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Full Length Research Paper

In vitro antifungal activity of *Argemone ochroleuca* Sweet latex against some pathogenic fungi

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The *in vitro* antifungal activities of crude latex of *Argemone ochroleuca* Sweet against four clinical isolates of *Candida* (*Candida albicans, Candida glabrata, Candida krusei* and *Candida tropicalis)* and six isolates of plant pathogenic fungi *(Alternaria alternate*, *Drechslera halodes*, *Fusarium oxysporum*, *Macrophomina phaseolina, Pythium ultimum* and *Rhizoctoina solani)* were assessed using well diffusion method. The chemical compounds of the hexan extract of *A. ochroleuca* **latex were investigated using Perkin-Elmer Gas chromatography-Mass spectrometry. The latex of *A. ochroleuca* showed antifungal activity against *D. halodes* (10.60 mm) and** *Candida* spp. **(15.06 to 20.16 mm). GC-MS analysis of the hexan extract of *A. ochroleuca* latex revealed that the latex contains diethyl phthalate (81.57%), 6-Nitro-imidazo(1,2-a)pyridine compound (8.833%), cyclohexasiloxane, dodecamethyl- (5.607%), 4-(2,2-Dimethyl-6-methylenecyclohexylidene)-3-methylbutan-2-one compound (2.410%) and cycloheptasiloxane, tetradecamethyl- (1.574%). These findings indicate that the latex of *A. ochroleuca* could be a good source of antifungal agent against *D. halodes* and** *Candida* spp. **and the phytocomponents present in the latex could be used against fungal pathogens.**

Key words: *Argemone ochroleuca*, latex, pathogenic fungi, GC-MS analysis.

INTRODUCTION

Natural products from plants have played crucial roles in the discovery of new chemical entities for drugs. Approximately 28% of new chemical entities between 1981 and 2002 were natural products or natural product-derived (Newman et al., 2003). Many chemical compounds have been evolved from plants to prevent pathogenic attack including the production of antimicrobial and anti-infective compounds, leading to their use as drugs (Lewis and Ausubel, 2006). Invasive fungal infections are increasingly distinguished as one of the main causes of mortality and morbidity following hematopoietic stem cell transplantation (HSCT), with a case-fatality rate of up to 87% (Jantunen et al., 2004; Marr et al., 2002; Martino et al., 2002; Grow et al., 2002; Martino and Subira, 2002; Safdar et al., 2001; Lin et al., 2001). This indicates the need for intensified research into and the development of novel, more effective and sustainable antifungal medicines from natural products. Latex from several plant species has been shown to be effectively involved in wound healing, pain killing and antimicrobial activity (Thankamma, 2003; Narendra et al., 2009; Sequeira et al., 2009). For example, it has been reported that ethyl acetate and chlorophormic fractions of *Ficus carica* latex contain chemical compounds that exhibited antifungal, antibacterial and anticandidal effects (Aref et al., 2010). Other studies have demonstrated that ethyl acetate fraction from *Euphorbia royleana* latex has immunosuppressive properties (Sarang et al., 2005). Latex from *Calotrpois procera* and *Hevea brasiliensis* showed antimicrobial activity against a range of pathogenic microorganisms (Kareem et al., 2008; Kanokwiroon et al., 2008). The utilization of various plant latex in combination with antifungal drug such as amphotericin B or ketoconazole resulted in a reduction of the doses of such drugs as in ketoconazole and latex of *Euphorbia characias* (Giordani et al., 2001). We have previously shown that the latex of *Argemone ochroleuca* has antibacterial

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activities against some human pathogenic bacteria (Alamri and Moustafa, 2010). This prompted us to investigate its antifungal potential against a range of plant and human pathogenic fungi. In addition, since there is a direct relation between plant phytocomponents and antimicrobial activity (Pereira et al., 2006, 2007; Proestos et al., 2005; Puupponen-Pimiä et al., 2001; Shan et al., 2007; Sousa et al., 2006; Zhu et al., 2004), this also led us to investigate some of these compounds in *A. ochroleuca* latex, which may act as a good alternative to antibiotics. The objectives of this study were to demonstrate the antifungal activity of latex of *A. ochroleuca* against some pathogenic fungi and to determine some of its chemical compounds using GC-MS analysis.

MATERIALS AND METHODS

Latex samples were collected from numerous shoot system of *A. ochroleuca* Sweet that grows as a weed in Abha city, Aseer Region, Saudi Arabia. Fresh latex was collected from healthy plants by making small incisions near the youngest leaves, allowing the latex to flow off into a sterile tube which was then stored at 4°C until used.

Microbial strains

Crude of *A. ochroleuca* was tested for antifungal activity against a taxonomically diverse group of pathogenic fungi including, four strains of clinical isolates of *Candida* (*Candida albicans, Candida glabrata, Candida krusei* and *Candida tropicalis)* and six strains of plant isolates *(Alternaria alternate*, *Drechslera halodes*, *Fusarium oxysporum*, *Macrophomina phaseolina, Pythium ultimum* and *Rhizoctoina solani).*

Determination of antifungal activity

The antifungal activities of the crude latex of *A. ochroleuca* were determined using the well diffused method as previously described (Rubio et al., 2003; Ghalem and Mohamed, 2009). Briefly, approximately 10 ml of Sabouraud dextrose agar was poured into sterilized Petri dishes and the plates were left overnight at room temperature to check for sterility. Each fungal spore suspension was prepared in 2 ml of sterilized distilled water and 100 µL of spore suspension was poured and uniformly spread on the sterile agar. An agar well of 5 mm diameter in the centre of each plate was prepared with the help of a sterilized stainless steel cork borer and then each well was loaded with 100 µL of crude latex of *A. ochroleuca.* Negative controls were inoculated with dimethyl sulfoxide (DMSO), and Fluconazole (30 µg/disc) was used as a positive control. The plates were incubated at 35°C for 48 h for the *Candida* spp. and at 30°C for 4 days for the other tested fungi. The antifungal activity was assessed on the basis of the diameter of the zone of inhibition, which was measured at the cross-angles of each well. The experiments were repeated three times.

Statistical analysis

Data were analyzed using SPSS version 10, for windows software. Statistical analysis was undertaken by one-way analysis of variance (ANOVA) tests by using post hoc test LSD (Least Significant Difference test) to calculate individual mean significant difference, standard deviation and 95% confidence interval for mean to the obtained data. *P*-value <0.05 were considered statistically significant

GC-MS analysis

The GC – MS analysis was carried out using a Clarus 500 Perkin – elmer (Auto system XL) Gas Chromatograph equipped and coupled to a mass detector Turbo mass gold – Perkin Elmer Turbomass 5.1 spectrometer with an Elite – 1 (100% Dimethyl poly siloxane) and TR-V1 column (30 m x 0.32 mm x 1.8 um) was used. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 6 mL/min with an injection volume of 2 µL (a split ratio of 5:1) and injector temperature 200°C. The oven temperature was programmed to temperature of 35°C -4 min hold then 30°C/min to 90°C then 30°C/min to 110°C -No hold, then 45°C/min to 170°C hold for 1 min. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da (Ezhilan and Neelamegam, 2012). Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

RESULTS

Antifungal activity of the crude latex

The antifungal activities of the crude latex of *A. ochroleuca* against the ten pathogenic fungi are presented in Table 1 and Figure 1. It was found that the crude latex of *A. ochroleuca* exhibited a significant activity against all *Candida* spp., less activity against *D.* halodes, but there was no antifungal activity against *A. alternate*, *F. oxysporum*, *M. phaseolina, P. ultimum* and *R. solani.* The zones of inhibition exhibited by the crude latex of *A. ochroleuca* against *Candida* spp. ranged between 15.06 to 20.16 mm, while *D. halodes* had a zone of inhibition of 10.60 mm as shown in Table 1 and Figure 1. The negative control did not show any effect on fungal growth, while the positive control showed halo indicative of lack of fungal growth in all plates (Table 1).

Phytochemical constituents present in the crude latex of *A. ochroleuca* Sweet

GC-MS chromatogram analysis of the hexan extract of *A. ochroleuca* latex (Figure 2) showed five peaks indicating the presence of five phytochemical constituents. Characterization and identification of the five phytochemical constituents by comparison the mass spectra of the constituents with the NIST library (Table 2 and Figure 2). Among the five compounds identified, diethyl phthalate was quantitatively the most dominant compound found (81.57%) followed by 6-Nitro-imidazo(1,2-a) pyridine compound (8.833%) and cyclohexasiloxane, dodecamethyl- (5.607%), while a small amount of 4-(2,2-Dimethyl-6-methylenecyclohexylidene)-3-methylbutan-2-one compound (2.410%) and cycloheptasiloxane, tetradecamethyl- (1.574%) could be detected in the hexan extract *A. ochroleuca* latex.

DISCUSSION

In many developing countries, medicinal plants play im-

Table 1. *In vitro* antifungal activity of the *Argemone ochroleuca* latex.

Pathogenic fungi	Mean diameter of zone of inhibition (mm) (95% Confidence Interval for Mean; Lower Bound- Upper Bound).		
	Crude Latex	DMSO	Fluconazole (30 µg/ disc)
Candida albicans	15.36±0.472**(14.19 - 16.54)	NI	32.43±0.81(30.41- 34.45)
Candida glabrata	15.06±0.808**(13.05 - 17.07)	NI	32.70±1.20(29.71 - 35.68)
Candida krusei	17.30±0.700**(15.56 - 19.03)	NI	33.73±0.90(31.47- 35.98)
Candida tropicalis	20.166±1.66**(16.03 - 24.29)	NI	35.90±1.41(32.39 - 39.40)
Alternaria alternate	NI	NI	33.36±0.75(31.50 - 35.23)
Drechslera halodes	10.60±5.80**(-03.81 - 25.01)	NI	30.83±0.85(28.72- 32.94)
Fusarium oxysporum	NI	NI	32.10±2.30(26.37- 37.82)
Macrophomina phaseolina	NI	NI	31.53±0.85(29.42 - 33.64)
Pythium ultimum	NI	NI	32.86±0.72(31.06- 34.66)
Rhizoctoina solani	NI	NI	34.60±1.60(30.60- 38.59)

NI = Not determined; DMSO, dimethyl sulfoxide = (negative control); Fluconazole = (positive control). Values are expressed as mean ± standard deviation (SD) from three experiments; **Significant at LSD *P*-value <0.01.

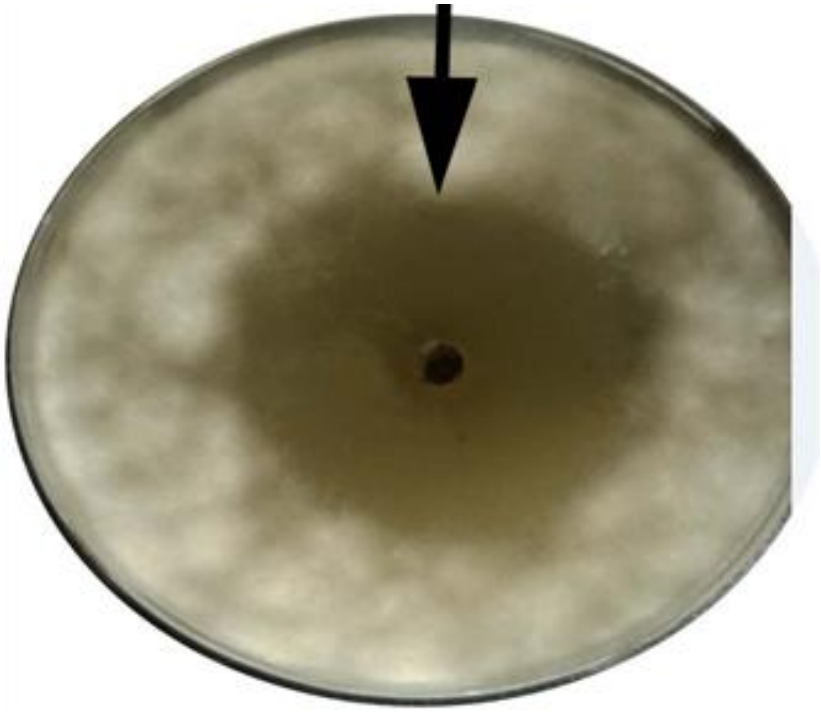

Figure 1. Inhibition zone of *Drechslera halodes* caused by crude latex of *Argemone ochroleuca*.

Table 2. GC-MS analysis of hexan extract of the *Argemone ochroleuca* latex.

Compound	Rt Min.	% Area	M.W.	Chemical Formula
Cyclohexasiloxane, dodecamethyl-	12.72	5.607	444.92	$C_{12}H_{36}O_6Si_6$
6-Nitro-imidazo(1,2-a)pyridine	14.91	8.833	163.04	$C_7H_5N_3O_2$
Cycloheptasiloxane, tetradecamethyl-	15.07	1.574	519.07	$C_{14}H_{42}O_7Si_7$
4-(2,2-Dimethyl-6-methylenecyclohexylidene)-3-methylbutan-2-one	15.55	2.410	206.32	$C_{14}H_{22}O$
Diethyl phthalate	16.66	81.57	222.23	$C_{12}H_{14}O_4$

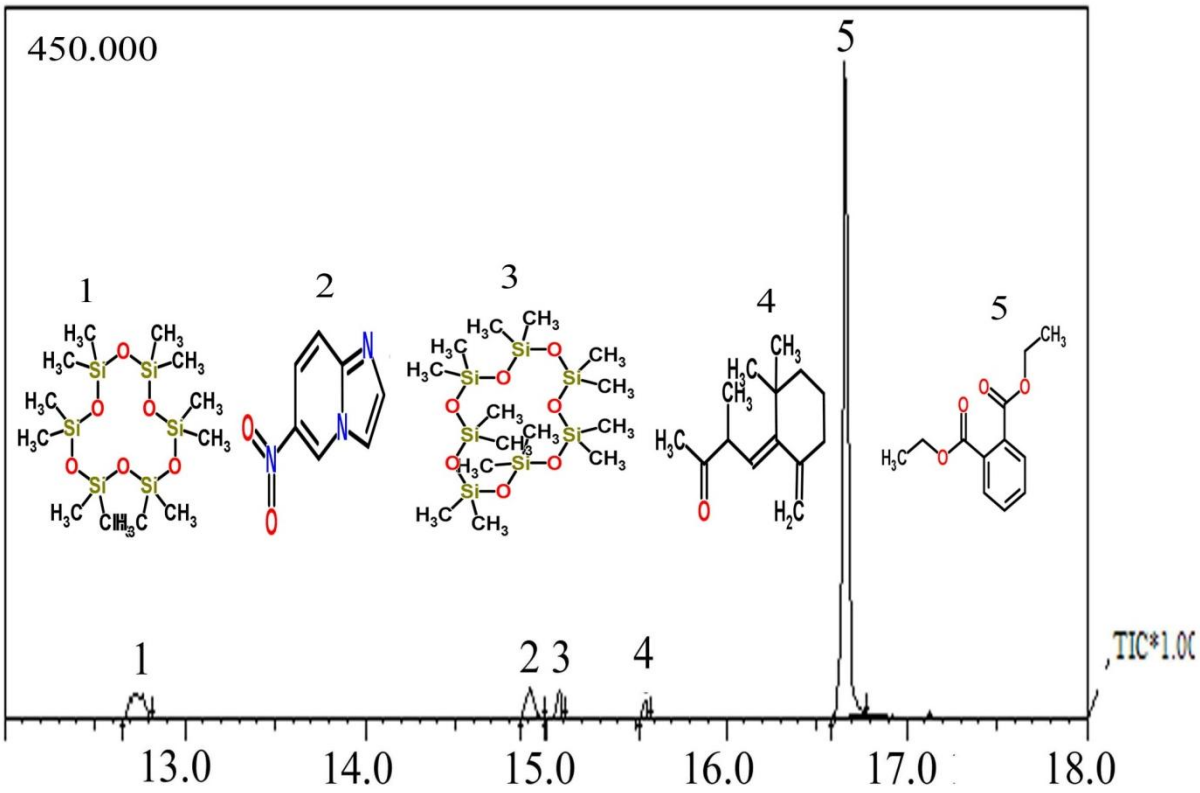

Figure 2. Mass spectrum and structure of phytocomponents identified by GC-MS in the hexan extracts of *Argemone ochroleuca* latex. 1, cyclohexasiloxane, dodecamethyl-; 2, 6-Nitro-imidazo(1,2-a)pyridine;3, cycloheptasiloxane, tetradecamethyl-; 4, 4-(2,2-Dimethyl-6-methylenecyclohexylidene)-3-methylbutan-2-one; 5, Diethyl phthalate.

portant roles in basic health needs, and may offer a novel source of antibacterial, antifungal and antiviral agents (Muñoz-Mingarro et al., 2003). In the present investigation, well diffusion assay revealed that the crude latex of *A. ochroleuca* has an antifungal activity against *Candida* spp. and *D. halodes*. The results indicate that the latex has antifungal properties, which explain the use of many plant latexes in traditional medicine for the treatment of various diseases whose symptoms might involve microbial infections. Many researchers have demonstrated the importance of the ethnobotanical approach for the selection of plants in the discovery of new drugs (Lin et al., 2001; Thankamma, 2003; Narendra et al., 2009). In this regard, the latex of *A. ochroleuca* can be used to facilitate the development of novel antibiotics, which are urgently needed in the wake of multidrug resistance in *Candida* spp. and *D. halodes* infections. As reported by De hoog et al. (2000), *Drechslera* is considered to be a potentially pathogenic species, causing many kinds of disease such as eye infections (Feghhi et al., 2010), allergic fungal sinusitis (AFS) (Rupa et al., 2002), and liver disease (Aslani et al., 2006). *Candida* spp. have been reported as potential human pathogens (Rainer et al., 2001), and being as the main agent for the incidence rate (63.9%) among the yeast isolates studied (Richter et al., 2005; Tatfeng et al., 2003). Furthermore, *Candida* was listed by the Center for Disease Control (CDC) as a main microorganism that are causing sexually transmitted infection (Prescott et al., 2008), such as vaginal candidiasis (Okungbowa et al., 2003), infection of the male glans penis (Prescott et al., 2008), and urinary tract infections (Tatfeng et al., 2003).

The evaluation of bioactive compounds in hexan extract of *A. ochroleuca* latex revealed the existence of the diethyl phthalate, 6-Nitro-imidazo(1,2-a)pyridine, cyclohexasiloxane, dodecamethyl-,4-(2,2-Dimethyl-6-methylenecyclohexylidene)-3-methylbutan-2-one and cycloheptasiloxane, tetradecamethyl compounds, suggesting that these chemicals compounds may have a potent antifungal properties. A recent research has indicated that many chemical compound, which present in rich amounts in several plants exhibited antioxidant, antifungal, antibacterial and anti-inflammatory properties (Shalini and Srivastava, 2009). All compounds identified in the latex of *A. ochroleuca* were reported to have antimicrobial activities. It was reported that imidazo[1,2-a]pyridine derivatives has a wide range of pharmaceutical, biological, and medicinal applications (Wisniewska et al., 2012; Hayakawa et al., 2007; Singhaus et al., 2010). Its derivatives have been found to possess antimicrobial (Al-Tel et al., 2011; Gueiffier et al., 1996; Gueiffier et al., 1998), antiulcer (Katsura et al., 1992), antitubercular (Moraski et

al., 2012), anti-inflammatory (Flores et al., 2012), anti-cancer (Ducray et al., 2011), antiparasitic (Martínez et al., 2010) and antiprotozoa (Ismail et al., 2004) activities. Complexes derived from diethyl phthalate have antifungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Trichoderma harizanum*, *Trichoderma viridae* and *Rhizoctonia solani* (Raman and Parameswari, 2007). The oil derived from *Pterocephalus canus* showed significant antimicrobial activity against *Staphylococcus saprophyticus* and *Escherichia coli* that have Dodecamethyl cyclohexasiloxane (Vahedi et al., 2011).

GC-MS analysis of volatile oils from *Bupleurum chinense* showed it contained 4-(2,2-Dimethyl-6-methylenecyclohexylidene)-3-methylbutan-2-one and the plant has been used in Traditional Chinese Medicine for thousands of years (Bensky et al., 1993; Zekun and Haixia, 2012). Aqueous extracts from the leaves of *Casimiroa edulis* effectively control *in vitro* development of the post-harvest fungi *Alternaria* spp., *Fusarium* spp., *Pestalotiopsis* spp. and *Rhizopus* spp (Bautista-Baños et al., 2000), and GC-MS analysis showed it contains cycloheptasiloxane tetradecamethyl (Barakat, 2011). Initial examination by light microscopic of the morphology of aerial hyphae and conidia of *D. halodes* growing at the edge of the zone of inhibition revealed that *A. ochroleuca* latex inhibited their growth (data not shown). These findings agree with previous studies that some phenolic compounds affect the growth, morphology and ultrastructure of the *Phytophthora megasperma* and *Cylindrocarpon destructans* (Baidez et al. 2006).

In conclusions, the results demonstrated that the *A. ochroleuca* latex possesses antifungal activities that could be a promising antibiotic source. Future research should focus on the more elucidation of the chemical constituents and their mechanism of action to facilitate efficient uses of important plant resources as antimicrobial drugs.

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