (5.58%) were categorized as moderately abundant ones. Five compounds namely 12-methyl-E,E-2,13-octadecadin-1-ol **(12)** (4.55%), 1-hexacosene **(14)** (3.44%), 9,12-octadecadienoyl chloride, (Z,Z) **(11)** (3.21%), phenol, 2,4-bis (1,1 dimethylethyl)- **(3)** (3.46%), and 2-ethylnon-1-en-3-ol **(2)** (3.09%) were categorized as less abundant. The two least abundant compounds in this sub-fraction were heptadecanoic acid, 16-methyl-, methyl ester **(9)** and 2-decenal, (Z)- **(1)** with peak areas of 2.88 and 2.76%, respectively. Antifungal activity of the identified compounds as reported in previous literature is presented in Table 2 and their structures are given in Fig. 6.

Discussion

In initial screening bioassays, methanol was used for extraction of phytoconstituents in different parts of *S. oleraceous.* Recently, this solvent has also been used for the extraction of other plant species such as *Eucalyptus citriodora*, *Chenopodium quinoa* and *Carthamus oxycantha* in various recent studies (Javaid *et al.* 2020; Khan and Javaid 2020; Rafiq *et al.* 2020). There were various reasons of using this solvent in these bioassays. First, it has preference over aqueous extracts because of its antiseptic nature that prevents contamination in the antifungal bioassays (Elzain *et al.* 2019). Secondly, generally higher

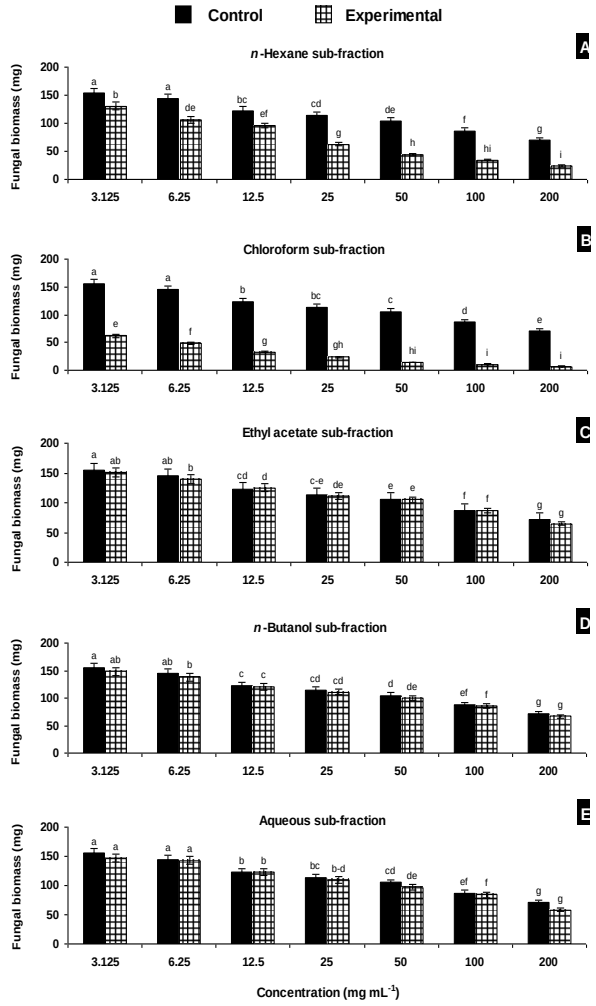

Fig. 3: Effect of different sub-fractions of methanolic stem extract of *S. oleraceous* on growth of *M. phaseolina*. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Tukey's HSD Test

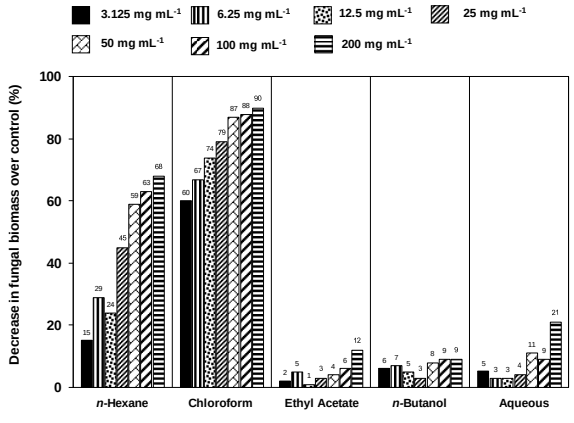

Fig. 4: Percentage decrease in biomass of *M. phaseolina* due to different fractions of methanolic stem extract of *S. oleraceous* over control

extraction yield is obtained in methanol as compared to other solvents like ethanol and acetone (Ngo *et al.* 2017). Thirdly, methanolic extracts generally show higher biological activities as compared to other extracts due to extraction of more number of phytoconstituents (Truong *et al.* 2019). In the present study, the entire selected concentration range of methanolic inflorescence, stem, leaf and root extracts generally controlled the growth of *M. phaseolina.* However, stem and root extracts showed the maximum inhibitory efficacy against the targeted pathogen with the evidence of differences in their antifungal nature. Earlier, the similar effects of ethanolic stem and leaf extracts of *S. oleraceous* against the plant pathogenic fungus *Aspergillus niger* were reported (Al-Hussaini and Mahasneh 2011).

Stem extract was fractionated using organic solvents possessing variable polarities. When bioassays were conducted, the chloroform sub-fraction depicted the best antifungal potential in arresting the growth of *M. phaseolina* followed by *n*-hexane. As the solvents had different polarities starting from non-polar *n*-hexane to a very polar *n*-butanol, therefore, different groups of compounds were extracted and collected in different solvents and showed variable antifungal activities. Previous studies have also shown the best antifungal activity of chloroform sub-fraction of methanolic extracts of *Chenopodium murale* and *Sisymbrium irio* against *Fusarium oxysporum* (Naqvi *et al.* 2019; Akhtar *et al.* 2020). However, by contrast some researchers reported otherwise where *n*-hexane sub-fraction of methanolic leaf extract of *Melia azedarach*, ethyl acetate sub-fraction of *Cenchrus pennisetiformis* and *n*-butanol sub-fraction of methanolic shoot extract of *Coronopus didymus* demonstrated the best antifungal activities against *Alternaria alternate*, *F. oxysporum* and *Sclerotium rolfsii*, respectively (Javaid and Samad 2012; Javaid and Iqbal 2014; Khurshid *et al.* 2018). It indicates diverse nature of antifungal compounds distributed in plant kingdom. In some plants like *S. oleraceous* in the present study as well as in *C. murale* and *S. irio*, moderately polar compounds of chloroform sub-fraction were antifungal in nature. On the other hand, in leaves of *M. azedarach*, *n*-hexane soluble antifungal compounds were non-polar in nature, while in case of *C. pennisetiformis* and *C. didymus*, ethyl acetate and *n*-butanol soluble antifungal compounds were highly polar in nature.

Chloroform sub-fraction was chosen for GC-MS analysis to identify antifungal phytoconstituents. Literature survey was carried out which showed that many compounds identified in the present study had inhibitory effects against the growth of some other fungal pathogens. Kumar *et al.* (2011) isolated the compound **5** from *Opuntia lindheimeri* ethanolic leaf extract with effective antifungal potential against *A. solani* and *F. oxysporum*. Similarly, compounds **10** and **13** were assessed against *A. fumigatus*, *A. niger*, *A. flavus* and *Candida albicans* with promising growth inhibition potential towards all the tested fungal pathogens

Table 1: Compounds identified from chloroform sub-fraction of methanolic stem extract of *S. oleraceous* through GC-MS analysis

Comp. No.	Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
1	2-Decenal, (Z)-	$C_{10}H_{18}O$	154	10.968	2.76
2	2-Ethylnon-1-en-3-ol	$C_{11}H_{22}O$	170	12.242	3.23
3	Phenol, 2,4-bis (1,1-dimethylethyl)-	$C_{14}H_{22}O$	206	14.392	3.46
4	1-Docosene	$C_{22}H_{44}$	308	17.390	6.56
5	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	18.809	13.26
6	1-Elcosene	$C_{20}H_{40}$	280	19.429	5.58
7	9,12-Octadecanoic acid, methyl ester, (E,E)-	$C_{19}H_{34}O_2$	294	20.457	12.95
8	11-Octadecanoic acid, methyl ester	$C_{19}H_{36}O_2$	296	20.534	13.12
9	Heptadecanoic acid, 16-methyl-, methyl ester	$C_{19}H_{38}O_2$	298	20.704	2.88
10	1-Docosanol	$C_{22}H_{46}O$	326	21.298	8.62
11	9,12-Octadecadienoyl chloride, (Z,Z)-	$C_{18}H_{31}ClO$	298	22.377	3.21
12	12-Methyl-E,E-2, 13-octadecadien-1-ol	$C_{19}H_{36}O$	280	22.785	4.55
13	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	24.314	8.28
14	1-Hexacosene	$C_{26}H_{52}$	364	24.560	3.74

Table 2: Antifungal properties of compounds identified from chloroform sub-fraction of methanolic stem extract of *S. oleraceous* through GC-MS analysis

Comp. No.	Names of compounds	Target fungus	Reference
1	2-Decenal, (Z)-	No activity reported	-
2	2-Ethylnon-1-en-3-ol	No activity reported	-
3	Phenol, 2,4-bis (1,1-dimethylethyl)-	*Phytophthora cinnamomi*	Rangel-Sanchez *et al.* (2014)
4	1-Docosene	*Candida albicans*	Seow *et al.* (2012)
5	Hexadecanoic acid, methyl ester	*Alternaria solani*, *Fusarium oxysporum*	Kumar *et al.* (2011); Bergaoui *et al.* (2007)
6	1-Elcosene	No activity reported	-
7	9,12-Octadecanoic acid, methyl ester, (E,E)-	*Aspergillus niger*	Krishnaveni *et al.* (2014); Wei and Wee (2011)
8	11-Octadecanoic acid, methyl ester	*C. albicans*	Dos Reis *et al.* (2019); Shobier *et al.* (2016); Orishadipe *et al.* (2012)
9	Heptadecanoic acid, 16-methyl-, methyl ester	No activity reported	-
10	1-Docosanol	*A. fumigatus*, *A. flavus*, *C. albicans*	Semwal and Painuli, (2019); Radulovic *et al.* (2012)
11	9,12-Octadecadienoyl chloride, (Z,Z)-	*C. albicans*	Omoregie *et al.* (2018)
12	12-Methyl-E,E-2, 13-octadecadien-1-ol	No activity reported	-
13	1,2 Benzenedicarboxylic acid, diisooctyl ester	*A. niger*, *A. flavus*, *C. albicans*	Balasundari and Boominathan, (2018)
14	1-Hexacosene	*A. alternata*, *Curvularia lunata*	Zhang *et al.* (2015)

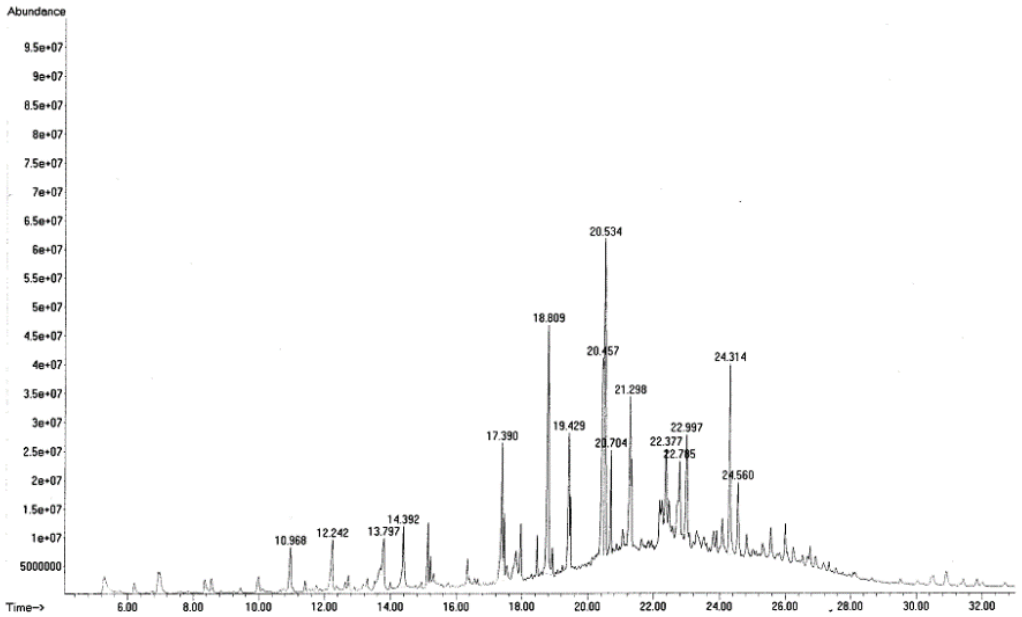

Fig. 5: GC-MS chromatogram of chloroform subfraction of methanolic stem extract of *S. oleraceous*

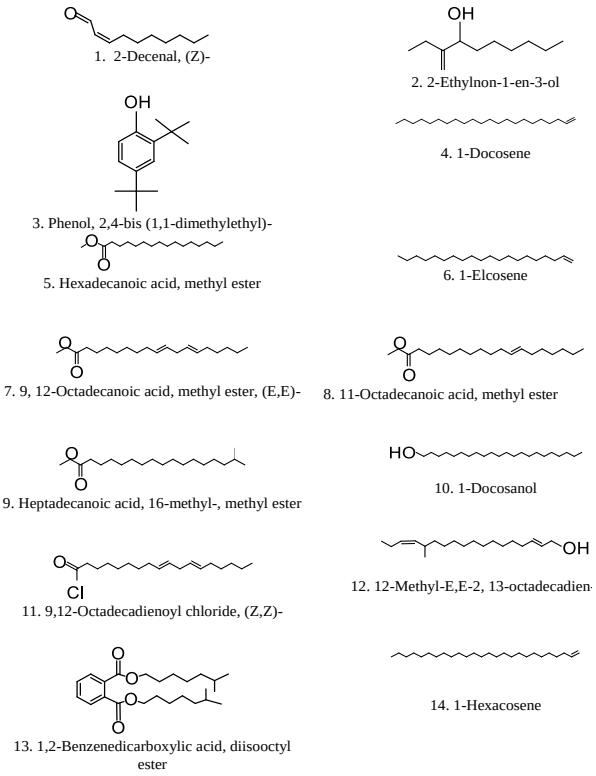

Fig. 6: Structures of compounds identified in chloroform subfraction of methanolic stem extract of *S. oleraceous* through GC-MS analysis

(Balasundari and Boominathan 2018; Semwal and Painuli 2019). Zhang *et al.* (2015) identified the compound **14** from an endophytic fungus *Epichloe gansuensis* and evaluated its antifungal potential against *A. alternata* and *Curvularia lunata* with notable results. Likewise, compounds **4**, **8** and **11** were isolated from ethanolic extracts of a marine seaweed *Ulva fasciata* and *Gynura segetum*, and tested against *C. albicans* with excellent antifungal properties (Seow *et al.* 2012; Omoregie *et al.* 2018; Dos Reis *et al.* 2019). Compounds **3** and **7** also showed the maximum inhibitory potential towards *Phytophthora cinnamomi* and *A. niger*, respectively (Krishnaveni *et al.* 2014; Rangel-Sanchez *et al.* 2014).

Conclusion

All parts of *S. oleraceous* have ability to significantly suppress the growth of *M. phaseolina*. However, stem extract exhibited the best antifungal activity possibly because of antifungal compounds such as 2-decenal, (Z); 1-docosene; hexadecanoic acid, methyl ester; 11-octadecanoic acid, methyl ester; and 1,2 benzenedicarboxylic acid, diisooctyl ester.

Author Contributions

SB did experimental work; AJ gave idea, did statistical analysis, prepared graphs and supervised the work while IHK contributed in paper write up.

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