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Article

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BIOACTIVE CONSTITUENTS OF SHOOT EXTRACTS OF *Sisymbrium irio* L. AGAINST *Fusarium oxysporum* f. sp. *cepae*

*Constituintes Bioativos de Extratos de Broto de **Sisymbrium irio** L. contra **Fusarium oxysporum** f. sp. cepae*

ABSTRACT - The present study was carried out to check the antifungal potential of *Sisymbrium irio* L. shoot extract against *Fusarium oxysporum* f. sp. *cepae* (FOC). In preliminary bioassays, different concentrations (1 to 5%) of leaf, stem and fruit extracts were evaluated against FOC. All the extracts were effective against the pathogen. However, the leaf extract was found the most effective causing 25-41% decrease in FOC biomass. The fractionation of methanolic leaf extract was done by two organic solvents namely *n*-hexane and chloroform. Different concentrations (1.56 to 200 mg mL^{-1}) of these fractions were tested against FOC. The *n*-hexane and chloroform fractions showed inhibitory activity against the pathogen and resulted in 77-93% and 80-96% reduction in biomass of FOC, respectively. GC-MS analysis showed the presence of 24 compounds in *n*-hexane and 4 compounds in chloroform fraction. In *n*-hexane fraction, β-sitosterol (18.64%) was the most abundant compound followed by orotic acid, bis(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester (12.18%), 10-octadecenoic acid, methyl ester (7.90%) and 1,2-benzenedicarboxylic acid, diisooctyl ester (6.05%). Major compounds identified in chloroform fraction were 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester (50.82%) and di-*n*-octyl phthalate (33.00%). This study concludes that *n*-hexane and chloroform fractions of methanolic leaf extract of *S. irio* contain potent antifungal constituents for the management of FOC.

Keywords: basal rot of onion, brassicaceous weed, methanolic extract.

*RESUMO - O presente estudo foi realizado para verificar o potencial antifúngico do extrato de caule de **Sisymbrium irio** L. contra **Fusarium oxysporum** f. sp. cepae (FOC). Em bioensaios preliminares, diferentes concentrações (1% a 5%) de extratos de folhas, caules e frutos foram avaliadas contra o FOC. Todos os extratos foram eficazes contra o patógeno. No entanto, o extrato de folhas foi o mais eficaz, causando 25-41% de redução na biomassa FOC. O fracionamento do extrato metanólico das folhas foi feito por dois solventes orgânicos: n-hexano e clorofórmio. Diferentes concentrações (1,56 a 200 mg mL^{-1}) dessas frações foram testadas contra o FOC. As frações de n-hexano e clorofórmio mostraram atividade inibitória contra o patógeno e resultaram em redução de 77-93% e 80-96% na biomassa de FOC, respectivamente. A análise por CG-EM mostrou a presença de 24 compostos em n-hexano e 4 compostos em fração clorofórmica. Na fração **n**-hexano, o β-sitosterol (18,64%) foi o composto mais abundante, seguido pelo ácido orótico, éster bis (terc-butildimetilsilil) terc-butildimetilsilílico (12,18%), éster de ácido 10-octadecenoico metil (7,90%) e Ter de di-isoctilo do ido 1,2-benzenodicarboxico (6,05%). Os principais compostos identificados na fração clorofórmica foram o*

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ácido 1,3-benzenodicarboxílico, o éster bis (2-etil-hexílico) (50,82%) e o ftalato di-n-octilo (33,00%). Este estudo conclui que as frações de n-hexano e clorofórmio do extrato metanólico das folhas de S. irio contêm potentes constituintes antifúngicos para o manejo do FOC.

Palavras-chave: podridão basal de cebola, planta daninha Brassicácea.

INTRODUCTION

Onion (*Allium cepa* L.), family Alliaceae, is an important horticultural crop widely cultivated all over the world for domestic use (Rose et al., 2005). It has been used as spices in roasted, fried and grilled form. It has great economic importance for having enormous medicinal values (Fayos et al., 2018). The genus *Allium* contains organosulphur compounds namely isoallin and methiin which are responsible for its pungency (Slimestad et al., 2007). Phenolics and flavonoids present in onion have shown anti-inflammatory, anti-cancerous and anti-oxidant properties (Yang et al., 2004; Slimestad et al., 2007). Onions are attacked by numerous fungal and bacterial pathogens. Among these, basal rot of onion caused by *F. oxysporum* f. sp. *cepae* causes serious damage and crop failure (Bayraktar, 2010; Akhtar and Javaid, 2018). The disease occurs in major growing areas of the world causing important yield losses (Bayraktar, 2010; Esfahani, 2018). The pathogen spreads through infected seeds or soil. It completely degrade the basal stem portion of the onion leaving the tissues watery and degraded (Cramer, 2000). Leaves become yellow, curved and dieback occurs from the tips. Eventually the whole plant collapsed due to the degradation of basal plate and this primary infection led to the occurrence of secondary infection during storage conditions (Cramer, 2000).

Several strategies have been adopted to manage and control soil-borne pathogens including cultural practices, seed treatments with fungicides, and cultivation of resistant varieties (Katan et al., 1980; Havey, 1995). Seeds treated with thiram, carbendazim and thiophanate methyl gave promising results in reducing incidence of basal rot disease of onion (Mishra et al., 2014). Propineb, copper oxychloride and metalaxyl + mancozeb have also contributed in the management of basal rot disease (Behrani, 2015). However, due to bad impact of synthetic chemicals on atmosphere, development of resistant mutants, more expensive pesticides and many health hazards (Westlund et al., 2018), such agrochemicals should be replaced with environmental friendly natural compounds (Javaid et al., 2018). Numerous recent studies have shown that crude plant extract and isolated purified compounds from plants have tremendous potential in management of fungal plant pathogens (Javaid et al., 2015, 2017, 2018; Karim et al., 2017; Khurshid et al., 2017).

There are many plant families including Brassicacae which possess antifungal properties. Its members produce sulfur compounds that break down into isothiocyanates which helps in the process of biofumigation (Mayton et al., 1996). Numerous studies carried out using extracts of crops and weeds of Brassicaceae such as *Brassica* spp., *Raphanus sativus* and *Coronopus didymus* revealed significant reduction in growth of the fungal pathogens namely *Alternaria alternata*, *Fusarium oxysporum* f. sp. *lycopersici, Sclerotium rolfsii* and *Verticillium dahliae* (Subbarao et al., 1994; Troncoso et al., 2005; Javaid and Iqbal, 2014; Javaid and Bashir, 2015). *S. irio,* a weed of Brassicacae, has shown such antifungal properties against *Macrophomina phaseolina* (Javaid et al., 2017). However, studies regarding the antifungal activities of this weed against *F. oxysporum* f. sp. *cepae* are lacking. The present work was, therefore, conducted to confirm the potential of methanolic extracts of aerial parts of *S. irio* against this fungal pathogen.

MATERIALS AND METHODS

Bioassays with methanolic extracts

In a survey to nearby areas of the University of the Punjab, Lahore, Pakistan, aerial parts of *S. irio* plants were collected. They were washed, separated into various parts and ground into fine powder. Two hundred grams of each part were soaked in 1.0 L of methanol and left for 2 weeks at room temperature. Thereafter, materials were filtered with muslin cloth followed by

through filter papers to get the extracts. Methanolic extracts were subjected to rotary evaporation to get crude leaf, stem and fruit extracts of 22 g, 18 g and 14 g, respectively (Khurshid et al., 2018).

In vitro screening bioassays were conducted in 100 mL flasks following Javaid et al. (2017). The weighed amount (9 g) of crude extract from each part was dissolved in 5 mL of dimethyl sulphoxide (DMSO) and stock solutions (15 mL each) were prepared by adding appropriate quantity of autoclaved distilled water. Different concentrations (0, 1, 2, 3, 4 and 5%) were prepared by adding 0, 1, 2, 3, 4, 5 mL stock solution and 5, 4, 3, 2, 1, 0 mL control solution (prepared by adding 5 mL DMSO to 10 mL distilled H_2O) to 55 mL autoclaved malt extract broth. This solution was then carefully divided into volume of 15 mL parts constituting four replicates of each concentration. From freshly grown (7 days old) pure culture of FOC, 5 mm plugs were inoculated into each flask of all the treatments. The experiment was kept at 27 °C for 7 days for growth of the fungus.

Bioassays with sub-fractions of methanolic leaf extract

Fractionation of crude methanolic leaf extract with different organic solvents was carried out by adopting the procedure of Javaid et al. (2017). The methanolic extract of leaf was prepared by extracting 1 kg of dried leaf material in 6 L methanol. It was kept for two weeks and then filtered with muslin cloth and material was re-extracted in methanol for 7 days. The material was then filtered and combined with the previous one. The final filtration was done by using filter papers and evaporated in rotary evaporator to get its crude methanolic extract (55 g). The methanolic leaf extract was mixed in 200 mL of autoclaved distilled water and then 300 mL *n*-hexane was added and mixed to obtain *n*-hexane soluble compounds. This process was repeated again and again to completely separate *n*-hexane soluble components. Afterwards the aqueous fraction was further subjected to fractionation with chloroform. Each fraction was evaporated to obtain crude extracts of *n*-hexane (4.2 g) and chloroform (1.5 g) fractions.

To confirm the antifungal activity of each sub-fraction against the pathogen, 1.2 g of each fraction of methanolic leaf extract was dissolved in 1 mL of DMSO and stock solution (6 mL) was prepared by adding autoclaved malt extract broth. Three milliliters were used in antifungal bioassays while the rest was used to prepare lower concentrations (100 to 1.562 mg mL^{-1}) by serially double dilution method. For control treatments, the DMSO (1 mL) was mixed in 5 mL of malt extract broth and serially double diluted. Experiment was conducted in 10 mL volume test tubes with 1 mL growth medium in each tube. Each treatment was replicated thrice. Tubes were incubated at 27 °C for 7 days. After the incubation period, the fungal biomass was filtered, dried and weighed (Javaid et al., 2017).

GC-MS analysis

n-Hexane and chloroform sub-fractions showed the highest inhibitory effect against FOC, therefore, these were selected for GC-MS analysis to find out the possible inhibitory compounds. Analysis was carried out with a chromatographic system consisting of a Shimadzu GC-2010 plus serial number 020525274726, installed with auto injector AOC-20i, auto sampler AOC-20s and gas chromatograph equipped with a QP2010 ultra mass-selective detector (Shimadzu).

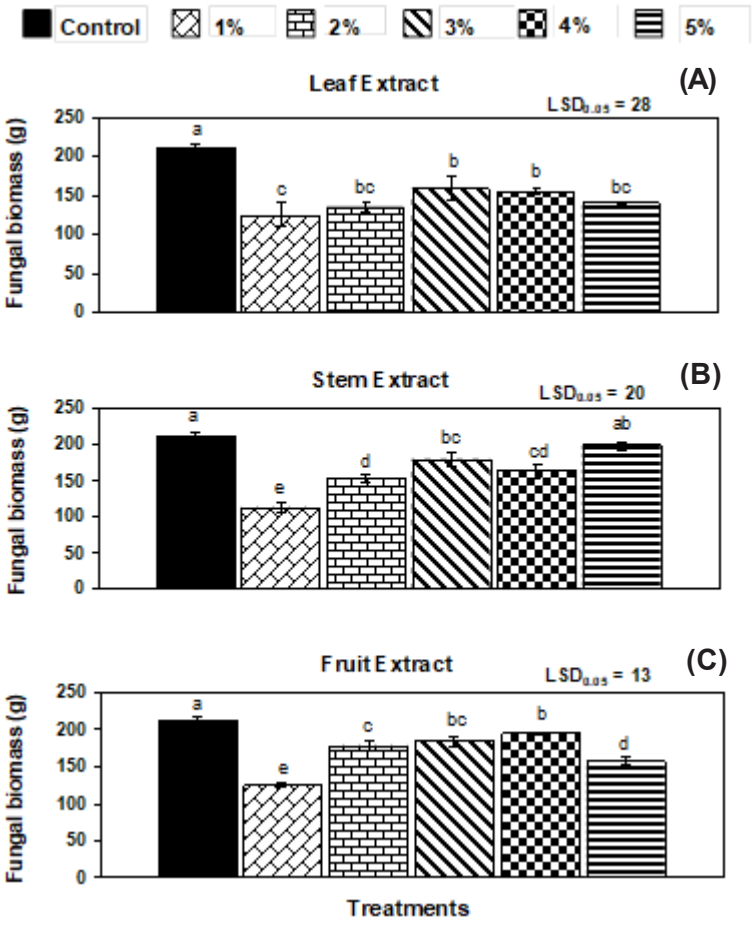
Statistical analysis

The collected data were analyzed by analysis of variance (ANOVA) followed by LSD ($P \leq 0.05$) using Statistix 8.1 software.

RESULTS AND DISCUSSION

Bioassays with methanolic extracts

The effect of methanolic leaf, stem and fruit extracts of *S. irio* on fungal growth is presented in Figure 1. All the concentrations of leaf extract significantly reduced the target fungal biomass



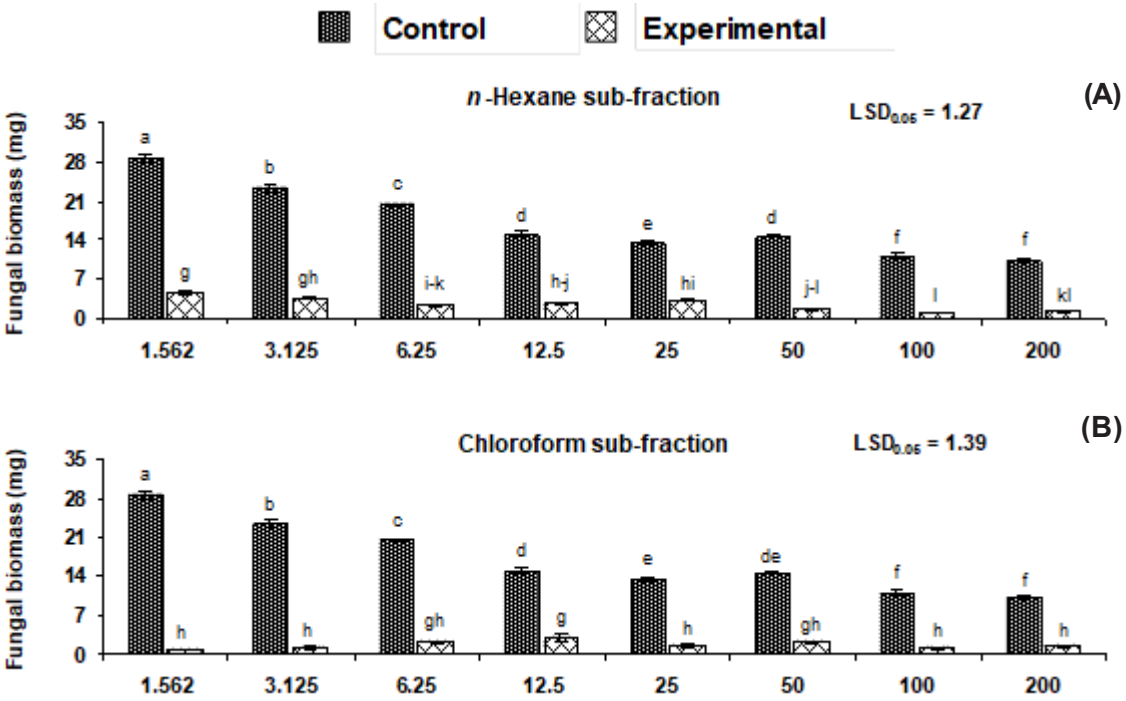
Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by LSD Test.

Figure 1 - Effect of different concentrations of methanol extract of aerial parts of *Sisymbrium irio* on biomass of *Fusarium oxysporum* f. sp. *cepae*.

by 25-41% over control. Similarly, reduction in fungal biomass recorded due to stem extract was 7-47% as compared to control. The inhibitory effect of fruit extract was also significant where 8-48% decrease in fungal biomass was noted due to various concentrations of the extract. Earlier, Javaid et al. (2017) also reported inhibitory effect of methanolic leaf extract of *S. irio* on growth of *Macrophomina phaseolina*. The presence of glucosinolates and flavonoids in the methanolic shoot extracts of brassicas (Al-Qudah and Abu Zarga, 2010; Sun et al., 2011), are responsible for their antifungal potential (Kanwal et al., 2010). The inhibitory response was dependent on various antimicrobial chemical constituents present in aerial parts of *S. irio*. Al-Qudah and Abu Zarga (2010) isolated precursors of isothiocyanates i.e. dioctyladipate; N-(*n*-proyl) acetamide; 3,7,11,15-tetramethyl-2-hexadecen-1-ol and palmitic acid as major compounds from *S. irio* shoot extract.

Bioassays with fractions of methanolic extracts

The effect of *n*-hexane and chloroform sub-fractions of methanolic leaf extract of *S. irio* on growth of FOC is shown in Figure 2. All the concentrations of *n*-hexane sub-fraction showed significant inhibitory effect against the pathogen. The lower concentrations (1.562-25 mg mL^{-1}) showed 77-90% reduction whereas higher concentrations (50-200 mg mL^{-1}) showed more adverse effect and fungal biomass was reduced from 90-93%. Similarly, all the concentrations of chloroform sub-fraction significantly (P≤0.05) suppressed growth of the pathogen. Chloroform sub-fraction was comparatively more effective against FOC than *n*-hexane sub-fraction causing 80-96% decline in FOC biomass over control (Figure 3). Previous study also support findings of the present study where *n*-hexane and chloroform sub-fractions of methanolic extract of *S. irio* also showed adverse effects on growth of *M. phaseolina* (Javaid et al., 2017).



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 LSD Test.

Figure 2 - Effect of different concentrations of *n*-hexane and chloroform sub-fractions of methanol leaf extract of *Sisymbrium irio* on biomass of *Fusarium oxysporum* f. sp. *cepae*.

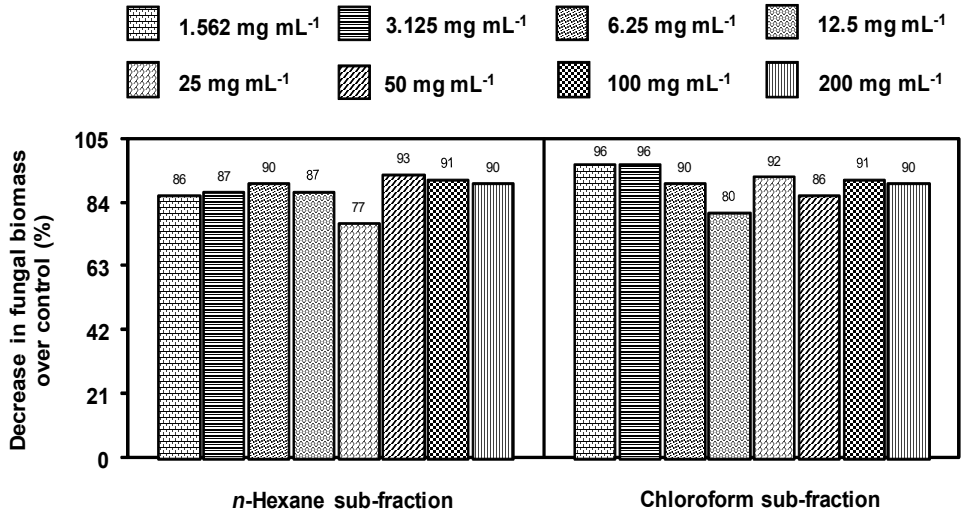


Figure 3 - Percentage decrease in biomass of *Fusarium oxysporum* f. sp. *cepae* due to different concentrations of *n*-hexane and chloroform sub-fractions of methanolic leaf extract of *Sisymbrium irio*.

GC-MS analysis

GC-MS chromatograms of *n*-hexane and chloroform sub-fractions are shown in Figure 4 while the compounds identified from these sub-fractions are presented in Table 1 and 2. GC-MS results revealed 24 compounds identified from *n*-hexane sub-fraction in which β-sitosterol (18.64%) and 4-pyrimidinecarboxylic acid,2,6-bis[(tert-butyldimethylsilyl)oxy]-,tert-butyldimethylsilyl ester (12.18%) were abundantly present. 1,2-Benzenedicarboxylic acid, diisooctyl ester (6.05%), 10-octadecenoic acid, methyl ester (7.90%), phytol (5.14%), campesterol (4.66%), hexadecane (3.80%), 1,3,4-tri-O-acetyl-2,5-di-O-methylribitol (3.75%), dodecane (3.36%), 6-ethyl-3-trimethylsilyloxydecane (3.05%), and tetradecane (3.29%),were moderately abundant.

Cyclopentanol (2.12%), decane (2.06%), eicosane (2.15%), 2-undecanone 6,10-dimethyl- (1.76%), hexadecanoic acid, methyl ester (2.12%), 2-hexyl-1-octanol (2.25%), 2[1-(R)-pantetheinyl]-myristoyl-glycinamide (1.86%), Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy] propyl ester (2.71%), Docosane, 1,22-dibromo- (1.73%), erythro-9,10-Dibromopentacosane (1.38%), hentriacontane-10,14,16-trione, mono-TMS (1.44%) and colfosceril palmitate (1.51%) were present in lower amount. The highly abundant compound β-sitosterol was previously isolated from methanol extract of aerial parts of *Senecio lyratus* and found to have antifungal activity against *Fusarium* spp. (Kiprono et al., 2000). Likewise, a mixture of β-sitosterol and stigmasterol from dichloromethane extract of *Uvaria scheffleri* leaf extract was found against *Candida albicans* (Moshi et al., 2004). In addition, various fatty acid methyl esters present in this sub-fraction are also known to posses antifungal properties (Choi et al., 2010; Pinto et al., 2017).

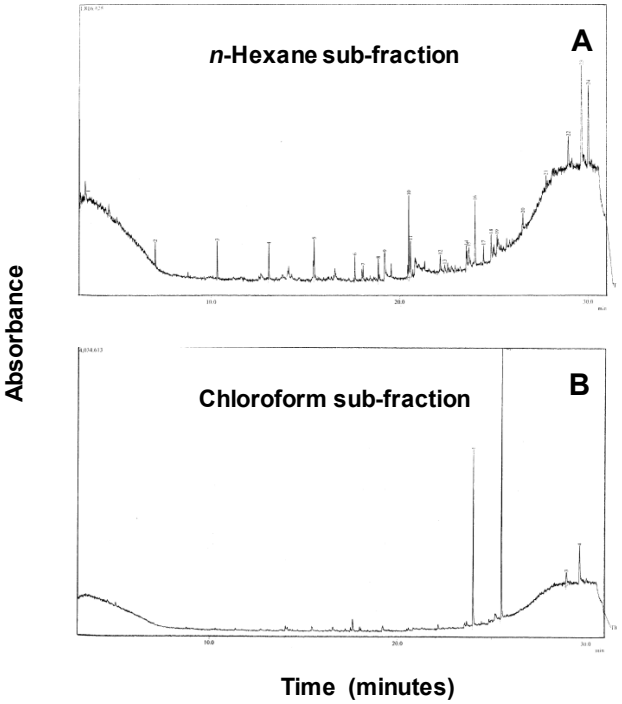


Figure 4 - GC-MS chromatogram of *n*-hexane and chloroform sub-fractions of methanolic leaf extract of *Sisymbrium irio*.

In chloroform sub-fraction, 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester (50.82%) and di-*n*-octyl phthalate (33.00%) were major compounds among the identified constituents. Campesterol and γ-Sitosterol occurred in low quantities viz. 5.22% and 10.96%, respectively. Structures of all the isolated compounds are shown in Figures 5 and 6. The most abundant compound 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester has already been identified as antifungal constituent in *Iris germanica* leaf

Table 1 - Compounds identified in *n*-hexane sub-fraction of methanolic leaf extract of *Sisymbrium irio* through GC-MS

Sr. no.	Name of compound	Formula	Weight	Retention time (min)	Peak area (%)
1	Cyclopentanol	$C_5H_{10}O$	86	3.080	2.12
2	Decane	$C_{10}H_{22}$	142	7.040	2.06
3	Dodecane	$C_{12}H_{26}$	170	10.322	3.36
4	Tetradecane	$C_{14}H_{30}$	198	13.065	3.29
5	Hexadecane	$C_{16}H_{34}$	226	15.467	3.80
6	Eicosane	$C_{20}H_{42}$	282	17.618	2.15
7	2-Undecanone, 6,10-dimethyl-	$C_{13}H_{26}O$	198	18.058	1.76
8	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	18.867	2.12
9	1,2-Benzenedicarboxylic acid, diundecyl ester	$C_{30}H_{50}O_4$	474	19.210	5.08
10	10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	20.490	7.90
11	Phytol	$C_{20}H_{40}O$	296	20.576	5.14
12	2-Hexyl-1-octanol	$C_{14}H_{30}O$	214	22.164	2.25
13	2[1-(R)-Pantetheinyl]-myristoyl-glycinamide	$C_{27}H_{52}N_4O_6S$	560	22.392	1.86
14	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy] propyl ester	$C_{25}H_{54}O_4Si_2$	474	23.541	2.71
15	1,3,4-Tri-O-acetyl-2,5-di-O-methylribitol	$C_{13}H_{22}O_8$	306	23.675	3.75
16	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	24.007	6.05
17	Docosane, 1,22-dibromo-	$C_{22}H_{44}Br_2$	466	24.459	1.73
18	6-Ethyl-3-trimethylsilyloxydecane	$C_{15}H_{34}OSi$	258	24.856	3.05
19	Erythro-9,10-Dibromopentacosane	$C_{24}H_{50}Br_2$	508	25.173	1.38
20	Hentriacontane-10,14,16-trione, mono-TMS	$C_{34}H_{66}O_3Si$	550	26.550	1.44
21	Colfosceril palmitate	$C_{40}H_{80}NO_8P$	734	27.779	1.51
22	Campesterol	$C_{28}H_{48}O$	400	28.957	4.66
23	β-Sitosterol	$C_{29}H_{50}O$	414	29.649	18.64
24	Orotic acid, bis(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	$C_{23}H_{46}N_2O_4Si_3$	498	30.008	12.18

Table 2 - Compounds identified in chloroform sub-fraction of methanolic leaf extract of *Sisymbrium irio* through GC-MS

Sr. no.	Names of compounds	Formula	Weight	Retention time (min)	Peak area (%)
1	Di-*n*-octyl phthalate	$C_{24}H_{38}O_4$	390	24.008	33.00
2	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	390	25.507	50.82
3	Campesterol	$C_{28}H_{48}O$	400	28.962	5.22
4	γ-Sitosterol	$C_{29}H_{50}O$	414	29.659	10.96

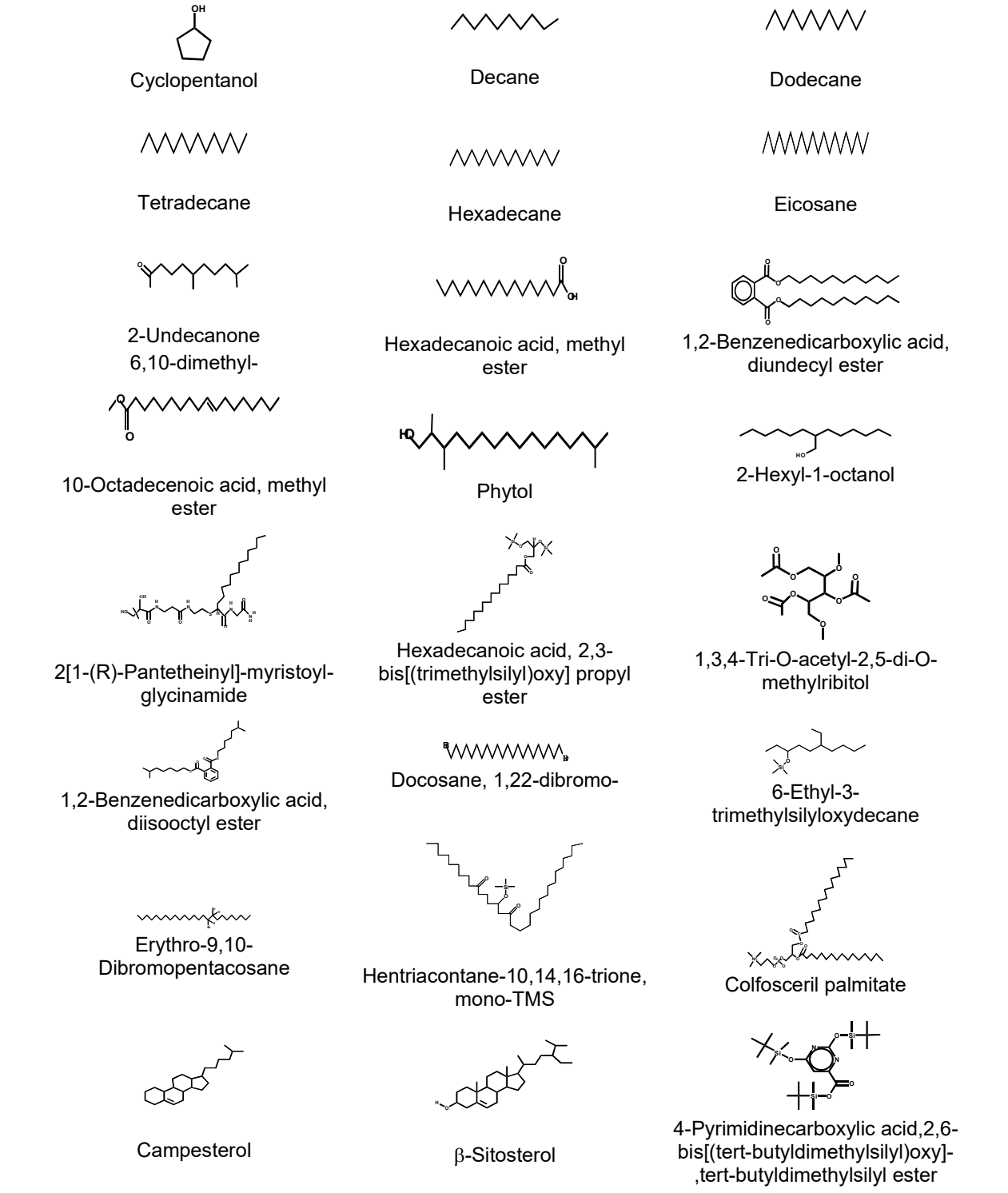


Figure 5 - Structures of compounds identified in *n*-hexane sub-fraction of methanolic leaf extract of *Sisymbrium irio.*

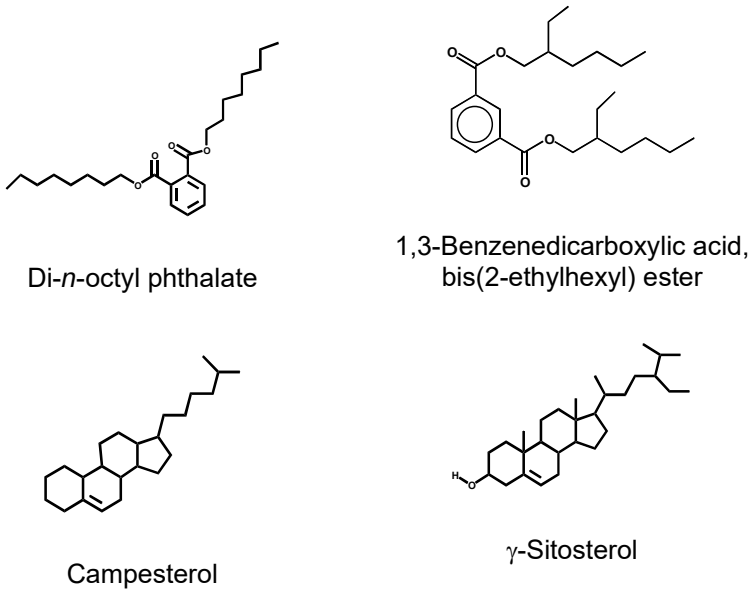


Figure 6 - Structures of compounds identified in chloroform sub-fraction of methanolic leaf extract of *Sisymbrium irio.*

extract (Asghar and Choudahry, 2011). Likewise, the second abundant compound di-*n*-octyl phthalate has been isolated from a number of plant species including *Schleichera oleosa, Dracaena cochinensis, Limonium bicolor* and *Caesalpinia sappan* (Romeh, 2013), and is known for its antifungal activity (Senthilkumar et al., 2011).

The present study concludes that leaf extract of *S. irio* contains potent antifungal compounds such as β-sitosterol; di-*n*-octyl phthalate and 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester responsible for control of *F. oxysporum* f. sp. *cepae*. However, further studies are needed to evaluate antifungal activity of these compounds individually.

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