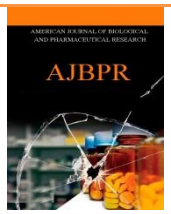
e - ISSN - 2348 - 2184
Print ISSN - 2348 - 2176

AMERICAN JOURNAL OF BIOLOGICAL AND PHARMACEUTICAL RESEARCH

Journal homepage: www.mcmed.us/journal/ajbpr



ANTI-CANDIDAL ACTIVITIES OF *Sepia aculeate* INK EXTRACT AGAINST MULTIPLE RESISTANT *Candida albicans* CAUSING ORAL CANDIDIASIS

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Article Info

Received 25/03/2014
Revised 15/04/2014
Accepted 18/05/2014

Keywords:*Sepia aculeate* ink, Ethanol fraction, Antimicrobial and Zone of inhibition

ABSTRACT

This study was carried out to evaluate the anti microbial activity of water, ethanol, methanol, acetone, hexane and butanol crude extract and fractions of Sepia aculeate ink. The antimicrobial activity was determined by the disc diffusion method and significant inhibitory concentrationagainst fungal strain. The combining ethanol with methanol at 2:18 concentration moderately inhibits (6 mm inhibition zone) Candida albicans than all individual extract and fractions. In the GC-MS analysis 65 bioactive phytochemical compounds were identified in the ethanolic fraction of Sepia aculeate ink.. The results indicated that fractions of Sepia aculeate ink were highly potent as anticandidal agent.

INTRODUCTION

Oral candidosis is the most common fungal infection encountered in general dental practice1 caused by *Candida albicans.* It is an opportunistic pathogen present in about 50-60% of the healthy human population, and becomes pathogenic when the host immune defense is undermined such as in HIV infection [1]. Liu et al reported 90% of AIDS patients affect oral and/or or opharyngeal candidiasis in various stages. However, synthetic drug treatments against *Candida albicans* can cause various side effects in chronic disease affected patients (i.e. HIV/AIDS, Cancer, Diabetes etc). Hence, the search for more effective agents with low side effect from marine source.

In nature, animals are provided with their own protective response against their predators, likewise marine mollusks are protected by their shells, but many of them are not fully protected by shells. Chemical defenses are used extensively by both shelled and shell-less mollusks. Among the chemical defenses, a class of defense including, squid, octopus and cuttlefish, have a striking defensive behavior-releasing ink when attacked. The ink of cephalopods functions as anti predatory visual stimuli either as like distracting “smoke screens” or as decoys [2] Most of the studies concerning antimicrobial activity includes specific compartments like egg masses, hemolymph or whole body extracts of mollusk [3]. Mollusks not only exhibit the anti-microbial activity, it constitutes many classes of bioactive compounds which includes antitumor, antileukemic and antiviral activities have been reported world-wide [4-6].

The bioactive compounds involved in sensory disruption and phagomimicry include free amino acids

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(FAA) and ammonium, which are extraordinarily concentrated in ink and opaline of the mollusc especially *Sepia sp* [7] and various simple amines and paralyzing proteins were found in the cephalopods [8]. In the past few decades, mining of bioactive compounds from marine sources are considered promising because of its rich species diversity. So the present study focuses the anti-candidal activities of *sepia aculeate* ink extract against multiple resistant *candida albicans*.

MATERIALS AND METHODS

Collection of Ink Samples

Marine mollusk namely cuttlefish (*Sepia aculeate*) was collected from the region of Gulf of Mannar (Thondi)., ink glands were removed and extracted with water. The ink fluid was obtained by disturbing the animals and extracted with water. All aqueous ink samples were centrifuged at 15,000 g for 15 min and the supernatant was taken and stored in -20°C for further use.

Extraction OF Marine Sample [9]

The ink fluid (1.0 kg) were extracted with different organic solvents viz., acetone, hexane, petroleum ether, chloroform and ethanol in a soxhlet apparatus for 8 h and the extract was concentrated in a rotary vacuum evaporator to yield crude extract.

Fractionation

Column was packed with ethanol with silica gel, sample was loaded as slurry of silica gel and the column was eluted with increasing concentration of water, ethanol, methanol, acetone, hexane and butanol solvent to increase polarity. After, active fraction was stored in a refrigerator until used for further usage.

Micro organisms

Clinical pathogenic fungal organisms *Candida albicans* was used for this study. These organisms were clinical isolates of patients isolated from clinical patients at dental clinics in and around Thanjavur and Chennai, Tamil Nadu, India.

Disc-Diffusion Method [10].

Various solvent fractions of *Sepia aculeate* were checked for anti-*Candida*l activity using disc-diffusion method. Multiple resistant *C. albicans* (C2 isolate) was swabbed on the surface of the sabouraud agar plates. The disc (Whatman No.1 filter paper with 9 mm diameter) was impregnated with the 50 µl of each test plant sample and it was placed on the surface of sabouraud agar plates. To compare the antifungal activities, nystatin (20µg/disc) used as standard antibiotic and blank disc impregnated with water act as negative control. The plates (triplicates) were incubated 28°C for 72 h. The antimicrobial potency of the test samples was measured by determining the diameter of the zones of inhibition in millimeter.

Determination of minimum fungicidal concentration (MFC)

The minimum inhibitory concentrations of *Sepia aculeate* against Multiple resistant *C. albicans* (C2 isolate) which was determined by serial dilution technique in the presence of standard nystatin.

Gas Chromatography – Mass Spectroscopy (GC-MS) analysis [11].

The ink sample was dissolved in 75 ml of methanol for 24 h. Then the filtrates were collected by evaporated under liquid nitrogen. 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 – Perking Elmer Turbomas 5.2 spectrometer with an Elite-1 (100% Dimethyl ply siloxane), 300 m x 0.25 mm x 1 x m df capillary column. The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period, the oven temperature was raised up to 280°C, at the rate of an increase of 5°C/min, and maintained for 9 min.

Injection port temperature was ensured as 250°C and Helium flow rate as 1 ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass Spectral scan range was set at 45-450 (mhz). The chemical constituents were identified by GC-MS. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using National Institute of Standards and Technology Mass Spectral database (NIST-MS). The percentage of each component was calculated from relative peak area of each component in the chromatogram.

RESULTS

ANTI-CANDIDAL ACTIVITY OF *Sepia aculeate*

Disc diffusion method

In the present study from the *S.aculeate* ink showed antifungal activity against *Candida albicans*. The individual fractions (water, ethanol, methanol, acetone, hexane and butanol) of *Sepia aculeate* ink showed moderate antifungal activity. The average zone of inhibition of 3, 8, 4, 2, 1 and 1 mm were observed. Among all the fractions, only ethanol and methanol showed maximum activity against *C. albicans.* Hence these two fractions were once again treated with multiple resistant *C. albicans* strain at various combinations, which showed effective results (Table 1 and 2). The significant anti-fungal activity of 9 mm zone of inhibition was observed with ethanol and methanol fraction of *S. aculeate* ink (2:18) against *C. albicans* strain (Fig 1).

Determination of minimum fungicidal concentration

The antifungal activities of *S.aculeate* ink against *C. albicans* were evaluated by minimal fungicidal concentration (MFC). The MFC of S.aculeate ink effectively inhibited the growth of *C. albicans* at concentration of 9.65 mg/ml (Table 3), which was higher than others.

Gas Chromatography – Mass Spectroscopy (GC-MS) analysis

The phytochemical compounds present in *S.aculeate* ink was identified by GC-MS analysis.GC-MS analysis of sepia ink extract displayed Thirteen peaks with the retention times ranging from 11.341 to 27.127 min (Figure 2). The positive electron impact mass spectrum of Thirteen compounds were obtained and characterized as 1-tetradecene ($C_{14}H_{28}$), phenol, 3,5–bis (1,1–dimethylethyl) ($C_{14}H_{22}O$), 2-tetradecene ($C_{14}H_{28}$), benzene, 1,1'–(1,2-cyclobutanediyl) bis-trans ($C_{16}H_{16}$), 1–octadecene ($C_{18}H_{36}$), 1H–indole–3–carboxylic acid ($C_{10}H_9NO_2$), nonadecanol–1 ($C_{19}H_{40}O$), methyl stearate ($C_{19}H_{38}O_2$), 1–docosene ($C_{22}H_{44}$), 1-heptacosanol ($C_{27}H_{56}O$), 1 – benzylindole ($C_{15}H_{13}N$), 1H–indole-2–methyl–3– phenyl ($C_{15}H_{13}N$), 5–methyl–2–phenylindolizine ($C_{15}H13N$). (Fig 2 and Table 4).

Table 1. Antifungal activity of *S.aculeate* ink tested against *C. albicans* by disk diffusion method.

Marine sample /Solvent	Zone of inhibition (mm)					
	Water	Ethanol	Methanol	Acetone	Hexane	Butanol
***S.aculeate* ink**	3	8	4	2	1	1

Table 2. Antifungal activity of Ethanol /Methanol fraction of *S.aculeate* ink tested against *C. albicans* by disk diffusion method.

Marine sample /Solvent	Zone of inhibition (mm)								
	18:2 (E/M)	16:4 (E/M)	14:6 (E/M)	12:8 (E/M)	10:10 (E/M)	8:12 (E/M)	6:14 (E/M)	4:16 (E/M)	2:18 (E/M)
***S.aculeate* ink**	1	2	2	1	8	2	0.5	-	9

Table 3. Determination of minimum fungal concentration of *S.aculeate*against*C. albicans*.

S.No	Marine sample	Diameter of zone of inhibition(in mm)
		Candida albicans
1	***S.aculeate* ink (mg/ml)**	9.65
4	**Nystatin(mg/ml)**	14

Table 4. Gas Chromatography –Mass Spectrometry analysis of *S.aculeate* ink

S. No	RT	Compound	MolecularFormula	Relative content (%)
1	11.341	1-Tetradecene	$C_{14}H_{28}$	2.79%
2	12.912	Phenol, 3,5–bis (1,1–dimethylethyl)	$C_{14}H_{22}O$	0.09%
3	13.845	2-tetradecene	$C_{14}H_{28}$	0.31%
4	15.766	Benzene, 1,1'–(1,2-cyclobutanediyl) bis-trans	$C_{16}H_{16}$	0.33%
5	16.074	1–Octadecene	$C_{18}H_{36}$	0.57%
6	16.938	1H–indole–3–carboxylicacid	$C_{10}H_9NO_2$	5.15%
7	17.440	Nonadecanol–1	$C_{19}H_{40}O$	1.27%
8	17.790	Methyl stearate	$C_{19}H_{38}O_2$	0.39%
9	18.087	1–Docosene	$C_{22}H_{44}$	93.44%
10	19.343	1-Heptacosanol	$C_{27}H_{56}O$	0.19%
11	21.620	1 – Benzylindole	$C_{15}H_{13}N$	0.10%
12	23.062	1H–Indole-2–methyl–3– phenyl	$C_{15}H_{13}N$	0.38%
13	27.122	5–methyl–2–phenylindolizine	$C_{15}H_{13}N$	2.55%

Fig 1. Disc Diffusion Method For *S.aculeate* ink Extract

Ethanolic extract of *S.aculeate* ink | **Methanolic extract of *S.aculeate* ink** | **E/M 2:18 combined fraction of *S.aculeate* ink**

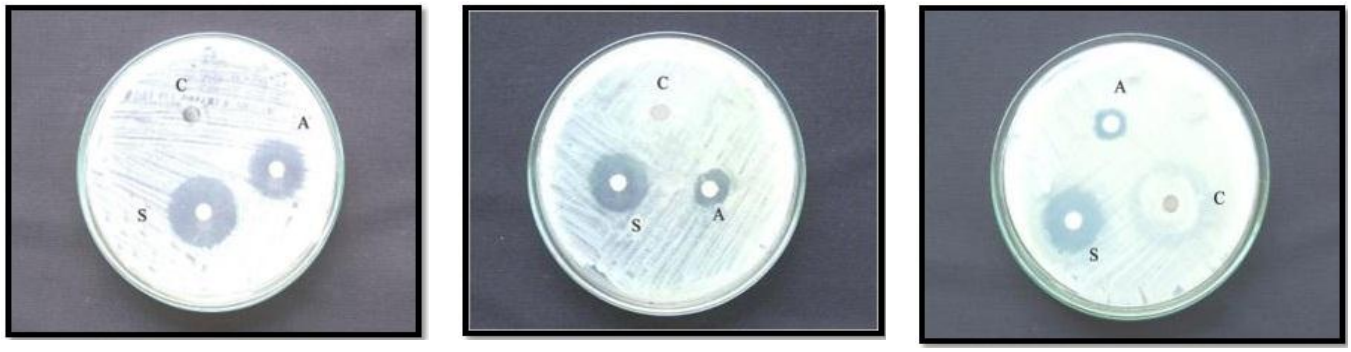
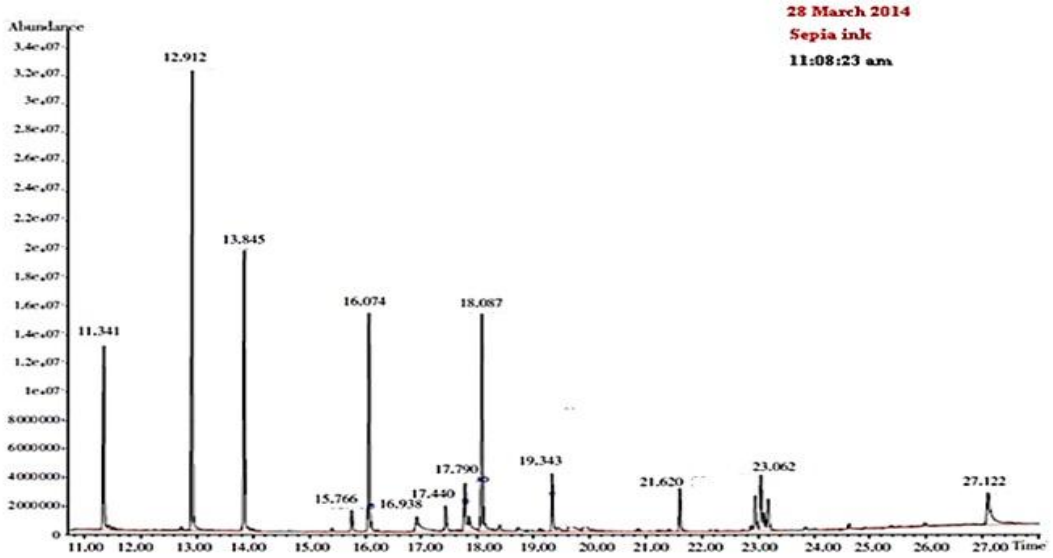


Fig 2. Gas Chromatography –Mass Spectrometry analysis of *S.aculeate* ink



DISCUSSION

C. albicans is capable of infecting all the parts of human body but only a limited number of anti *candida* drugs are available. In the meantime, multidrug resistance genes in *C. albicans* increased enormously and the treatment for this strains are very difficult especially in HIV patients, transplant recipients and patients with neoplasm [12,13].

Antifungal therapy is the recommended first line treatment for uncomplicated oral candidiasis. Treatment in the early part of the 20th century was with the gentian violet but because of developing resistance and side effects, such as staining of the oral mucosa, it was replaced by a polyene antibiotic, nystatin, discovered in 1951 and amphotericin B, discovered in 1956. They act by binding to sterols in the cell membrane of fungi and altering cell membrane permeability [14,15]. Miconazole has been used as a local applicant in the mouth but its use in this way is limited because of the potential side effects such as vomiting and diarrhoea. Other drugs belonging to this class are clotrimazole and ketoconazole. Nystatin is the most widely used antifungal agent for the treatment of oral candidiasis. Both, nystatin oral rinses and clotrimazole troches have high sucrose content resulted in tooth decay. Oral candidiasis is also complicated by diabetes, steroid use or an immunocompromised state, for which triazoles, which include fluconazole or itraconazole once per day has been found to be effective in these cases [16,17].

Ketoconazole is also as effective as fluconazole and itraconazole but its use in elderly patients is not recommended due to drug interactions and side effects, which include hepatotoxicity. Fluconazole is a potent and selective inhibitor of fungal enzymes involved in the synthesis of ergosterol, an important constituent of the plasma cell membrane. Therefore it disrupts cell wall formation leading to leakage of cellular contents and cell

death. Itraconazole has a wider spectrum of activity than fluconazole and is therefore valuable in salvage treatment of the immunocompromised patients with fluconazole resistant candidiasis. Increasing resistance to antifungals has become increasingly common since the introduction of fluconazole especially in patients with advanced HIV disease [18]. Thus *C. albicans* emerging as a fungus is characterized by resistance or lower susceptibility to standard antifungal agents. For these reasons, there is a need for new molecules which is less side effect and can serve as lead compounds for further development in antifungal therapy. Therefore there is a constant and urgent need to develop new anticandidal drugs for the treatment of infectious disease from medicinal plants. The best solution to such a problem is to use traditional method of treatment against pathogens. The medicines from marine organism have been used for the treatment of uncountable diseases. Marine based drug constituents can be used for many untreatable diseases [19]. More than 2000 marine organism known to produce chemical substances and valuable metabolites against microbial diseases.

In the present study from the *S.aculeate* ink showed antifungal activity against *Candida albicans*. The individual fractions (water, ethanol, methanol, acetone, hexane and butanol) of *Sepia aculeate* ink showed moderate antifungal activity. The average zone of inhibition of 3, 8, 4, 2, 1 and 1 mm were observed. Among all the fractions, only ethanol and methanol showed maximum activity against *C. albicans.* Hence these two fractions were once again treated with multiple resistant *C. albicans* strain at various combinations, which showed effective results . The significant anti-fungal activity of 9 mm zone of inhibition was observed with ethanol and methanol fraction of *S. aculeate* ink (2:18) against *C. albicans* strain.

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