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In Vitro Efficacy Of Biologically Active Compounds Derived From Navicula Arenaria Against Soil Borne Phytopathogenic Macrophomina Phaseolina And Fusarium Oxysporum

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ABSTRACT

Navicula arenaria Donkin 1861 is a marine microalga belongs to class of bacillariophycea. Recently, microalgae and their products are used as biological control of diseases caused by phytopathogenic fungi. This is considered environmentally ecofriendly method to overcome the plant damage caused by soil borne pathogenic fungi and thereby economic loss. Therefore, this study aimed to investigate in *vitro* the antifungal activity of *N. arenaria* isolate PS 31 extracellular and intracellular metabolites against two taxa of soil borne phytopathogenic fungi; *Macrophomina phaseolina* and *Fusarium oxysporum*.

N. arenaria PS 31 hexane extract was the most effective extract on growth inhibition of both phytopathogenic fungi. There is no significant difference between miconazole and *N. arenaria* hexane extract of 5.6 mg/ml on growth inhibition of *F. oxysporum*. The inhibitory effect of hexane extract at 5.6, 4.2 and 2.8 mg/ml and ethyl acetate extract at 35% (v/v) was higher than controls. Ethyl acetate extract was effective on growth inhibition of *M. phaseolina* (29.67%). GC-MS analysis of *N. arenaria* hexane fraction revealed the presence of potent antifungal compounds such as Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-, di-n-octyl phthalate, cholestane-3,5-diol, 5-acetate,(3.beta.,5.alpha.), Cholestan-3-ol,(3.beta.,5.beta.)- and beta.-Sitosterol. These results suggest that *N. arenaria* hexane extract can be used in biological control of plant diseases caused by *M. phaseolina* and *F. oxysporum*.

Keywords:

Antifungal activity; Diatoms; *Fusarium oxysporum*; *Macrophomina phaseolina*; *Navicula arenaria* .

1. INTRODUCTION

Diatoms are unicellular microalgae with silica shields and belong to class of bacillariophycae. Diatoms are ubiquitous photosynthetic organisms that are found in freshwater, seawater, brackish water and terrestrial habitat [1]. Microalgae are naturally rich with several metabolites that have several biological activities such as antifungal, antibacterial, antiviral, anti-oxidative, antitumor and anti-inflammatory [2]. In recent years, algae are used in many applications such as biofuels and bioplastics production, nutritional supplements, cosmetics, bioremediation, animal feeds, agriculture branches as fertilizers, bio-stimulants or in biocontrol and pharmaceutical industry [2].

The plant damage caused by soil borne pathogenic fungi has led to the focusing of considerable effort for fighting plant pathogens using natural products extracts as safe alternatives to synthetic fungicide [3]. Conventional strategies that involves the utilization of chemical fungicides are used in the control of soil borne phytopathogenic fungi. However, the application of synthetic fungicides can cause harmful problems to the environment and other organisms [4]. As a consequence, there is an increased attention toward the utilization of algae as a biocontrol agent for plant disease management to reduce the use of fungicides and this considered ecofriendly method to environment for protecting crops from soil borne pathogenic fungi [5,6].

Okunowo [7] determine the antifungal activity of *Navicula* sp. methanol extract against *Candida albicans*. Qin *et al*. [8] evaluate the antimicrobial activity of marine diatom *Thalassiosira rotula* against three strains of Gram positive bacteria and six strains of Gram negative bacteria and *Candida albicans*. Sherief *et al*. [9] study the antimicrobial efficiency of marine diatoms (DE) and silver diatom nanoparticle (Ag-DE/NPs) against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger*. The results revealed that the antimicrobial activity of silver diatom nanoparticle was higher than the antimicrobial activity of marine diatoms.

The aim of this research is to evaluate the antifungal activity of *N. arenaria* metabolites against *Fusarium oxysporum* and *Macrophomina phaseolina* that are considered two sorts of soil borne pathogenic fungi.

2. MATERIALS AND METHODS

2.1. *Navicula arenaria* isolation and identification

Navicula arenaria was isolated and purified from sea water samples collected from Mediterranean Sea at Port Said city. F/2 medium has been used for *N. arenaria* isolation and purification [10]. The morphological identification of *N. arenaria* was accessed by light microscopy and imaged using optika 4083.B5 digital camera. The morphological identification was assessed according to Donkin, (1858) [11].

The molecular identification of *N. arenaria* was based on ITS-5.8s rRNA gene sequence analysis. *Navicula arenaria* chromosomal DNA was extracted by phenol/chloroform technique and precipitated by isopropanol followed by washing with ethanol [12]. The quality of the purified genomic was examined by running on agarose gel electrophoresis (1%) in TAE buffer pH 8.0 (0.04M Tris-acetate and 0.001M EDTA) and visualized by UV trans-illuminator after staining with ethidium bromide.

The ITS1 and ITS2 regions including 5.8S rRNA gene was amplified and sequenced using the universal eukaryotic primers ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') [13]. The amplified PCR products were

sequenced by an automated sequencer (Macrogen, South Korea) using the same previous primers. Basic local alignment search tool nucleotide (BLASTn) was performed for search alignment of the resulting ITS region of DNA sequence to match the best similarities with other related sequences on database [14]. The best DNA sequence similarities with the resulting regions were obtained from national center for biotechnology information (NCBI) GenBank and aligned using CLUSTAL Omega. Unaligned terminal regions were excluded manually and some sequences of the same species and unidentified organisms were discarded. Finally, phylogenetic tree analysis was viewed and analyzed using MEGA version 4 [15].

2.2. Antifungal activity of *N. arenaria* metabolites

N. arenaria PS 31 propagation was performed in 2L glass bottle media filled with 1800 mL media at 25^{O}C under 8:16 LD cycle with 36 µmol m^{-2} s^{-1} with cool white fluorescent lamps and continuous aeration by vacuum pump (3.5L/min). Biomass of *N. arenaria* and cultivation medium were harvested after 35 days by centrifugation at 10000 rpm [16]. *N. arenaria* biomass was washed with distilled water to remove salts and then air dried.

Four organic extraction solvents according to polarity; hexane, chloroform, acetone and methanol were used in extraction process of *N. arenaria* intracellular metabolites by maceration with continuous shaken for 24 hours at 200 rpm. The percentage of each extract was measured as follow [17,18]:

Extract % = (Weight of extract (g) / Weight of algal sample (g)) * 100

Each crude extract was dissolved in dimethyl sulfoxide (DMSO) then sterilized using micro filters (Millipore, 0.22µm) and stored in refrigerator for further use. The concentration of each extract was calculated [18,19] .

N. arenaria extracellular metabolites were extracted according to modified method from Bhore *et al.,* (2010) [20]. F/2 culture medium was extracted by absolute ethyl acetate (1:3 v/v). The mixture was shaken for 24 hours at 200 rpm. The mixture was imparted in a separating funnel until the separation of ethyl acetate from the aqueous layer. The extract was sterilized through micro filters (Millipore, 0.22µm) and stored in refrigerator for antifungal assay.

Antifungal efficiency of *N. arenaria* extracellular and intracellular metabolites was carried in *vitro* against *M. phaseolina* and *F. oxysporum* by agar diffusion method on potato dextrose agar medium [18,21]. Negative control was DMSO in case of intracellular metabolites and ethyl acetate in case of extracellular metabolites Positive control was performed by using miconazole and nystatin at 0.03 mg/mL.

Plates were incubated at 30^{O}C and the results of fungus growth inhibition were recorded after 96 hours in case of *M. phaseolina* and till 120 hours in case of *F. oxysporum*. Percentage of mycelium growth inhibition (MGI %) was measured using the following equation [18,22]:

$$MGI\% = [(Control - Test)/Control] \times 100$$

(Control= fungus radial growth from the middle of fungal disc toward the negative control well, Test= fungus radial growth from the middle of fungal disc toward the extract well).

Determination of MIC was achieved by using three diluting concentrations of each extract (75, 50 and 25%) [18]. All experiments were performed in triplicate.

2.3. Metabolic profile of *Navicula arenaria* PS 31

N. arenaria hexane fraction exhibited the highest antifungal activity against *M. phaseolina* and *F. oxysporum*. Hexane extract was analyzed using GC-MS.

Mass spectra were recorded using Shimadzu GCMS-QP2010 (Koyoto, Japan) equipped with Rtx-5MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Restek, USA) equipped with a split–splitless injector. This analysis was carried out in Faculty of Pharmacy, Ain Shams University. The initial column temperature was kept at 50^{O}C for 3 min (isothermal) and programmed to 200^{O}C at a rate of 15^{O}C/min, and kept constant at 200^{O}C for 5 min (isothermal). Temperature was programmed to 240^{O}C at a rate of 3^{O}C/min, and kept constant at 240^{O}C for 10 min (isothermal). Finally, the temperature was programmed to 300^{O}C at a rate of 4^{O}C/min, and kept constant at 300^{O}C for 10 min (isothermal). Injector temperature was 280^{O}C. Helium carrier gas flow rate was 1.41 ml/min. All the mass spectra were recorded applying the following condition: (equipment current) filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 220^{O}C. Diluted samples (1% v/v) were injected with split mode (split ratio1:5).

2.4. Statistical analysis

The results were expressed as mean of three replicates ± standard error. IBM SPSS software version 25 was used for results statistical analysis. Data was subjected to analysis of variance using one-way ANOVA at $P < 0.05$ in order to assess the significance among treatments and means were separated using the least significant difference (LSD) [23].

3. RESULTS AND DISCUSSION

3.1. Identification of the *N. arenaria* isolate

N. arenaria is mobile, oblong, narrow and acute cell. It has slightly convergent opposite central nodule. Cells are 22.83 ± 0.186 µm long and 4.14 ± 0.586 µm wide (**Fig. 1**).

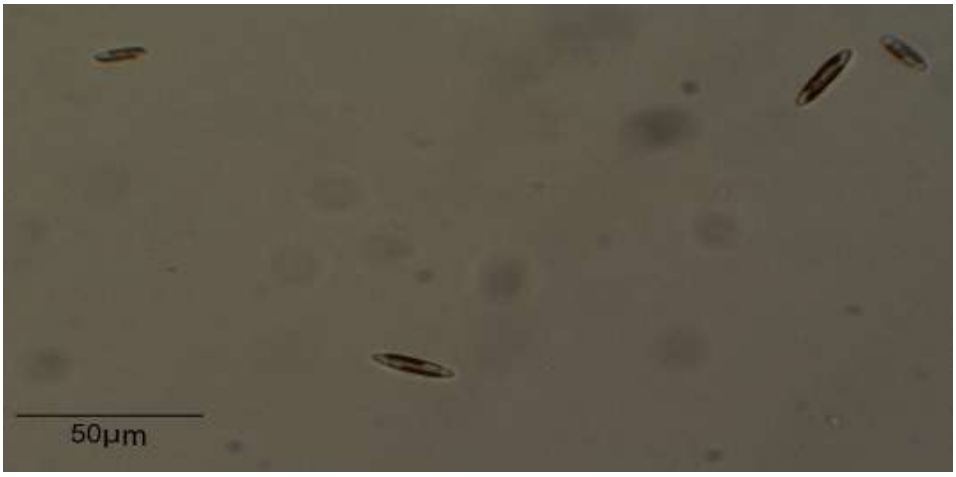

Fig. 1. Microscopic image of *Navicula arenaria* PS 31.

ITS-5.8s rRNA gene sequence alignment (NCBI-Blastn) of *Navicula arenaria* PS 31 isolate with other related *Navicula* genera exhibited the highest identity with *Navicula arenaria* isolate CCY 0228 (reached 82.10% similarity with query cover 95% and bootstrap value 100). Less query cover sequences with *Navicula gregaria* isolate K7 and K8 (39% and 38% respectively) revealed 89% similarity but clustered away from *Navicula arenaria* PS 31. The other *Navicula* species were grouped in a separate cluster away from the studied isolate as represented in the phylogenetic tree **(Fig. 2).**

The ITS-5.8S rRNA gene sequence confirmed the identification of *Navicula* isolate PS 31 as it clustered with *Navicula arenaria* isolate CCY 0228 in the same clade with similarity reached 82.10%. Also, its morphological characteristics match with the other *Navicula*

arenaria. On the other hand, cells of *N. gregaria* are broadly lanceolate and apiculate while cells of *N. arenaria* are narrowly lanceolate and acute and both habitat are marine [11]. Cells of *Navicula arenaria* PS 31 isolate are 22.83 ± 0.186 µm long and 4.14 ± 0.586 µm wide., while *N. gregaria* cell was 13 to 42 µm long and 5 to 10 µm wide [24].

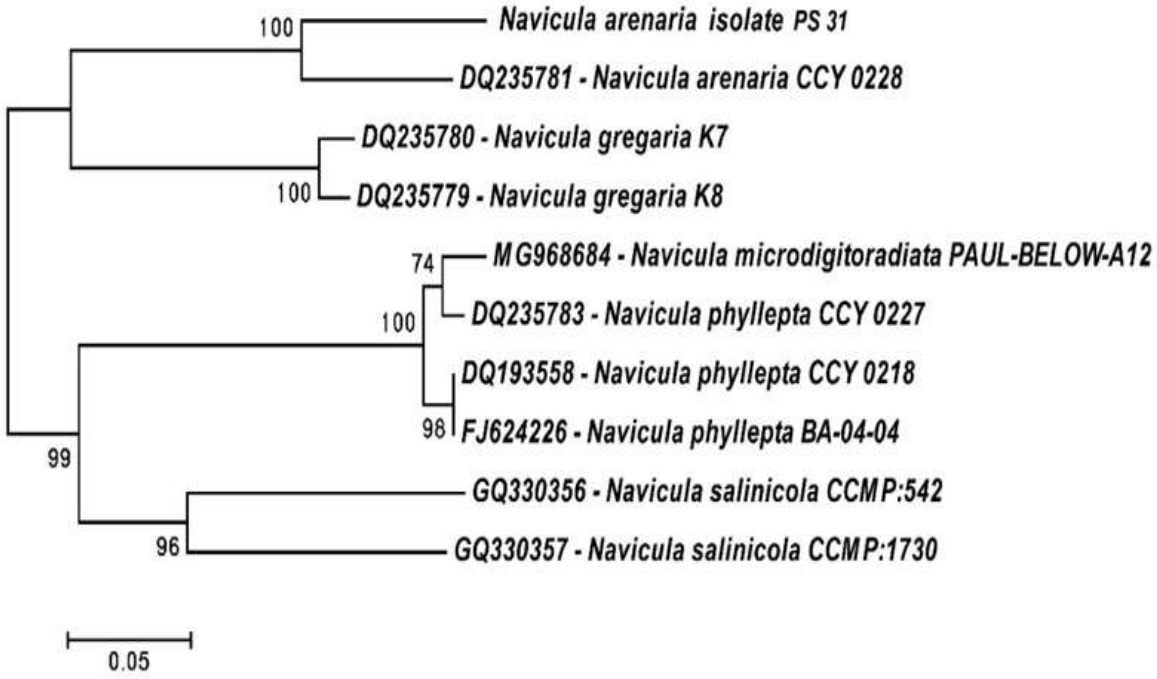

Fig. 2. Phylogenetic tree analysis based on ITS-5.8s rRNA gene sequence alignment of *Navicula arenaria* isolate PS 31 with some other related *Navicula* species. The values next to branches are confidence levels estimated by 1000 bootstrap replicates.

3.2. Antifungal activity of *N. arenaria* extracts (In- *vitro* assay)

Organic extraction solvents have an obvious effect on extraction yield. The highest concentration of *N. arenaria* extraction yield was obtained by acetone followed by methanol. The lowest extract concentration was obtained by hexane (**Fig. 3**). In consistent with our observations Lotfi *et al*. [18] who reported that the highest percentage of extraction yield in *Ulva fasciata*, *Ulva lactuca* and *Cladophora sericea* was obtained by methanol followed by acetone. Also, the results disagree with Abdel-Aal *et al*. [17] who found that the highest extraction yield from *Spirogyra longata* was obtained by methanol.

The highest MGI% against *M. phaseolina* and *F. oxysporum* was observed by hexane extract. Both of soil-borne fungi are sensitive to *Navicula arenaria* hexane extract and resistant to methanol extract of this alga (**Table. 1**). This observation was contrasted to Okunowo [7] who found that *Navicula* sp. methanol extract inhibit the growth of all tested clinical isolates. *N. arenaria* hexane extract at 5.6 mg/ml showed 31.02 MGI% to *M. phaseolina* and 21.56 MGI% against *F. oysporum* (**Table. 2**). *M. phaseolina* was resistant to chloroform, acetone and methanol extracts. In consistent with our observations Qin *et al*. [8]

who found that *Candida albicans* was resistant to all tested extraction solvents of a marine diatom *Thalassiosira rotula*.

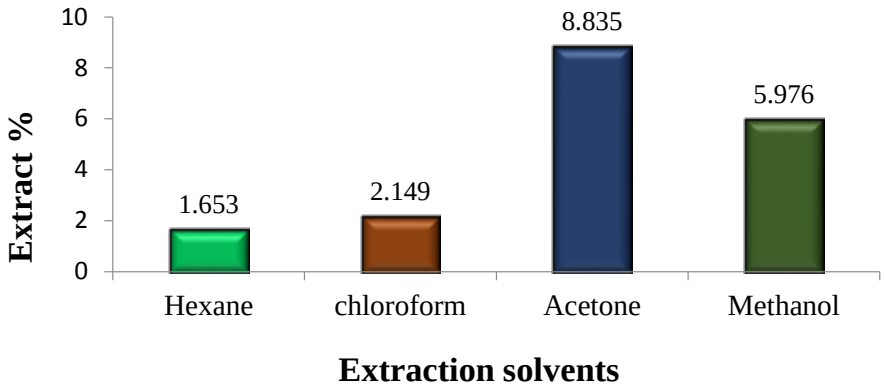

Fig. 3. Variation in crude extract % of *N. arenaria* with different organic solvents.

Antifungal activity of *N. arenaria* hexane extract of 5.6, 4.2 and 2.8 mg/ml concentrations against *M. phaseolina* was significantly higher than positive control (**Table. 2**). *F. oxysporum* was sensitive to hexane, chloroform and acetone extract at high concentrations. Methanol and ethyl acetate extract were not effective in growth inhibition of *F. oxysporum*. There is no significant difference in mycelium growth inhibition of *F. oxysporum* by miconazole and *N. arenaria* hexane extract. *N. arenaria* ethyl acetate extract was effective in growth inhibition of *M. phaseolina* (**Fig. 5**). The inhibitory effect of *N. arenaria* extracellular metabolites to *M. phaseolina* reached to 29.67%. About 33.33% of ethyl acetate extracts from the cultivation medium inhibit the growth of tested fungi. Similarly, was reported by Mundt *et al*. [16] who found that 38% of the ethyl acetate extracts obtained from the cultivation medium inhibit the growth of tested fungi and bacteria. The whole difference between results of the present study and other researches result is due to the difference in tested microorganism, extraction solvent and method, type of alga and chemical composition of algae that differ according to species and environmental conditions [25].

Table 1. Determination of MIC for different concentrations of extraction solvents of *N. arenaria* against phytopathogenic fungi.

Hexane	Concentration (mg/ml)	**1.4**	**2.8**	**4.2**	**5.6**
	F. oxysporum	-	-	-	+
	M. phaseolina	+	+	+	+
Chloroform	Concentration (mg/ml)	**1.42**	**2.84**	**4.26**	**5.68**
	F. oxysporum	-	-	-	+
	M. phaseolina	-	-	-	-
Acetone	Concentration (mg/ml)	**0.61**	**1.23**	**1.84**	**2.46**
	F. oxysporum	-	-	-	+
	M. phaseolina	-	-	-	-
Methanol	Concentration (mg/ml)	**5.75**	**11.5**	**17.25**	**23**
	F. oxysporum	-	-	-	-
	M. phaseolina	-	-	-	-
Ethyl acetate	Concentration (v/v)	**5%**	**15%**	**25%**	**35%**
	F. oxysporum	-	-	-	-
	M. phaseolina	-	-	-	+

(+) means that the pathogen was sensitive to the extract and (–) means that the pathogen was resistant to the extract.

Table 2. MGI % of phytopathogenic fungal candidates by different extraction solvents of *N. arenaria*.

Treatments	**Extract Conc.**	**MGI %**	
		F. oxysporum	***M. phaseolina***
Miconazole	0.03 mg/ml	**24 ± 1.76** ^{a}	**10.76 ± 2.98** ^{h}
Nystatin	0.03 mg/ml	**12.2 ± 2.32** ^{b}	**14.86 ± 0.63** ^{g}
Hexane	5.6 mg/ml	**21.56 ± 0.46** ^{a}	**31.02 ± 0.88** ^{e}
Hexane	4.2 mg/ml	0 ^{c}	**27.27 ± 0.30** ^{e}
Hexane	2.8 mg/ml	0 ^{c}	**17.04 ± 0.19** ^{g}
Hexane	1.4 mg/ml	0 ^{c}	**6.813 ± 0.07** ^{i}
Chloroform	5.68 mg/ml	**18.07 ± 0.22** ^{d}	0 ^{f}
Acetone	2.46 mg/ml	**13.54 ± 1.01** ^{b}	0 ^{f}
Methanol	23 mg/ml	0 ^{c}	0 ^{f}
Ethyl acetate	35% v/v	0 ^{c}	**29.67 ± 0.32** ^{e}
LSD at p ≤ 0.05		**3**	**2.92**

All values are mean (n=3) ± standard error.
Values with the same letters in the same column are not significantly different at $p \leq 0.05$.

Fig. 4. Mycelial growth inhibition of *F. oxysporum* by *N. arenaria* hexane extract; A. represents DMSO only (negative control) and B. represents *N. arenaria* hexane extract dissolved in DMSO**.**

Fig. 5. Mycelial growth inhibition of *M. phaseolina* by extracellular metabolites of *N. aenaria*; A. represents ethyl acetate only (negative control) and B. represents extracellular metabolites of *N. arenaria* dissolved in ethyl acetate.

3.3. Metabolic profile of *Navicula arenaria* PS 31

The bioactivity of *N. arenaria* hexane extract was proved against two tested phytopathogenic fungi. Eight compounds were identified from *N. arenaria* hexane extract and belong to different chemical classes like steroids, diterpene alcohols, esters, fatty alcohols, phenols, phytosterols and phthalic acid derivatives (**Table. 3, Fig. 6**). Some of them were recorded as antifungal compounds like phenol, 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl- [26], di-n-octyl phthalate [27], beta.-Sitostero [27], cholestan-3-ol, (3.beta.,5.beta.)- [28] and Cholestane-3,5-diol, 5-acetate, (3.beta.,5.alpha.)- [28]. The highest peak area in *N. arenaria* hexane fraction was identified as di-n-octyl phthalate (**Fig. 7**).

Table 3. Identified compounds from *N. arenaria* hexane extract by GC/MS.

Peak No.	R_t	Metabolites	Molecular formula	Molecular weight	Chemical groups	Base peak m/z	Peak area %	Biological activities	References
1	20.826	1-Eicosanol	$C_{20}H_{42}O$	298	Fatty alcohol	43.05	3.02	Antimicrobial activity	[28]
2	21.571	Phytol	$C_{20}H_{40}O$	296	Diterpene alcohol	43.05	3.69	Antimicrobial activity	[29,30]
3	26.167	2-Propenoic acid, pentadecyl ester	$C_{18}H_{34}O_2$	282	Acid	55.05	14.35	-	-
4	29.605	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	$C_{23}H_{32}O_2$	340	Phenol	177.10	3.85	Antifungal, Antibacterial activity, germicidal	[26]
5	32.901	Di-n-octyl phthalate	$C_{24}H_{38}O4$	390	Phthalic acid derivative	149.05	38.32	Antifungal activity	[27]
6	52.077	Cholestane-3,5-diol, 5-acetate, (3.beta.,5.alpha.)-	$C_{29}H_{50}O_3$	446	Steroids	43.05	4.98	Antifungal activity	[28]
7	52.265	Cholestan-3-ol, (3.beta.,5.beta.)-	$C_{27}H_{48}O$	388	Steroids	43	8.20	Antifungal activity	[28]
8	56.453	beta.-Sitosterol	$C_{29}H_{50}O$	414	Phytosterols	43.05	15.83	Antifungal activity	[27]

(-) No reported biological activity

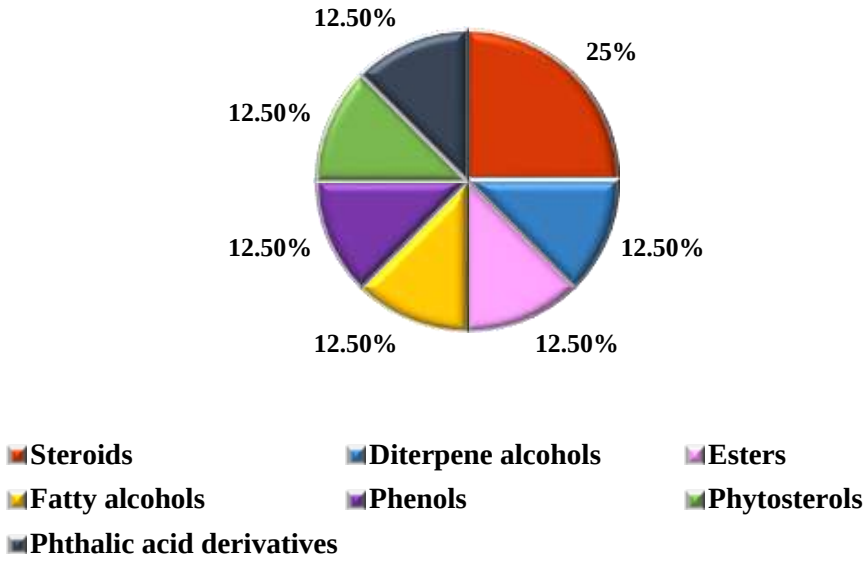

Fig. 6. Percentage of metabolites chemical groups identified by GC/MS in *N. arenaria* hexane extract.

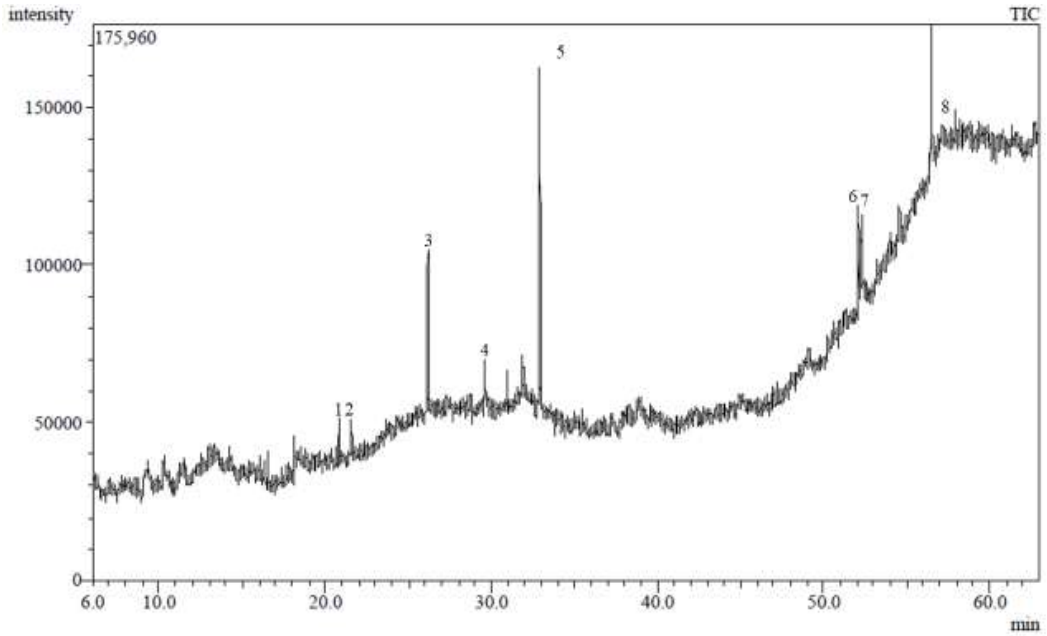

Fig. 7. Total ion chromatogram of GC-MS of *N. arenaria* hexane extract.

4. CONCLUSION

As *N. arenaria* hexane extract was effective in mycelium growth inhibition of *Macrophomina phaseolina* and *Fusarium oxysporum*, it would be considered a promising bio-agent source for biological control of diseases caused by those two fungi. This is considered ecofriendly method to the environment.

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