Evaluating the *In Vitro* Antagonism of Secondary Metabolites Fractionated from the Brown Algae, *Sargassum swartzii* against Human *Candida* spp.

Aseer Manilal1*, Gemechu Ameya1, Tigist Gezmu1, Behailu Merdekios1, Sabarathnam Balu2, Akbar Idhayadhulla3 and R. Surendra Kumar2

1College of Medicine and Health Sciences, Arba Minch University, Arba Minch, Ethiopia
2Department of Microbiology, Bharathidasan University, Trichy, Tamil Nadu, India
3PG and Research Department of Chemistry, Nehru Memorial College, Puthanampatti -621007, Tamil nadu, South India

Corresponding author: Aseer Manilal, Department of Medical Laboratory Sciences, College of Medicine and Health Sciences, Arba Minch University, Arba Minch, Ethiopia, Tel: 251-919904201; E-mail: aseermanilal@gmail.com

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**Abstract**

**Objective:** To inspect the antifungal potency of brown algae *S. swartzii* and GC-MS analysis to delineate its bioactive principles.

**Methods:** The marine brown algae *S. swartzii*, was extracted and fractionated in organic solvents and quantitatively analyzed for its *in vitro* antifungal activity against a battery of five clinically relevant species of *Candida*.

**Results:** The fractionation of the crude algal extract yielded a bioactive algal fraction that exhibited broadest spectra of activity. It impeded the growth of all the evaluated Yeast pathogens in variable degrees. The maximal activity was recorded against the *Candida albicans*. The GC-MS studies of active algal fraction evinced the presence of three chemical constituents. Thence, the potent broad spectra of activity against the human *Candida* could be due the presence of major principle 1,2-Benzenedicarboxylic acid, disoocystyl ester, or could be pertained to the synergistic activity all the components.

**Conclusion:** The overall results of this study implicates that the bioactive principles found in this algal fraction could be utilized as a lead molecule to develop natural antifungal drug to combat pathogenic *Candida* species.

**Keywords:** Algal fraction; Anticandidal activity; Secondary metabolites; Antimicrobials

**Introduction**

Fungi are the diverse group of ubiquitous eukaryotes which intercedes vital ecological processes. Nevertheless, many of them are primary or opportunistic pathogens capable of inflicting wide spectra of ailments in humans [1]. Amid the 1.5 million estimated species of fungi, around 317 species are known to smite diseases in human [2,3]. The fungal scourges have broad and variable clinical manifestations ranging from superficial skin and nails infections to disseminated life threatening infections [1]. In the developing countries, opportunistic fungi are oftentimes causing infections in immunocompromised patients which therefore need to control promptly. Of the different infective species of fungi, Yeasts of the *Candida* genus inflict fatal systemic infections which increased substantially over the last decade. For instance, it is noted that the *Candida* spp. are the most common opportunistic pathogen in AIDS patients [4]. In addition, infections due to non-albicans species have also egressed over the past two decades, and a switch from *C. albicans* to species such as *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis* has alarmingly increasing [5].

During the last decades, pharmaceutical industries have produced limited number of new therapeutic drugs for the management of *Candida*. However, the prolonged/inadequate usage of exciting antifungal drugs has precipitated the emergence of drug resistant *Candida* spp. and poses an extra concern [6]. With the increased incidence of *Candidal* infections coupled with the setback associated with the overuse and misuse of antibiotics demonstrates the insistent need of finding safe, novel and effective antifungal agents. Therefore, novel antifungal drugs with increased potency are required in lieu of conventional antibiotics for the management of fungal diseases.

In comparison to the chemicals and drugs used for synthetic treatments, allelo-chemicals from marine origin are less associated with negative effects and have enormous therapeutic potentials to heal many infectious maladies in human [7]. Currently, marine organisms are an enormous reserve of novel drugs and drug leads for the pharmaceutical industry. Marine natural products have been discovered from a wide array of organisms including sponges, algae, bryozoans, mollusks, cnidarians, tunicates, echinoderms, sea worms and microorganisms. It is a confirmed fact that, the marine algae
are prime candidate in producing antimicrobial metabolites to thwart the invaders in their natural habitat. Plurality of algal species have been reported to possess multitude of bioactivities and thence, potential for elaborating antimicrobial agents. For instance, literature addressed that species of genus *Sargassum* presented diverse activities such as anti-Herpetic [8], antiretroviral [9], antifungal [10], antibacterial [11], and anticancer [12].

Retrospective examination evidenced that antifungal actions of marine algae were exposed as earlier as 1915s [13]. Various marine algae from the Southwest littoral of India has been recently corroborated for exerting diverse bioactivities such as antibacterial [14], antifungal [15], antiviral [16], anticoagulant [17] and cytotoxicity [18]. In so far, there is no precedence of research being conducted to inspect the antagonistic potential of brown algae, *Sargassum swartzii* against human *Candida*. In our preliminary experiments, the crude methanolic extract of *S. swartzii* evinced pronounced antibacterial activity against clinical and biofilm forming bacteria. In this regard, the brown algae *S. swartzii* was preferred to explore its antifungal potency and GC-MS analysis to delineate its bioactive principles.

**Materials and Methods**

**Collection of algal specimens**

Live and healthy thalli of marine algae, *S. swartzii* were handpicked at ebb tide from the rugged intertidal zone of Kollam coast, South India (08°54' N and 76°38' E). Garnered specimens were then successively rinsed with seawater to remove dirt and transferred to laboratory in plastic bags containing seawater to prevent evaporation. The rinsed thalli were air dried under a stream of air flow for one week at room temperature to prevent photolysis and thermal degradation of metabolites. Dried fronds of algae were powdered in a grinder, packed in polyethylene bags and stored in moisture free place until extraction.

**Extraction of algae**

Algal bioactives were extracted from dried algal powder according to the parameters previously optimized [14]. Briefly, a definite quantity (200 g) of dried algal powder was submerged in conical flasks (2000 mL) containing 1000 mL of methanol (MeOH) and placed at 35°C on a shaker at 120 rpm for two weeks to permit full extraction of the bioactive components. After two weeks, algal material was filtered using Whatman filter paper No 1. The filter residue was collected in a round-bottom flask and the solvent was concentrated in a rotary vacuum evaporator at 45°C for the elimination of MeOH. The resultant gummy dark coloured extract was collected in airtight plastic vials and stored in the refrigerator for further studies.

**Fractionation of *Sargassum swartzii***

A known morsel of dried extract of the algae (crude solid residue collected after vacuum evaporation) was adsorbed to silica gel and applied in a column developed with petroleum ether and eluted step-wise with petroleum ether and ethyl acetate (9:1 to 1:9 and 100% ethyl acetate) followed by ethyl acetate and methanol (9:1 to 1:9 and 100% methanol) [15]. Column elute was collected in 10 mL screw cap bottles. Preliminary experiments confirmed that the fraction eluted using ethyl acetate: methanol (4:6) retained antibacterial activity (data not shown). Other fractions that displayed meagre activity were not more considered in the present study. Hence, the same fraction (4:6) was used to investigate antifungal activity and GC-MS analysis.

**Gas chromatographic and mass spectroscopic (GC-MS) analysis**

The active fraction was chemically analysed through GC-MS method as described elsewhere [14].

**Antifungal assay**

To determine antifungal activity, the pre-selected algal fraction was evaluated against a battery of clinically relevant five species of *Candida* those previously used and continuously maintained in our laboratory [15]. The antifungal assay was performed as described elsewhere [15]. The Sabouraud dextrose agar (Himedia) was used for bioactivity screening and routine propagation of human fungus respectively. Cell suspensions containing 10^7 CFU/ml cells for yeasts, were prepared and aseptically besmeared onto the surface of the agar plates of Sabouraud dextrose medium using sterile swab sticks. Thereafter, wells with five millimeter of diameter were prepared using a sterile cork borer. The resultant wells were carefully filled with 100 μl (15 mg/ml) of algal fraction. The well with binary solvents used for fractionation was considered as negative control. The assay was performed in triplicates of individual Petri dishes. The clear zones of inhibition formed around wells after 48 h at 30°C were considered to be an indicative of antifungal activity. The inhibitory activity was recorded by calculating the area of clear zone and anti-biogram was statistically analyzed.

**Statistical analysis**

The results are expressed as means ± SE of three experiments. Mean values were assessed using One-way analysis of variance using SPSS for Windows version 20.0 (Statistical Package for Social Services, Chicago, IL, USA).

**Results**

Our preliminary study posited that, the methanolic extract of *S. swartzii* was excellent for subduing the growth of clinical and biofilm forming bacteria *in vitro* (data not shown). And there is an extreme paucity of studies pertains to the antifungal efficacy of *S. swartzii* from the Indian littoral.
Thence, in the present study, anticandidal activity of \textit{S. swartzii} was explored against the panel of five species of \textit{Candida}. The overall results emphasize the potentiality of using \textit{S. swartzii} for the development of chemotherapeutic agents against \textit{Candida} spp. \textbf{Figure 1} illustrates the inhibitory spectra produced by the \textit{S. swartzii} against \textit{Candida} spp. It is evident that the \textit{S. swartzii} exerted broad spectra of activity against all the tested species of \textit{Candida} in varying degrees. The active algal fraction produced mean zones of inhibition ranged between 102.17 ± 10.03 mm² to 117.51 ± 22.28 mm² against the \textit{Candida} sp. The antifungal action was very high against \textit{C. albicans} to the extent of 117.51 ± 22.28 mm². The nearly active range was displayed against \textit{C. tropicalis} (110.37 ± 17.7) and \textit{C. glabrata} (107.03 ± 23.45). The activity range of \textit{C. krusie} and \textit{C. parapsilosis} accounted for 100.86 ± 20.5 and 102.17 ± 10.03 mm² respectively. The overall results exhibited that potent antifungal constituent can be isolated from \textit{S. swartzii}.

Bioactive molecules of marine algal origin have high potentiality to subjugate the growth of many infectious organisms. In fact, several \textit{in vitro} studies are demonstrated the anticandidal activity of many marine algae [15,20-22]. Albeit, diverse species of \textit{Sargassum} from the Indian coast has been recognized as a potential source of antibacterial agents, the antifungal activity has seldom been reported [23]. Therefore, in the present study, secondary metabolites fractionated from \textit{S. swartzii} were quantitatively examined anticandidal efficacy. The algal fraction exerted wider spectrum of activity, since it impeded the growth of all the evaluated Yeast pathogens in variable degrees. Antifungal activity was found to be positively skewed toward \textit{C. albicans} as compared to other Yeast spp. This type of variation in the efficacy was analogous to that observed for red algae, \textit{A. taxiformis} against human \textit{Candida} spp. [15]. In accordance with the present study, the crude extract of other sp. of \textit{Sargassum} exhibited antifungal activity against \textit{C. albicans} [24]. The demonstration of antifungal efficiency is an indication that the \textit{S. swartzii} is a potential source for bioactive-compounds with broad spectra of activity. The secondary metabolites extracted from the algae can incapacitate the growth of Yeast by mechanisms that are unlike those of antifungal agents currently available. Hence, it is posited to have significant clinical value in the management of resistant yeast strains. Howbeit, further detailed studies are necessary to verify the \textit{in vivo} efficacy and mechanism of action.

The antimicrobial compounds responsible for the antifungal efficacy are not elucidated in this study. Howbeit, GC-MS analysis of active fraction evinced the presence of three compounds such as, 1,2-Benzenedicarboxylic acid, diisooctyl ester (390 g/mol), \textit{n}-Hexadecanoic acid (256 g/mol) and 3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol (296 g/mol). Thence, it was envisaged that the growth inhibition of \textit{Candida} spp. displayed by the active algal fraction could be due to the presence of major principle 1,2-Benzenedicarboxylic acid, diisooctyl ester, or could be related with synergistic activity of all components, since antimicrobial activities are pertained to the presence of secondary metabolites. The bioactive phytochemicals of diverse species of genus \textit{Sargassum} were well reviewed by Liu et al. [25]. The same author noted the presence of 1,2-Benzenedicarboxylic acid; Dioctyl ester in \textit{S. wightii}. Similarly, the GC-MS results are in line with recent studies that annotated the similar bioactive chemical constituents in other plant specimens [26-29].

GC-MS analysis of active fraction of \textit{S. swartzii}

The active fraction was subjected to GC-MS analysis to explicate its bioactive chemical constituents. The spectral data has brought a single prominent peak to fore with the retention time and molecular weight of 25.73 and 390 respectively (\textbf{Table 1}). MS data has perfectly matched a compound of molecular formula \textit{C_{22}H_{36}O_{4}} that is analogous to 1,2-Benzenedicarboxylic acid, diisooctyl ester in the NIST library.

**Discussion**

Globally, diseases caused by the species of genus \textit{Candida} are leading health problems with high morbidity in immuno-competent and immuno-compromised patients [19]. The application of chemotherapeutics for the management of clinically relevant \textit{Candida} spp. is currently restricted by the development of drug resistance. In this viewpoint, an antifungal agent with broad efficacy and minimal side effect is needed to mitigate the plights of vast masses of immunocompetent and immuno-compromised patients.
anticandidal chemical defences. In this context, more studies pertaining to mode of action of algal bioactives and interaction with pathogenic fungi may bring forth new drug leads for the control of fungal pathogens.

Table 1 Components identified in the active fraction of S. swartzii by GC-MS study

<table>
<thead>
<tr>
<th>No</th>
<th>RT</th>
<th>Name of the compound</th>
<th>MF</th>
<th>MW</th>
<th>PA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.44</td>
<td>3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol</td>
<td>C29H44O2</td>
<td>296</td>
<td>6.10</td>
</tr>
<tr>
<td>2</td>
<td>16.24</td>
<td>n-Hexadecanoic acid</td>
<td>C18H36O2</td>
<td>256</td>
<td>16.36</td>
</tr>
<tr>
<td>3</td>
<td>25.73</td>
<td>1, 2-Benzenedicarboxylic acid, disoocetyl ester</td>
<td>C24H30O4</td>
<td>390</td>
<td>77.54</td>
</tr>
</tbody>
</table>

RT - Retention time; MF - Molecular formula; MW - Molecular Weight; PA - Peak Area

References

8. Zonaria tournefortii


