



Article

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BIOASSAYS GUIDED FRACTIONATION OF *Senna occidentalis* FOR IDENTIFICATION OF NATURAL ANTIFUNGAL CONSTITUENTS AGAINST *Macrophomina phaseolina*

Fracionamento Baseado em Bioensaios de Senna occidentalis para Identificação dos Componentes Antifúngicos Naturais Contra Macrophomina phaseolina

ABSTRACT - *Macrophomina phaseolina* is one of the most destructive soil-borne fungal plant pathogens. In the present study, different aerial parts of *Senna occidentalis* were analyzed for their antifungal activity against *M. phaseolina*. In initial screening bioassays, 0.5-3.0% concentrations of methanolic extracts of fruit, leaf and stem were tested against *M. phaseolina*. The methanolic leaf extract showed the greatest activity causing up to 29% suppression in biomass production of *M. phaseolina*. Fractionation of leaf extract of *S. occidentalis* was carried out with four organic solvents. Bioassays with a range of concentrations (2.34-150 mg mL⁻¹) of these sub-fractions revealed that the chloroform sub-fraction was the most effective, causing 93-98% reduction in the biomass of *M. phaseolina*, followed by 59-92% suppression in fungal biomass due to the *n*-hexane sub-fraction. The ethyl acetate and *n*-butanol sub-fractions were only effective at higher concentrations. GC-MS analysis of chloroform sub-fraction was performed to identify different compounds. Six compounds were identified in this fraction; 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester was the predominant compound (53.55%) followed by 9,10-dimethyltricyclo[4.2.1.1 (2,5)]decane-9,10-diol (22.68%), cyclohexanol, and 2-(2-hydroxy-2-propyl)-5-methyl (9.87%).

Keywords: Antifungal activity, leaf extract, *Macrophomina phaseolina*, Natural fungicide, *Senna occidentalis*.

RESUMO - *Macrophomina phaseolina* é um dos mais destrutivos agentes patogênicos fúngicos de plantas com origem no solo. No presente estudo, as diferentes partes aéreas de *Senna occidentalis* foram analisadas em relação à atividade antifúngica contra *M. phaseolina*. Em bioensaios iniciais de triagem, concentrações de 0,5 a 3,0% dos extratos metanólicos de frutas, folhas e caule foram testadas contra *M. phaseolina*. O extrato metanólico das folhas mostrou a maior atividade, causando supressão de 29% na produção de biomassa de *M. phaseolina*. O fracionamento do extrato das folhas de *S. occidentalis* foi realizado com quatro solventes orgânicos. Os bioensaios com diversas concentrações (2,34 a 150 mg mL⁻¹) dessas subfrações revelaram que a subfração de clorofórmio foi a mais eficaz, causando redução de 93-98% na biomassa de *M. phaseolina*, seguida da supressão de 59-92% na biomassa fúngica devido à subfração de *n*-hexano. As subfrações de acetato de etila e *n*-butanol foram eficazes apenas nas concentrações mais elevadas. A análise por GC-MS da subfração de

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clorofórmio foi feita para identificar compostos diferentes. Seis compostos foram identificados nesta fração; 1,3-benzenodicarboxílico, bis (2-etilhexil) éster foi o composto predominante (53,55%), seguido por 9,10-dimetil triciclo [4.2.1.1 (2,5)] decano-9,10-diol (22,68%), cicloexanol, e 2-(2-hidroxi-2-propil)-5-metil (9,87%).

Palavras-chave: atividade antifúngica, extrato de folhas, *Macrophomina phaseolina*, fungicida natural, *Senna occidentalis*.

INTRODUCTION

Soil-borne fungal pathogen *Macrophomina phaseolina* is the causal agent of root diseases of more than 500 different plant species including maize, mungbean, chickpea, sorghum, alfalfa etc. (Wyllie, 1993; Ma et al., 2010). The pathogen exists in soil as sclerotia, which are compact hardened structures of mycelium. Sclerotia can survive in the soil for many years and with the advent of appropriate conditions, they can produce hyphae which then cause infection in plant roots. It has been reported that *M. phaseolina* can exhibit high variation in genetic diversity or pathogenicity or both, enabling itself to carry on and adapt in different set of environmental conditions (Abawi and Corrales, 1990; Baird et al., 2003). Due to its adaptability and availability of such a large number of hosts, it is very difficult to effectively control *M. phaseolina*. So far, no resistant host cultivar or any approved fungicide has been identified for this pathogen. Soil fumigation with methyl bromide and chloropicrin can provide some control of soil-borne pathogens (Kiran et al., 2006), but these fumigants also destroy beneficial microorganisms (Gamliel et al., 2000). Moreover, large-scale fumigation is neither practical nor economical. Increased public concern about the use of synthetic chemicals has diverted the attention of scientists towards environment-friendly management systems, which are based on naturally-occurring compounds in plants and microorganisms (Cuthbertson and Murchie, 2005; Babu et al., 2008).

Nature provides the source of pesticides in plants that need to be discovered. Recent studies have shown that the use of various plant crude extracts or isolated purified compounds can effectively control *M. phaseolina* and other fungi (Kanwal et al., 2010; 2012; Banaras et al., 2015). These compounds can be further used to prepare pesticides. Antifungal activity of extracts of various plants such as *Cymbogon citratus*, *Syzygium cumini*, *Eucalyptus citriodora*, *Azadirachta indica*, *Melia azedarach*, *Datura metel* and *Chenopodium album* have been checked against this pathogen (Bankole and Adebajo, 1995; Javaid and Saddique, 2012; Javaid and Rauf, 2015). The weed *Senna occidentalis* belongs to family Fabaceae and is commonly found in tropics, and it can easily be grown from seeds (Kaey, 1989). It is a medicinal plant useful for measles in children, convulsions, whooping cough, poisonous snake bites, hepatitis, toothache, headache and jaundice (Bin-Hafeez and Hussaini, 2001; Nuhu and Aliyu, 2008). Different parts are also reported to possess antiplasmodial, anti-inflammatory and antimicrobial activities (Kuo et al., 1996; Sadiq et al., 2012). However, studies regarding its antifungal activities against *M. phaseolina* are lacking. Therefore, in the present study, the antifungal potential of *S. occidentalis* has been evaluated for management of *M. phaseolina*.

MATERIALS AND METHODS

Screening bioassays

These bioassays were conducted ~~while~~ following the method of Javaid and Akhtar (2015). Thoroughly crushed, 200 g of different parts of *S. occidentalis* were separately soaked in one liter of methanol at room temperature and left for two weeks. Afterwards, the soaked materials were filtered first through cheesecloth and then passed through filter papers. Methanol in the filtrates was then evaporated in a rotary evaporator to obtain 10.4 g leaf, 9.47 g fruit and 5.68 g stem extracts. *In vitro*, bioassays with these extracts were carried out in 100 mL conical flasks. The methanolic extract (4.725 g) of each part was mixed in 4 mL of dimethyl sulfoxide (DMSO), and 10.5 mL of stock solution was prepared by adding distilled water. Similarly, a control solution was made by mixing DMSO and distilled water at 4:6.5. Malt extract broth (42 mL) was sterilized by autoclaving at 121 °C and 103.4 kPa pressure for 30 minutes. After cooling, six concentrations

viz., 0.5, 1, 1.5, 2, 2.5, 3% were prepared by adding 0.5, 1, 1.5, 2, 2.5, 3 mL stock solution and 2.5, 2, 1.5, 1, 0.5, 0 mL control solution, respectively, to each flask. These 45 mL of each treatment were equally divided into three portions and poured into 100 mL flasks. In the control treatment, 42 mL of malt extract broth and 3 mL of control solution were mixed. One fungal disc of 5 mm in diameter was added in each flask aseptically from an actively growing culture of *M. phaseolina*. After one week of incubation at 28 °C, fungal biomass was collected on pre-weighed filter papers, dried at 60 °C and weighed.

Bioassays with fractions of methanolic leaf extract

Methanolic leaf extract of *S. occidentalis* was selected for fractionation using various organic solvents following the method of Javaid and Bashir (2015) with some modifications. For this purpose, 2 kg of dry and powdered leaves of *S. occidentalis* were extracted in 6 L. of methanol for two weeks. After filtration, material was re-extracted in methanol for a week and filtered. Filtrates were combined and evaporated on a rotary evaporator at 45 °C. After evaporation of methanol, the remaining material was mixed with 300 mL distilled water and partitioned with *n*-hexane again and again for complete separation of *n*-hexane soluble materials from the aqueous phase. The remaining extract was further partitioned with chloroform, ethyl acetate and *n*-butanol. Solvents were evaporated and 12 g of *n*-hexane, 0.83 g of chloroform, 0.99 g of ethyl acetate, 12 g of *n*-butanol and 15 g of aqueous sub-fraction were obtained.

In order to test antifungal activity, 0.75 g of each sub-fractions was dissolved in 0.3 mL DMSO followed by addition of 4.7 mL malt extract broth. This stock solution (150 mg mL⁻¹) was serially double diluted to prepare 75, 37.5, 18.75, 9.38, 4.69 and 2.343 mg mL⁻¹ concentrations. A series of control treatments was prepared by adding 0.3 mL DMSO to 4.7 mL malt extract broth followed by serially double dilution to maintain same amount of DMSO in the control and experimental treatments. The experiment was conducted in 10 mL test tubes and replicated thrice. After incubation with one drop of *M. phaseolina* culture suspension, tubes were incubated at 28 °C for one week. Thereafter, fungal biomass was filtered, dried at 60 °C and weighed.

GC-MS analysis

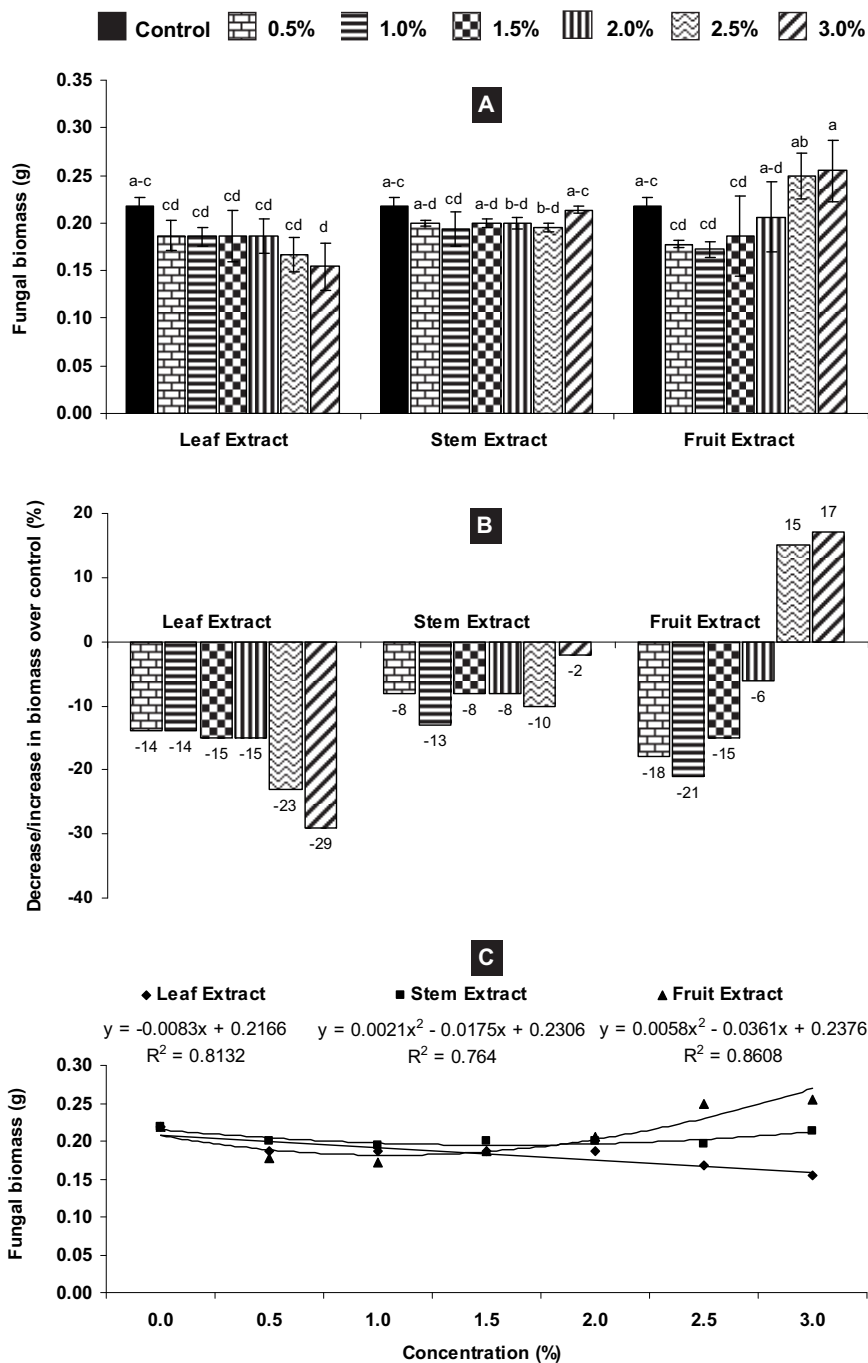
The chloroform sub-fraction of the methanolic leaf extract was found to be highly effective against the target fungus and, thus, selected for GC-MS analysis. GC-MS analysis was performed using an Agilent 7890A GC system with 5975C mass Spectrometer fitted with a HP5-MS capillary column.

Statistical analysis

Data regarding germination and plant growth in laboratory and pot experiments were analyzed by ANOVA followed by the LSD test ($P < 0.05$) using the software Statistix 8.1.

RESULTS AND DISCUSSION

ANOVA indicated that the effect of plant parts was significant for fungal biomass production. Leaf extract showed remarkable antifungal activity. Its different concentrations suppressed biomass of *M. phaseolina* by 14-29% over control. The highest concentration (3%) of leaf extract significantly reduced the fungal biomass by 29%, while the effect of other concentrations was not significant. The effect of all the concentrations of methanolic stem extract was insignificant where 2-13% reduction in fungal biomass was noted. Lower concentrations (0.5% to 2%) of fruit extract reduced fungal biomass by 6-21%. By contrast, higher concentration (2.5% and 3%) stimulated fungal growth and enhanced the production of fungal biomass by 15-17% over control. However, in general, the effect of all the concentrations of fruit extract was insignificant as compared to the control (Figure 1A and B). There was a linear relationship between fungal biomass and different concentrations of leaf extract with $R^2 = 0.8132$ (Figure 1C). Earlier studies have shown variation in antifungal properties of different parts of *Syzygium cumini*, *Coronopus didymus* and *Withania somnifera*, against *Ascochyta rabiei* and *Sclerotium rolfsii* (Jabeen and Javaid, 2010; Iqbal and Javaid, 2012; Javaid and Munir, 2012). This variation may be attributed to the presence



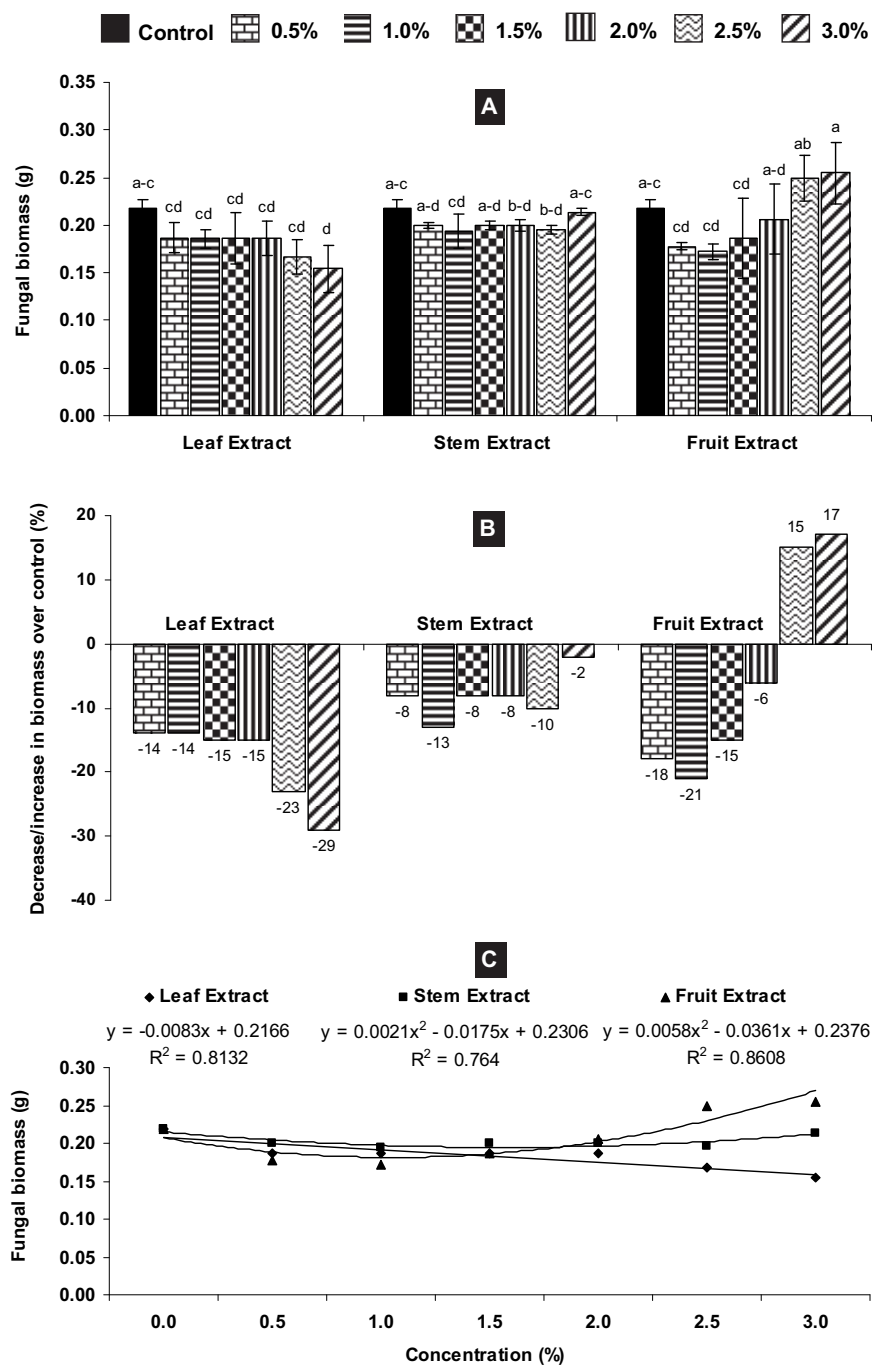
Vertical bars show standard errors of means of three replicates. Values with different letters at the top show significant difference ($P \leq 0.05$) as determined by the LSD Test.

Figure 1 - A - Effect of different concentrations of methanolic leaf, stem and fruit extracts of *Senna occidentalis* on biomass of *Macrophomina phaseolina*. B - Percentage decrease/increase in fungal biomass due to different concentrations of methanolic extracts over control, C) Relationship between different concentrations of methanolic extracts of *S. occidentalis* and biomass of *M. phaseolina*.

of different compounds in various plant parts. Use of methanolic extracts has been proved very effective in such antifungal bioassays. Methanolic extracts are preferred over aqueous extracts in antifungal bioassays because there is no chance of contamination during extraction of plant materials in methanol (Amin et al., 2012; Rauf and Javaid, 2013).

The methanolic leaf extract was further fractionated because of its best antifungal activity. Among the various sub-fractions, the chloroform sub-fraction showed the maximum growth inhibition of *M. phaseolina*. All of its concentrations significantly declined fungal growth resulting

in 93-98% reduction in its biomass. Likewise, all the concentrations of the *n*-hexane sub-fraction reduced biomass of *M. phaseolina* significantly by 59-92% (Figure 2A and B). In case of the ethyl acetate and *n*-butanol sub-fractions, generally lower concentrations were ineffective while higher concentrations showed significant antifungal activity. Higher concentrations (18.75 mg mL⁻¹ to 150 mg mL⁻¹) of the ethyl acetate sub-fraction significantly reduced the fungal biomass by 92-96% over control. In contrast, the lowest concentration (2.34 mg mL⁻¹) increased fungal growth



Vertical bars show standard errors of means of three replicates. Values with different letters at the top show significant difference ($P \leq 0.05$) as determined by the LSD Test.

Figure 1 - A - Effect of different concentrations of methanolic leaf, stem and fruit extracts of *Senna occidentalis* on biomass of *Macrophomina phaseolina*. B - Percentage decrease/increase in fungal biomass due to different concentrations of methanolic extracts over control, C) Relationship between different concentrations of methanolic extracts of *S. occidentalis* and biomass of *M. phaseolina*.

significantly by 23%. In a similar way, higher concentrations of the *n*-butanol sub-fraction (37.5 to 150 mg mL⁻¹) showed pronounced inhibitory effect on the growth of *M. phaseolina* and reduced its growth by 86-92% as compared to the control. Conversely, its lower concentrations (2.34 to 18.75 mg mL⁻¹) stimulated fungal growth up to 10% (Figure 2C and D). None of the concentration of aqueous sub-fraction showed antifungal activity. In contrast, there was 8-206% increase in the fungal biomass as a result of various concentrations of this sub-fraction (Figure 2E). Fractionation using a series of organic solvents of different polarities is a very useful technique to separate large number of compounds in methanolic extracts into small groups on the basis of their polarity nature. Similarly to that of results of the present study, many previous studies have shown that compounds of low polarity in *n*-hexane and chloroform sub-fractions generally possess greater antifungal properties than sub-fractions with high polarity. Aqueous fractions containing compounds with the highest polarity are generally either less antifungal or stimulatory for fungal growth (Javaid and Iqbal, 2014; Javaid et al., 2015).

The typical total ion chromatogram (TIC) of the chloroform sub-fraction of the methanolic leaf extract of *S. occidentalis* is shown in Figure 3. Six major peaks can be seen in the chromatogram. The retention time of these peaks ranged from 9.456 to 22.904 min. Names, molecular formula, molecular weight and percent peak area of the identified compounds are shown in Table 1. The six identified compounds were 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (53.549%), 9,10-dimethyltricyclo[4.2.1.1 (2,5)]decane-9,10-diol (22.684%), cyclohexanol, 2 (2-hydroxy-2-propyl)-5-methyl- (9.869%), 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester (6.644%), octanal, 7-hydroxy-3, 7-dimethyl- (4.620%), and 5,6,6-trimethyl-5-(3-oxobut-1-enyl)-1-oxaspiro[2.5]octan-4-one (2.634%). The structures of these compounds are shown in Figure 4. Although it is difficult to define the activity of a particular component in a mixture of compounds, antifungal activity of the mixture may be attributed to the involvement of dominant compounds. The most prevalent compound 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester has also been documented in leaf extracts of many medically important plants such as *Iris germanica* (Asgar et al., 2011), *Mallotus tetracoccus* (Ramalakshmi and Muthuchelian, 2011) and *Piper longum* (Das et al., 2012), with antibacterial, antioxidative and radical scavenging activities (Save et al., 2015). Antifungal activity may occur as well. The second most frequently occurring compound 9,10-dimethyltricyclo[4.2.1.1 (2,5)]decane-9,10-diol also occurs in medicinal plants such as *Oldenlandia umbellata* and *Achillea fragrantissima* and it has various bioactivities (Alenad et al., 2013; De et al., 2013).

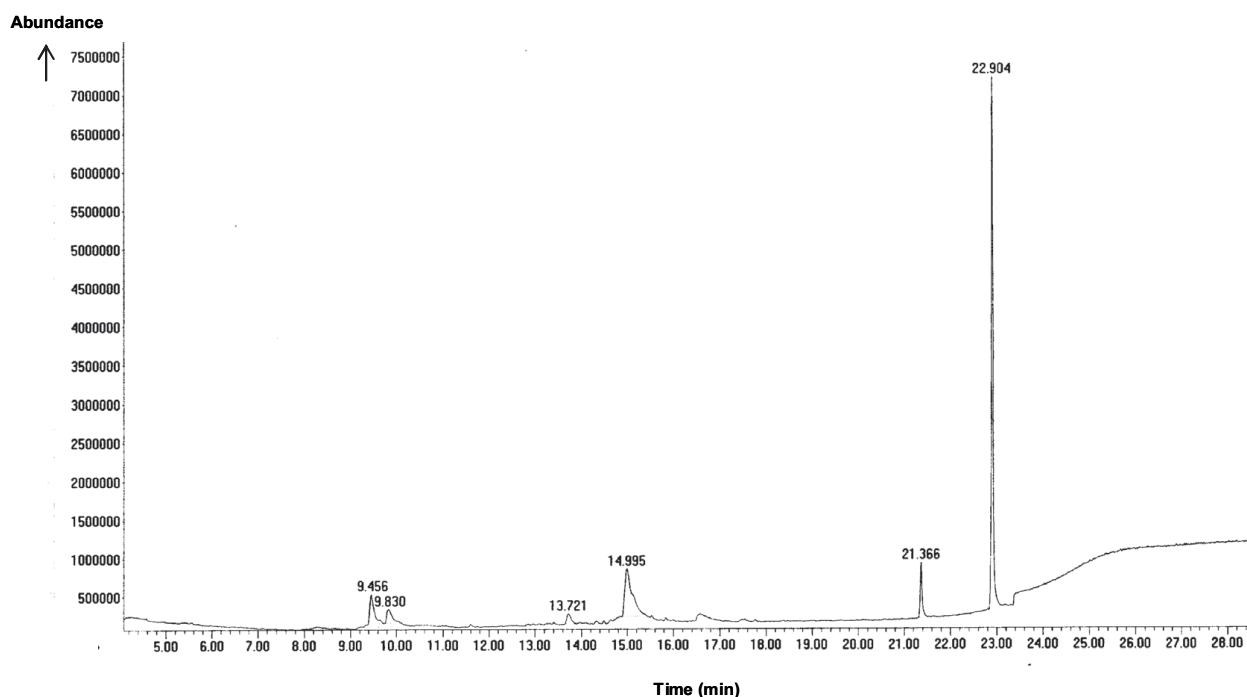
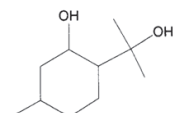


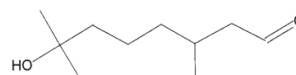
Figure 3 - GC-MS chromatogram of the chloroform sub-fraction of the methanolic leaf extract of *S. occidentalis*.

Table 1 - Compounds identified from chloroform sub-fraction of methanolic leaf extract of *S. occidentalis* through GC-MS analysis

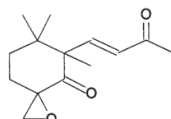
No.	Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
1	Cyclohexanol, 2-(2-hydroxy-2-propyl)-5-methyl-	C ₁₀ H ₂₀ O ₂	172	9.456	9.87
2	Octanal, 7-hydroxy-3, 7-dimethyl-	C ₁₀ H ₂₀ O ₂	172	9.830	4.62
3	5,6,6-Trimethyl-5-(3-oxobut-1-enyl)-1-oxaspiro[2.5]octan-4-one	C ₁₄ H ₂₀ O ₃	236	13.721	2.63
4	9,10-Dimethyltricyclo[4.2.1.1 (2,5)]decane-9,10-diol	C ₁₂ H ₂₀ O ₂	196	14.995	22.68
5	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	C ₁₆ H ₂₂ O ₄	278	21.366	6.64
6	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	C ₂₄ H ₃₈ O ₄	390	22.904	53.55



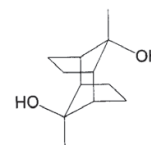
1- Cyclohexanol, 2-(2-hydroxy-2-propyl)-5-methyl-



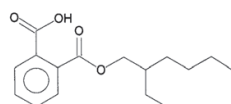
2- Octanal, 7-hydroxy-3, 7-dimethyl-



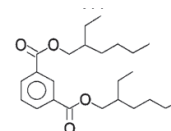
3- 5,6,6-Trimethyl-5-(3-oxobut-1-enyl)-1-oxaspiro[2.5]octan-4-one



4- 9,10-Dimethyltricyclo[4.2.1.1 (2,5)]decane-9,10-diol



5- 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester



6- 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester

Figure 4 - Structures of compounds identified from the chloroform sub-fraction of the methanolic leaf extract of *S. occidentalis* through GC-MS analysis.

The present study concludes that the methanolic leaf extract of *S. occidentalis* contains potent antifungal constituents in its chloroform sub-fraction. The most abundant compounds in this sub-fraction, namely 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester; 9,10-dimethyltricyclo[4.2.1.1 (2,5)]decane-9,10-diol and cyclohexanol, 2-(2-hydroxy-2-propyl)-5-methyl-, may be responsible for the antifungal activity of the leaf extract against *M. phaseolina*.

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