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Article

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ANTIFUNGAL ACTIVITY OF *Cirsium arvense* EXTRACTS AGAINST PHYTOPATHOGENIC FUNGUS *Macrophomina phaseolina*

*Atividade Antifúngica dos Extratos de **Cirsium arvense** contra o Fungo Fitopatogênico **Macrophomina phaseolina***

ABSTRACT - Leaves, stems, roots and inflorescence of the asteraceous weed *Circium arvense* were extracted in methanol for two weeks. Methanol was evaporated in a rotary evaporator. Different concentrations (1, 2, 3, 4 and 5%) of methanolic extracts were prepared, and their antifungal activities were studied against *Macrophomina phaseolina*, using malt extract broth as growth medium. In general, extracts of all plant parts showed antifungal activities to variable extents. The highest antifungal activity occurred due to methanolic extract of leaves, followed by stem and root extracts, resulting in 10-74%, 6-57% and 11-39% reduction in fungal biomass over control, respectively. Inflorescence extract showed the least antifungal activity, resulting in 2-30% reduction in fungal biomass over control. There was a linear and inverse relationship between extract concentrations and fungal biomass for extracts of all the four parts. GC-MS analysis showed that there were 10 compounds in most effective methanolic leaf extract. Among these, 10-octadecanoic acid, methyl ester (26.442%), 2H-1-benzopyran, 6,7-dimethoxy-2-2-dimethyl (20.195%), hexadecanoic acid, methyl ester (15.752%) and 9,12-octadecadienoic acid (Z,Z)-, methyl ester (12.628%) were predominant compounds in the extract that may be responsible for antifungal activity. This study concludes that methanolic leaf extracts of *C. arvense* can be used for the management of *M. phaseolina*.

Keywords: antifungal activity, asteraceous weed, *Cirsium arvense, Macrophomina phaseolina*.

*RESUMO - Foram extraídos em metanol, por duas semanas, as folhas, caules, raízes e inflorescência de uma planta daninha da família Asteraceae, a **Circium arvense**. A evaporação do metanol se fez com o auxílio de um evaporador rotativo. Foram preparadas diferentes concentrações (1, 2, 3, 4 e 5%) de extratos de metanol, e suas atividades antifúngicas contra o **Macrophomina phaseolina** foram analisadas através da utilização de caldo extrato de malte como ambiente de crescimento. De forma geral, ocorreram graus variáveis de atividades antifúngicas nos extratos de todas as partes da planta. A atividade antifúngica mais significativa ocorreu ao extrato de metanol das folhas, seguido dos extratos do caule e da raiz, com redução de 10-74%, 6-57% e 11-39% de biomassa fúngica em relação ao grupo controle, respectivamente. No extrato de inflorescência ocorreu a menor atividade antifúngica, resultando em redução de 2-30% de biomassa fúngica em relação ao controle. Houve uma relação inversamente proporcional entre concentração de extrato e biomassa fúngica para todas as quatro partes de extratos. O método GC-MS de análise revelou 10 compostos no extrato de metanol da folha, que foi mais eficaz. Dentre estes, o ácido 10-octadecanóico, éster de metilo*

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(26,442%), 2H-1-benzopirano, 6,7-dimetoxi-2-2-dimetil (20,195%), ácido hexadecanóico, éster de metilo (15,752%) e ácido 9-12-octadecadienóico (Z,Z)-, éster de metilo (12,628%) predominaram no extrato que pode ter sido responsável pela atividade antifúngica. Este estudo conclui que extratos de metanol de folhas de ***C. arvense*** *podem ser utilizados para controlar o* ***M. phaseolina****.*

Palavras-chave: atividade antifúngica, ervas daninhas da Asteraceae, *Cirsium arvense*, *Macrophomina phaseolina*.

INTRODUCTION

The notorious soil and seed-borne fungus *Macrophomina phaseolina* is responsible for infecting over 500 hosts, including legumes and cereal crops, and it generally causes rot diseases, especially charcoal rot disease, in arid and water-deficient regions of the world (Das et al., 2008). Many economically important oilseed plants, such as *Glycine max*, *Gossypium herbaceum*, *Zea mays*, *Sesamum indicum*, *Phaseolus vulgaris* and *Helianthus annuus*, also become victims of this fungus, with great yield loss (Mayek-Perez et al., 2002; Beas et al., 2006; Abdel-Kader et al., 2010). Besides being a destructive phytopathogen, *M. phaseolina* has also been documented as an opportunistic pathogen to human with the potential to weaken plant, animal, and human immunity (Arora et al., 2012). Moreover, strains that can infect plants and humans have been interestingly reported. Similarly, it is possible that it could spread widely, causing unpredictable losses to every living being (Srinivasan et al., 2009).

M. phaseolina has shown to be comparatively a hard pathogen to control, and cultural, chemical or biological disease management strategies have failed to provide expected results (Abdel-Kader et al., 2010). So far, no registered fungicide has become available to fight *M. phaseolina* (Srinivasan et al., 2009), although some fungicides such as quintozene (PCNB) and captan have been used against this pathogen to some extent (Frison et al., 1990; Dubey et al., 2012). However, the plead for sustainable agriculture in an eco-friendly manner cannot be sustained with such health hazard chemicals (Oruc, 2010). An alternative strategy could be applied through the exploration of natural antimicrobial compounds from plant bark, stems, leaves, flowers and fruits (Ikram and Dawar, 2013; Silva et al., 2014; Javaid and Akhtar, 2015). These can act directly as pesticides or may provide structural lead for the development of pesticides (Kilani, 2006; Tapwal et al., 2011).

Cirsium arvense is a perennial plant belonging to family Asteraceae (Orhan et al., 2013). It is native to Europe, parts of North Africa and Asia, including Pakistan, Iran Afghanistan and China (Jacobs, 2006). It is often found as a noxious weed in almost all types of crops, including pastures and range lands (Khan et al., 2011a). Its diuretic, anti-inflammatory and hemostatic, astringent, anti-phlogerstic and hepatic medicinal values have been documented to be associated with the presence of flavonoid and coumarin (Khan et al., 2011b). Likewise, antioxidant and antimicrobial aspects have also been previously reported as positive properties of *C. arvense* (Maria et al., 2010; Orhan et al., 2013). However, there are only a few studies addressing the antifungal activity of *C. arvense* against *M. phaseolina*. Therefore, in this present study, the antifungal activity of methanolic extract of different parts of *C. arvense* has been assessed against *M. phaseolina*.

MATERIALS AND METHODS

Fungal pathogen isolation and culturing

M. phaseolina was isolated from infected mash bean stem. The 2 mm pieces of infected stem were surface sterilized with 1% sodium hypochlorite solution, by soaking them for 2 min, followed by washing with autoclaved water. The pre-sterilized samples were placed on 2% malt extract agar medium and incubated at 27 °C for 7 days. Fungus identification was confirmed through macro and microscopic features.

Plant collection and identification

C. arvense was collected from barren land and fields at the University of the Punjab, Lahore, Pakistan during March and April, 2014. Taxonomic identification of the plant was confirmed by

Dr. Arshad Javaid at the Institute of Agricultural Sciences (IAGS), in the University of the Punjab, Lahore.

Different plant parts i.e., leave, stems, roots and inflorescence, were isolated and washed thoroughly with water, shade dried, ground to coarse powder, and stored in polythene bags at room temperature.

Methanolic extract preparation

Methanol (2 L) was used for soaking 200 g of each of the four parts of dried plant. After 14 days, soaked materials were filtered through muslin cloth, and double filtered with filter paper, to make it dust free. The methanolic filtrate was evaporated on rotary evaporator at 45 °C to remove methanol, and the dark brown gummy mass was obtained. To completely remove methanol, the gummy materials were taken in pre-weighed beakers and incubated at 45 °C. Finally, the amount of extract was measured for the stems (14.5 g), leaves (15 g), roots (10.78 g) and inflorescences (11.8 g). Afterwards, it was stored in cool and dry place for further use.

Antifungal assays

Stock solutions of methanolic extracts were made by dissolving 9.0 g of each of the extracts of leaf, stem, root and inflorescence in 5 mL dimethyl sulphoxide (DMSO), followed by the addition of sterilized distilled water to make volume up to 15 mL. Control solution was prepared by mixing DMSO and water in 5:10. Pre-sterilized malt extract broth (55 mL) was supplemented with antibacterial chloromycetein (25 mg 100 mL^{-1}). Five concentrations viz. 0, 1, 2, 3, 4, 5 g 100 mL^{-1} were prepared in each 250 mL flasks by adding measured quantities of stock and control solutions to achieve a 60 mL final volume of the medium. Medium was divided into three equal portions. Flasks were inoculated with mycelial plugs (5 mm diameter) from a 7 day old colony of *M. phaseolina* and incubated for one week. Thereafter, fungal biomass was filtered, dried at 70 °C in an oven, and weighed (Javaid and Iqbal, 2014). The experiment was carried out using completely randomized design.

GC-MS of methanolic fraction

Methanolic root extract of *C. arvense* was filtered through Millipore filter paper with the help of filter assembly to remove any kind of suspended particles. Samples were analyzed by GC-MS for identification of various chemical constituents on Agilant technologies model GC 7890A coupled with MS 5975C mass spectrometer.

Statistical analysis

Acquired experimental data were assessed by analysis of variance. Means were sorted through the LSD method at 5% level of significance, using the Statistix 8.1 software. The relationship between extract concentrations and fungal biomass was calculated using MS Excel.

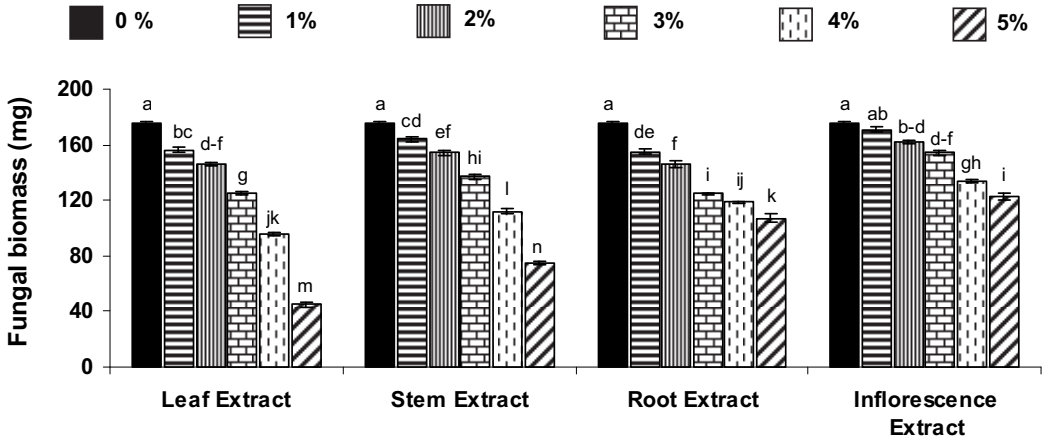
RESULTS AND DISCUSSION

The analysis of variance showed significant (P≤0.001) effect of plant parts, extract concentrations, as well as their interaction for fungal biomass (Table 1). Among different plant parts, methanolic leaf extract of *C. arvense* showed the highest antifungal activity. Fungal biomass significantly dropped by 10-74%, due to various concentrations of this extract over control (Figure 1A and 2). There was a linear relationship between extract concentrations and fungal biomass with R^2 = 0.9297 (Figure 3A).

Table 1 - Analysis of variance (ANOVA) for the effect of different concentrations of methanolic leaf, stem, root and inflorescence extracts of *Cirsium arvense* on the biomass of *Macrophomina phaseolina*

Sources of variation	Df	SS	MS	F values
Plant parts (P)	3	10416	3472	332*
Concentration (C)	5	83986	16797	1607*
P × C	15	10673	712	68*
Error	72	753	10.5	
Total	95	105827		

* Significant at P≤0.001.



Vertical bars show mean standard errors of three replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Tukey's HSD Test.

Figure 1 - Effects of different concentrations of *Cirsium arvense* methanol extracts on the biomass of *Macrophomina phaseolina.*

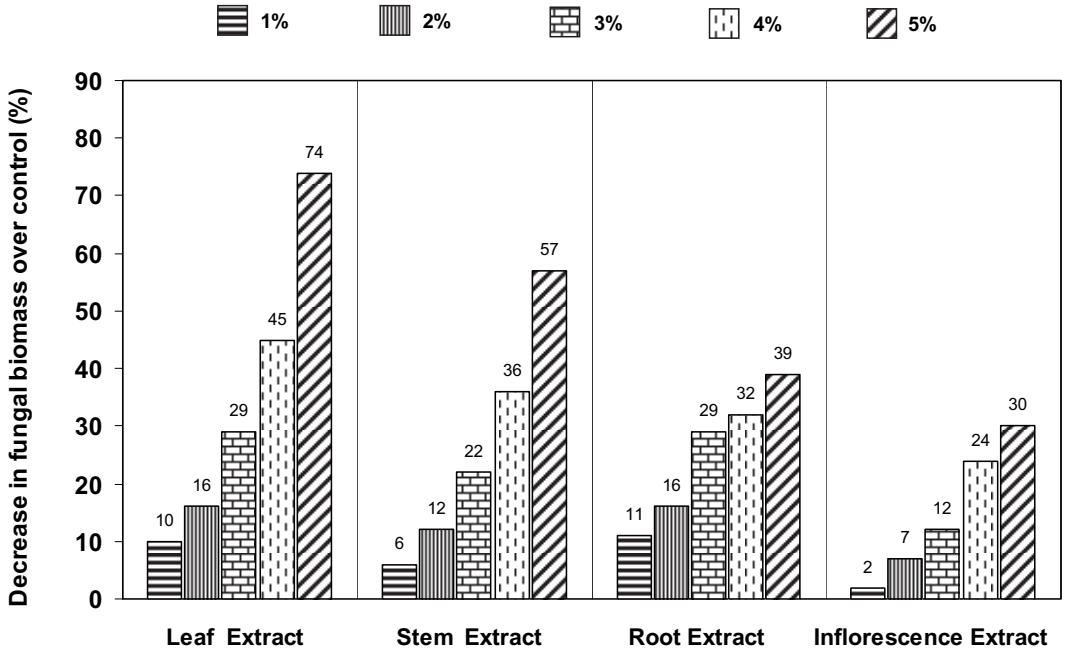


Figure 2 - Percentage decrease in the biomass of *Macrophomina phaseolina* due to different concentrations of *Cirsium arvense* methanolic leaf, stem, root and inflorescence extracts over control.

The highest antifungal activity in leaf extract could be due to the presence of the highest content of polyphenol and flavonoid as compared to the other plant parts (Mihaela, 2014). It has been documented that flavonoid content of leaves is enough to exhibit antifungal property compared to other plant parts. So far, flavonoid has been enlisted amongst the most diverse and widespread groups of natural compounds with greater antimicrobial and radical scavenging properties (Karasakal et al., 2015).

Similar to those of the leaf extract, all the concentrations of methanolic stem extract showed significant inhibitory effects on fungal growth. However, the stem extract was found to be comparatively less inhibitory to fungal growth than the leaf extract (Figure 1B). There was 6-57% suppression in fungal biomass due to different concentrations of the stem extract compared to control (Figure 2). The relationship between the concentrations of stem extract and fungal biomass was linear with $R^2 = 0.935$ (Figure 3B). Although all concentrations of methanolic root

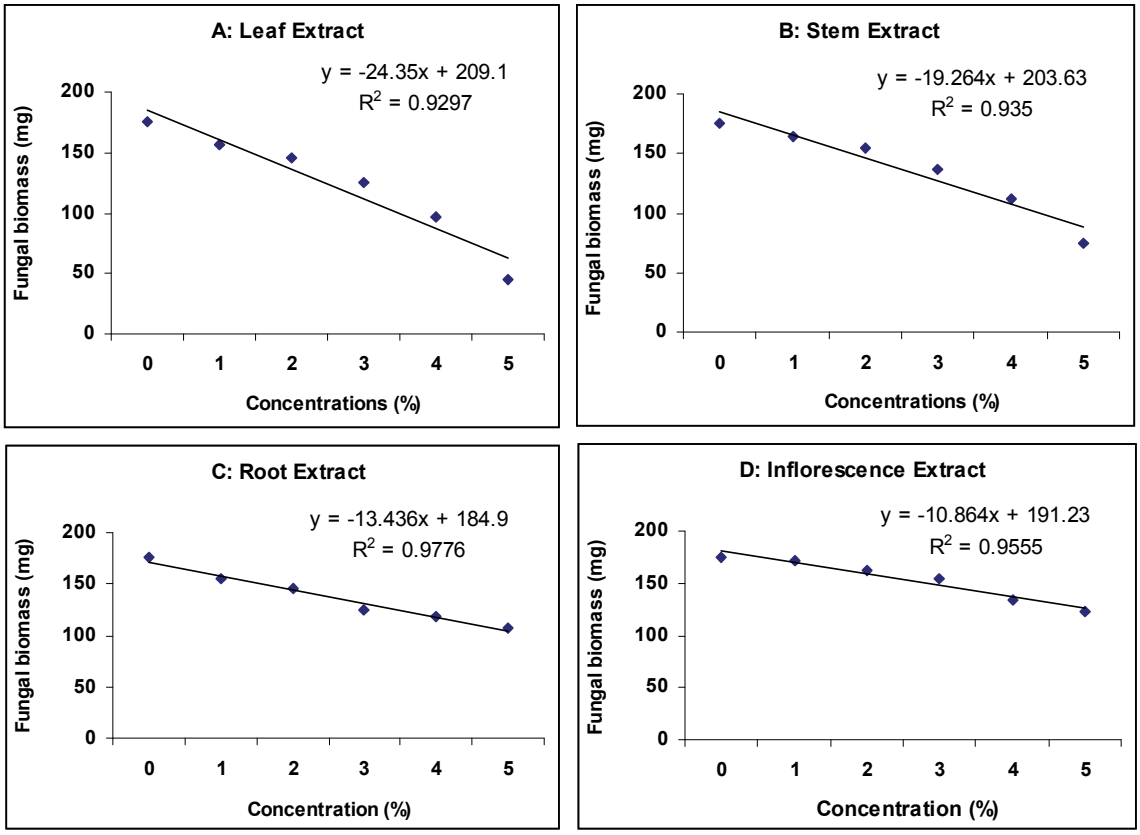


Figure 3 - Regression analysis of the effects of different concentrations of *Cirsium arvense* methanolic leaf, stem, root and inflorescence extracts on the biomass of *Macrophomina phaseolina*.

extract of *C. arvense* significantly reduced fungal growth over control, however, this extract was found comparatively less toxic against *M. phaseolina* than leaf and stem extracts (Figure 1C). There was significant decline of 11-39% in the target pathogen biomass due to various concentrations of this extract over control (Figure 2). Extract concentrations showed a linear relationship with the fungal biomass with $R^2 = 0.9776$ (Figure 3B). Inflorescence extract of *C. arvense* showed the least antifungal activity. The lowest concentration of 1% presented an insignificant effect on the growth of the target fungal pathogen. However, further increase in extract concentration significantly and gradually decreased fungal biomass, showing a linear relationship with $R^2 = 0.9555$ (Figure 3D). There was 2-30% reduction in fungal biomass due to different concentrations of the extract over control (Figure 2). Variation in antifungal activity in different parts of plants has also been reported in previous studies (Banaras et al., 2015; Javaid et al., 2015). This variation may be associated with the presence of different types or amounts of natural antifungal constituents in different parts of a plant species. Khan et al. (2011a) have previously reported antifungal activity of *C. arvense* against *Aspergillus niger*. It has been documented that polyacetylenic nature of compounds present in *C. arvense* along with tannin, gallic acid, taraxasterol, aplotaxene and their derivatives probably bestowed it with strong antifungal potential (Donald, 1994; Norton, 2005). Khan et al. (2011b) isolated ciryneol *C. scopoletin*, pectolinarigenin-7-O-glucopyranoside and acacetin from chloroform soluble fraction of *C. arvense* whole plant. All of these compounds showed variable antifungal activities against *Candida albicans*, *Aspergillus flavus*, *Microsporum canis*, *Candida glaberata* and *Fusarium solani*. Conversely, Borchardt et al. (2008) reported the absence of any antimicrobial activity in all parts of *C. arvense*. This contradiction could be explained by geographical differences that may contribute to such different plant traits (Wright and Tinker, 2012).

The GC-MS analysis of *C. arvense* methanolic leaf extract showed the presence of 10 phytochemical constituents (Figure 4). Table 2 shows the active constituents and their retention time, molecular formula, molecular weight, and percentage peak area. Four of the compounds

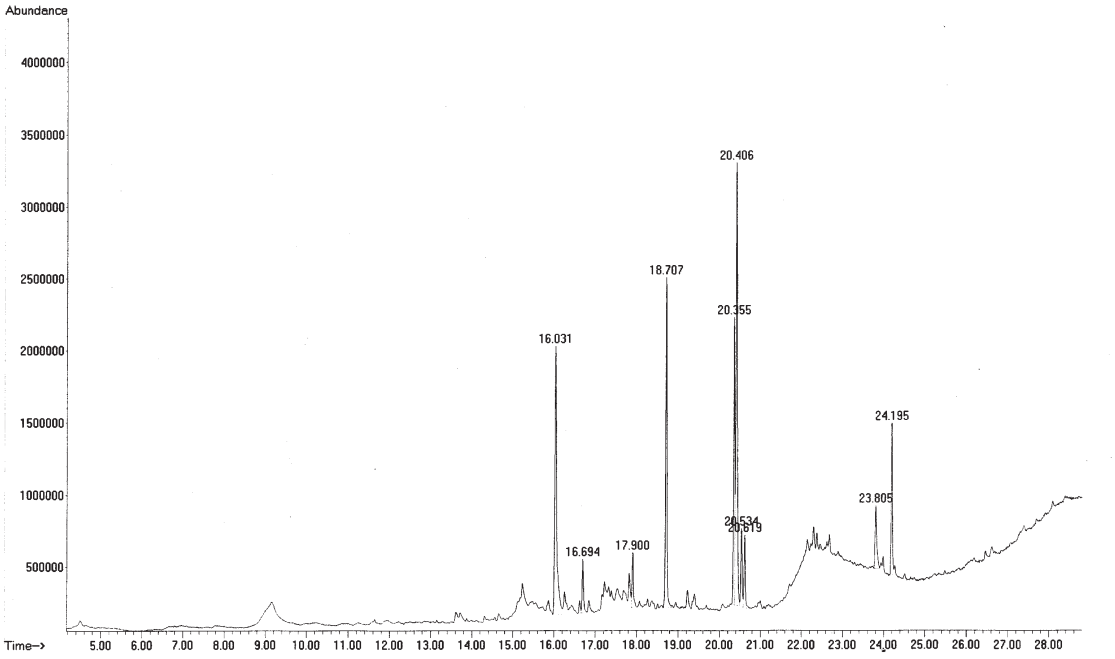
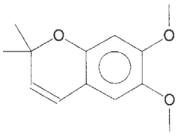
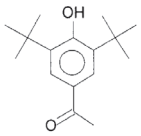


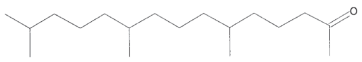
Figure 4 - *Cirsium arvense* methanolic leaf extract GC-MS chromatogtam.



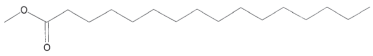
1- 2H-1-Benzopyran, 6, 7-dimethoxy-2-2-dimethyl



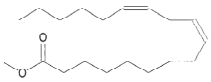
2- 3,5-ditert-Butyl-4-hydroxyacetophenone



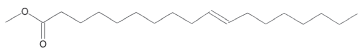
3- 2-Pentaadecanone, 6, 10, 14-trimethyl



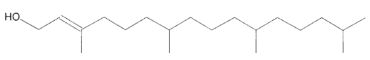
4- Hexadecanoic acid, methyl ester



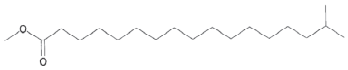
5- 9, 12-Octadecadienoic acid (Z,Z)-, methyl ester



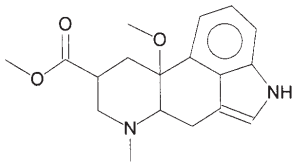
6- 10-Octadecanoic acid, methyl ester



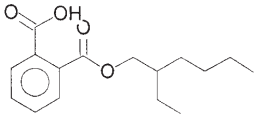
7- Phytol



8- Heptadecanoic acid, 16-methyl-, methyl ester



9- Ergoline-8-carboxylic acid, 10-mrthoxy-6-methyl-, methyl ester, (8 alpha)



10- 1, 2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester

Figure 5 - Compound structures in *Cirsium arvense* methanolic leaf extract identified through GC-MS analysis.

Table 2 - GC-MS analysis of *Cirsium arvense* methanolic leaf extract

Comp. No.	Compound Name	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
1	2H-1-Benzopyran, 6, 7-dimethoxy-2-2-dimethyl	$C_{13}H_{16}O_3$	220	16.031	20.195
2	3,5-ditert-Butyl-4-hydroxyacetophenone	$C_{16}H_{24}O_2$	248	16.694	2.858
3	2-Pentaadecanone, 6,10,14-trimethyl	$C_{18}H_{36}O$	268	17.900	2.966
4	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	18.707	15.752
5	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294	20.355	12.628
6	10-Octadecanoic acid, methyl ester	$C_{19}H_{36}O_2$	296	20.406	26.442
7	Phytol	$C_{20}H_{40}O$	296	20.534	4.048
8	Heptadecanoic acid, 16-methyl-, methyl ester	$C_{19}H_{38}O_2$	298	20.619	3.541
9	Ergoline-8-carboxylic acid, 10-mrthoxy-6-methyl-, methyl ester, (8 alpha)	$C_{18}H_{22}N_2O_3$	314	23.805	4.150
10	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278	24.195	7.420

Table 3 - Nature and properties of compounds found from the methanolic leaf extract of *Cirsium arvense* through GC-MS analysis

Comp. No.	Compound Name	Nature	Property	Reference
1	2H-1-Benzopyran, 6, 7-dimethoxy-2-2-dimethyl	Coumarin (chromene)	Antibacterial, antimicrobial	Thareja et al. (2010); Thomas and Zachariah (2013)
2	3,5-ditert-Butyl-4-hydroxyacetophenone	ketone	Antibacterial	Mitu et al. (2009)
3	2-Pentaadecanone, 6,10, 14-trimethyl	ketone	Antibacterial	Akpuaka et al. (2013)
4	Hexadecanoic acid, methyl ester	Palmitic acid methyl ester (fatty acid methyl ester)	Antimicrobial, pesticidal, nematicidal	Chandrasekaran, et al. (2011); Hema et al. (2011)
5	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Linoleic acid methyl ester (fatty acid methyl ester)	Potent antifungal, antibacterial	Godwin et al. (2015)
6	10-Octadecanoic acid, methyl ester	Stearic acid methyl ester (fatty acid methyl ester)	Antimicrobial	Abubakar and Majinda (2016)
7	Phytol	Diterpene	Antifungal, antibacterial	Hema et al. (2011)
8	Heptadecanoic acid, 16-methyl-, methyl ester	Margaric acid methyl methyl ester (fatty acid methyl ester)	Antimicrobial	Zheng et al. (2005)
9	Ergoline-8-carboxylic acid, 10-mrthoxy-6-methyl-, methyl ester, (8 alpha)	Clavine alkaloids	LSD related used in medicines of prolactinoma and Parkinsons disease	Yates et al. (1985); Sundar and Justin (2015)
10	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	Aromatic dicarboxylic ester	Antifungal, Antimicrobial, antitumor, anti diabetic, anticancer, antioxidant, antiscabies, antiinflammatory	Syeda et al. (2011); Balachandran et al. (2012); Bagavathi and Ramasamy (2012)

found were fatty acid methyl esters, 2 were ketones, one of coumarin, diterpene, alkaloids and dicarboxylic ester. The compounds found included 10-octadecenoic acid, methyl ester (26.442%), 2H-1,benzopyran, 6,7-dimethoxy-2, 2-dimethyl- (20.195%), hexadecanoic acid, methyl ester (15.752%), 9,12- octadecadienoic acid (Z,Z)-, methyl ester (26.442%), 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester (7.420%), ergoline 8-carboxylic acid, 10-methoxy-6-methyl-, methyl ester (4.150%), phytol (4.048%), heptadecanoic acid, 16-methyl,- methyl ester (3.541%), 3,5-di-tert-butyl-4-hydtoxyacetophenone (2.858%), 2H-1-benzopyran, 6,7-dimethoxy-2, 2-dimethyl- (2.858%). Figure 5 shows the structures of the isolated compounds. The most frequently occurring compound 10-octadecanoic acid, methyl ester is known to have antimicrobial activity (Zheng et al., 2005). Among others, 2H-1-benzopyran, 6,7-dimethoxy-2-2-dimethyl; hexadecanoic acid, methyl ester; 9,12-octadecadienoic acid (Z,Z)-, methyl ester; phytol and 1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester are known to have antifungal and antimicrobial activities, as shown in Table 3.

The present study concludes that all parts of *C. arvense* have antifungal potential against *M. phaseolina.* The leaf extract showed the highest antifungal activity, followed by the stem extract.

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