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Anti-Fungal Efficacy and Secondary Metabolite Analysis of the Methanolic Extract from *Colpomenia peregrina* towards Opportunistic Fungal Pathogens

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Running title: Anti-fungal efficacy from methanolic extract of *Colpomenia peregrina* towards opportunistic fungal pathogens.

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ABSTRACT:

The present study illustrates the antifungal efficacy of methanolic extract from marine brown seaweed *Colopomenia peregrina* gathered from Leepuram coast, South India, towards opportunistic fungal pathogens comprising of dermatophytes, non-dermatophytes, and yeasts. The opportunistic fungal pathogens used in the study are *Aspergillus flavus* (ATCC 27692), *Aspergillus fumigatus* (ATCC 19673), *Microsporum gypseum* (ATCC 24102), *Cryptococcus neoformans* (ATCC 14116), and *Candida albicans* (ATCC14053) which are commonly responsible for nosocomial infections. The NMR analysis revealed the presence of various chemical shifts showing the presence of protons containing Hydroxyl, Methoxy, Methyl groups, and –COO-CH_2 groups.The presence of phytochemicals from the extract of seaweed confirmed the nutritional profile. The results revealed greater efficacy of methanolic extract towards *Aspergillus fumigatus, Microsporum gypseum, Cryptococcus neoformans, and lower activity with Aspergillus flavus and Candida albicans* compared with the standard anti-fungal fluconazole.

Keywords:*Colopomenia peregrina*, Methanolic extract, NMR, antifungal efficacy, Opportunistic fungal pathogens.

INTRODUCTION:

Seaweeds arealso known as marine macroalgae are photosynthetic primarily found among the tidal and intertidal regions of the sea contribute to 60 % of the global renewable consumption. These seaweed macrophytes are classified into three divisions namelyChlorophyceae, Phaeophyceae, and Rhodophyceae which are widely used for industrial applications to make products such as agar, alginic acid, and carrageenans and also used as feed, fodder, and medicines across the world[1-3]. Seaweed consumption and utilization have been way back in history where they were used as traditional medicine in china 2500 years ago and by japan as food for their stable diet due to their rich nutritional content[4-6]. The secondary metabolites from the bioactive compounds with antioxidant nature present in seaweeds make it a viable source for

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many biologic activities[7,8]. The rich source of vitamins, minerals, proteins, and carbohydrates in seaweeds are utilized as dietary applications in pharmaceutical, feed, and fodder industries[9]. The anti-microbial, anti-tumor, and COX-inhibitorattributes of seaweed extracts play a crucial role and major contributionto the pharmaceutical industry[10]. The rise in nosocomial infections on fungi has increased manifold due to the deep treatments in immuno-compromised patients[11]. The stimulus rise in nosocomial fungal diseases are changing microbiomedue to prolonged corticosteroid treatments, less neutrophil for a prolonged period; weakened immune system; chemotherapy, transplantations, and extensive intensive care unit admit[12]. The search for new antifungals has been the current focus due to the rise in multidrug-resistant fungal strains posing a threat in nosocomial and immunocompromised patients. The seaweeds especially brown algae was studied extensively due to their high nutritional profile like carbohydrates, proteins, vitamins, and minerals[13,14] as well as the bioactive substances containing potent[1^0& 2^0]metabolites[15]. In the present study, the nutritional profile of the brown experimental algae *Colpomenia peregrina* has been investigated along with the antifungal efficacy of the methanolic extract from the seaweed to the opportunistic fungal pathogens which creates serious health problems in nosocomial infections.

EXPERIMENTAL METHODS:

Seaweed – Collection and Extraction:

Colpomenia peregrina was collected from the intertidal regions of Leepuram, Kanyakumari District (Latitude 8°16'23.12" N, Longitude 77°22'03.02"E); South India. Collected seaweed was rinsed with saline water for eliminating impurities such as sand, rocks, epiphytes, and epifauna. The washed samples were preserved with 5-10% formaldehyde in seawater and kept in slushed ice under laboratory conditions.The seaweed material was fixed and preserved by formalin fumes in the laboratory,post which the seaweed samples were washed with freshwater followed by deionized water to decant the salt and other impurities and dried at 37^0C for 12 to 15 days.The processed seaweed samples were made into a powder form and preserved in the anaerobic container.The methanol extraction was done by taking 20 g moisture-free seaweed powder in 250 ml of methanol (carbinol) (Merck) in a Soxhlet apparatus at 45^0C for 7 to 8 hrs. The final dried extract was further dissolved in aqueous methanol (90%) and evaporated using a rotary type evaporator and preservedusing colored bottles at 4 °C for experimental analysis[16,17].

Secondary Metabolite Analysis (SMA):

The estimation analysis of 2^0 active metabolites from the methanolic extract of marine brown seaweed *Colpomenia peregrina* was analyzed and the active substances were identified as per the standard protocols. The methods adopted

for estimation analysis areMayer's reagent test, Keller-Killani test, Shinoda's magnesium ribbon test, and Ferric chloride test[18-24].

Nuclear Magnetic Resonance (NMR):

The ^{1}H-NMR analyses (Proton magnetic resonance) were done using Bruker VIII 500MHZ from the sophisticated analysis instrument facility (SAIF), IIT Madras, Chennai, Tamilnadu, India. The methanolic extract of *Colpomenia peregrina* was dissolved using DMSO-d_6 solvent and the tetramethylsilane was used as the standard. The functional groups in the extract were determined by comparing with the standard chart[25,26].

Antifungal Assay:

The fungal cultures were obtained from LGC Promochem India Pvt. Ltd, Peenya, Bangalore, India. The fungal pathogens used for the efficacy studies against methanolic extract of *Colpomenia peregrina* are *Aspergillus flavus* (ATCC 27692), *Aspergillus fumigatus* (ATCC 19673), *Microsporum gypseum* (ATCC 24102), *Cryptococcus neoformans* (ATCC 14116), and *Candida albicans* (ATCC14053), and Fluconazole, (Hi-media) was used as the standard antifungal agent. The concentration potent (30 µg/mL) was used for extract and standard as zone interpretive criteria.The fungal strains were cultured using Mueller-Hinton agar supplied by Hi-media with 2% glucose for growth stability for the strains of fungus and 0.5 µg/L methylene blue dyewhich provides and sharpens the zone edges. The optical density of the fungal cultures wascompared with McFarland standard and the pH 7.3 at the ambient temperature of 37^{0}C was maintained. Finally, the Petri plates were incubated in a bacteriological incubator at 35^{0}C for 18 to 24 hrs, post which the plates were checked for growth, and in cases of insufficient growth, 48 hrs of incubation was kept and the zone interpretation was calculated [27].

Statistical analysis:

The mean ± SD triplicates of data were analyzed using MS Excel 2010. Mono-factorial analysis of variance (ANOVA) and post hoc Tukey's tests were used to analyze the difference in zone formation and the calculated means were significantly statistical if $p<0.05$

RESULTS:

The extract from the marine brown seaweed *Colpomenia peregrina* was analyzed for the presence of secondary metabolites (Table 1). The results revealed the presence of phenols (54.65 ± 0.01), alkaloids (13.32 ± 0.01), triterpenoids (3.25 ± 0.01), steroids (19.17 ± 0.01), tannins (16.55 ± 0.08), saponins (3.12 ± 0.01), flavonoids (2.53 ± 0.01), anthraquinones (3.37 ± 0.01) and glycosides (10.07 ± 0.01) in milligrams per gram dry weight.

The ^{1}H-NMR analysis of the methanolic extract from experimental *Colpomenia peregrina* exhibited various chemical shift values (Table 2). The occurrence of hydroxyl protons between δ5.2- 4.8, Protons of –COO-CH_2 between δ4.7-4.6, Methoxy protons between δ3.3-

3.18 and Methyl protons between δ1.1-1.4 was determined (Figure 1).

The Kirby Bauer method was adopted for evaluating the anti-fungal effect of the methanolic extract (30 µg/mL) from *Colpomenia peregrina*(Figure 2). The zone formation was measured in millimeters and the efficacy was determined against the opportunistic fungal pathogens. The methanolic extract from *Colpomenia peregrina* showed 15 ± 0.002 mm for *Aspergillus fumigatus*, 6 ± 0.001 mm for *Aspergillus flavus*, 12 ± 0.002mm for *Microsporum gypseum*, 5 ± 0.002 mm for *Candida albicans*, and 11 ± 0.001 mm for *Cryptococcus neoformans* (Figure 3). Similarly, the results were compared with the standard antifungal fluconazole and the zone of inhibition was compared with the methanolic extract from *Colpomenia sinuosa*. The standard fluconazole showed 10 ± 0.002 mm for *Aspergillus fumigatus*, 9 ± 0.002 mm for *Aspergillus flavus*, 2 ± 0.001 mm for *Microsporum gypseum*, 3 ± 0.001 mm for *Candida albicans*, and 11 ± 0.002 mm for *Cryptococcus neoforman*s (Table 3).

DISCUSSION

The antifungal efficacy of methanolic extract from *Colpomenia peregrina* showed a better zone of inhibition towards opportunistic fungal pathogens *Aspergillus fumigatus*, *Microsporum gypseum*, *Cryptococcus neoformans* whereas lesser activity was observed with *Aspergillus flavus* and *Candida albicans* compared to standard antifungal agent fluconazole. The fungal pathogen *Cryptococcus neoformans*exhibited similar efficacy with a methanolic extract from seaweed and the standard antifungal fluconazole. Moderate activity was observed with the fungal pathogen *Aspergillus flavus* both with methanolic extract and fluconazole and less activity was seen with the fungal pathogen *Candida albicans* with methanolic extract and fluconazole. Thus the opportunistic fungal pathogens *Aspergillus fumigatus*, *Microsporum gypseum*, *Cryptococcus neoformans*exhibited greater efficacy with methanolic extract compared with *Aspergillus flavus* and *Candida albicans* which were moderate and less respectively. The fungal pathogen *Candida albicans* exhibited the least zone of inhibition and was considered to have the Minimum fungicidal concentration (MFC) with methanolic extract and fluconazole. The ubiquitousness of active metabolites like phenolic compounds, alkaloids, triterpenoids, steroids, tannins, saponins, flavonoids, anthraquinones,and glycosides from the methanolic extract from the brown marine seaweed *Colpomenia per*egrina may have contributed to the anti-fungal efficacy towards the opportunistic fungal pathogens[28]. The effects of anti-fungal activity greatly depend upon the factors influencing the chemical composition of the seaweed, growth conditions, sampling period, species variation, and environmental variations like salinity, temperature, location, and climatic conditions[29-31].

The enhanced antimicrobial and antifungal activity were observed during the spring season due to the dominance of secondary metabolites during the period [32-34]. The secondary metabolites

especially alkaloids, triterpenoids, tannins, and phenols have better anti-fungal efficacy especially against dermatophytic fungi[35,36].The presence of triterpenoids in the extract may hinder the mitochondrial content of the opportunistic fungal pathogens thereby altering their ATP generation and reactive oxygen level inhibiting the growth of the fungal pathogen[37]. The alterations in the fungal morphology in consequence with active metabolites may restrict the fungal growth acting as inhibitory substances[38]. The phenols present in the seaweed extract may hinder the cell membrane and could stop the spore germination by denaturing the enzymes and proteins [39].

NMR is an important tool and high throughput non-destructive method for the evaluation of 2^0active metabolites in seaweeds and other bioticsamples [40 -42]. The presence of Hydroxyl proton, Protons of –COO-CH_2, Methoxy protons, and Methyl protons via ^{1}NMR studies may have inhibitory action against opportunistic fungal pathogens. The metabolic alterations in the opportunistic fungal pathogens *Cryptococcus neoformans*and*Candida albicans*due to osmotic pressure and heat stress have been reported by ^{1}NMR analysis[43-45]. Meroditerpenoids a compound from triterpenoids that is structurally complex in metabolic pathways was elucidated using ^{1}NMR studies and known to possess strong antifungal activity[46,47]. The anti-fungal effects of flavonoids and phenolic compounds using ^{1}NMR analysis have been documented and evaluated[48,49]. Thus the occurrence of 2^0 active metabolites in the methanolic extract of *Colpomenia peregrina* along with the presence of chemical shifts protons of hydroxyl, Methoxy, and methyl groups may be the possible reason for the antifungal efficacy towards the opportunistic fungal pathogens.

CONCLUSION

The methanolic extract from the seaweed *Colpomenia peregrina* exhibited a better efficacy towards the fungal pathogens whereas a moderate activity and lesser activity was observed with *Aspergillus flavus* and *Candida albicans* respectively when compared with standard antifungal agent fluconazole(30 µg/mL). The possible anti-fungal activity may be the due to the existence of 2^0 active metabolites and possible chemical shift proton groups present in the extract. This study forms the basis of future research for the bioactive compounds of seaweeds concerning anti-fungal efficacy towards the opportunistic fungal pathogens which pose a serious health condition in nosocomial infections and immunocompromised patients.

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TABLE 1: SECONDARY METABOLITE ANALYSIS OF *Colpomenia peregrina*

Secondary Metabolites	***C. peregrina (mg/g dry wt)***
Phenols	54.65 ± 0.01^{j}
Alkaloids	13.32 ± 0.01^{l}
Triterpenoids	3.25 ± 0.01^{n}
Steroids	19.17 ± 0.01^{m}
Tannins	16.55 ± 0.08^{h}
Saponins	3.12 ± 0.01^{k}
Flavonoids	2.53 ± 0.01^{g}
Anthraquinones	3.37 ± 0.01^{c}
Glycosides	10.07 ± 0.01^{j}
F-Value	3100
P-Value	0

Values are expressed as Mean ± SEM, n=3 as ANOVA test p <0.05% level.
Means in each column with superscripts(s) are significantly different (p<0.05)

TABLE 2: ^{1}H-NMR ANALYSIS OF THE EXTRACT FROM *Colpomenia peregrina*

Chemical Shift value (ppm)	**Presence of protons**
5.2-4.8	Hydroxyl proton
4.7-4.6	Protons of –COO-CH_2
3.3-3.18	Methoxy protons
1.1-1.4	Methyl protons

TABLE 3:ANTI-FUNGAL EFFICACY ZONE OF INHIBITION (MM) OF METHANOLIC EXTRACT FROM *Colpomenia peregrina* COMPARED WITH THE STANDARD.

Opportunistic Fungal Pathogens	**Methanolic extract [*C. peregrina*] (30 µg/mL)**	**Fluconazole (30 µg/mL)**
Aspergillus fumigatus	15	10
Aspergillus flavus	6	9
Microsporum gypseum	12	2
Candida albicans	5	3
Cryptococcus neoformans	11	11

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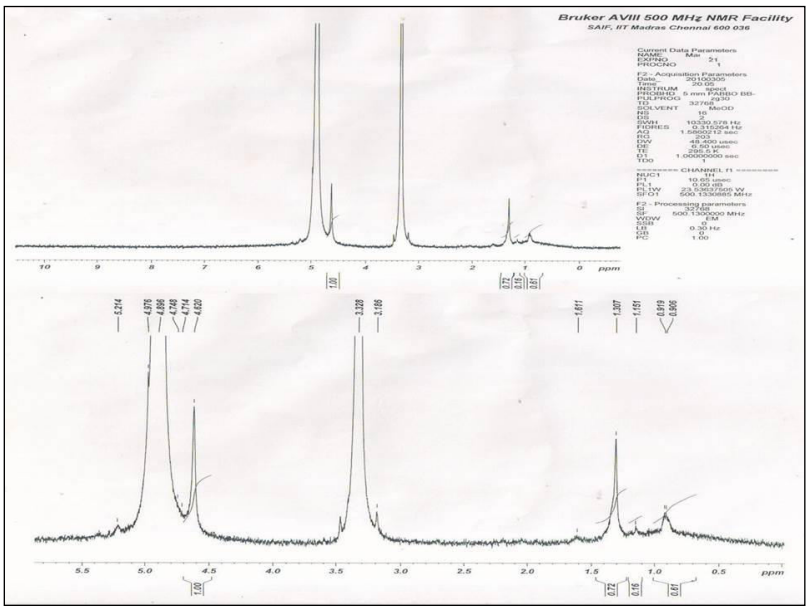

Fig. 1:Nuclear Magnetic Resonance of the extract of *Colpomenia peregrina*

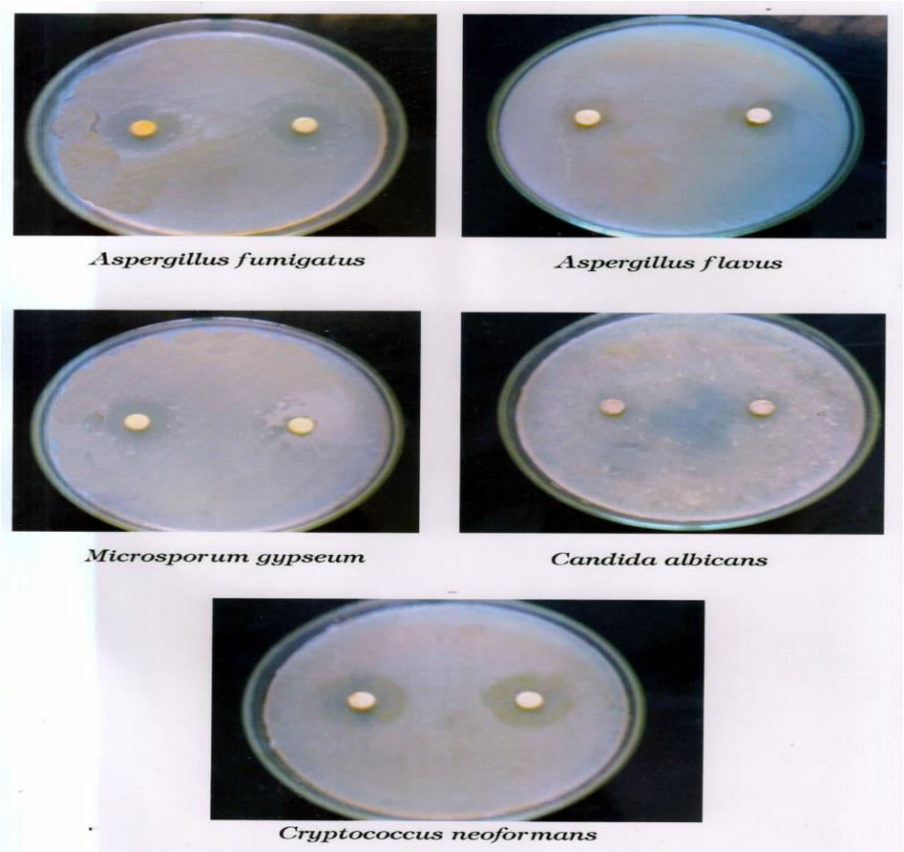

Fig. 2:Efficacy of methanolic extract from *Colpomenia peregrina* towards opportunistic fungal pathogens

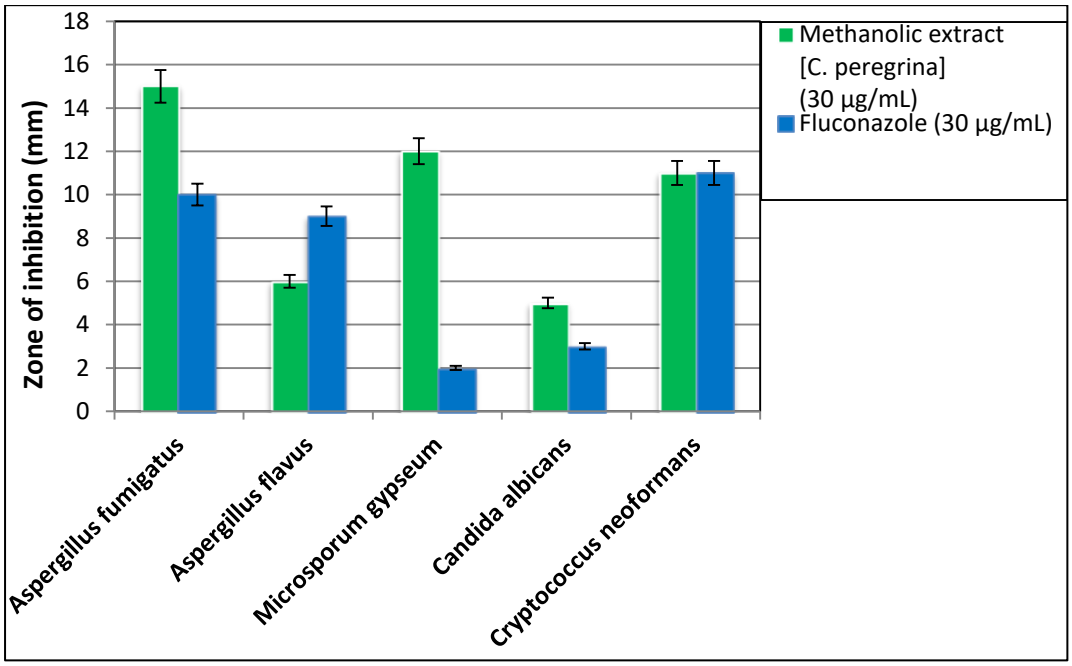

Fig. 3: Methanolic extract of *Colpomenia peregrina* and standard fluconazole efficacy towards opportunistic fungal pathogens

Tables and figure titles and legend:

TABLE 1: SECONDARY METABOLITE ANALYSIS OF *Colpomenia peregrina*

Values are expressed as Mean ± SEM, n=3 as ANOVA test p <0.05% level.
Means in each column with superscripts(s) are significantly different (p<0.05)

TABLE 2: ^{1}H-NMR ANALYSIS OF THE EXTRACT FROM *Colpomenia peregrina*
TABLE 3:ANTI-FUNGAL EFFICACY ZONE OF INHIBITION (MM) OF METHANOLIC EXTRACT FROM *Colpomenia peregrina* COMPARED WITH THE STANDARD

Fig. 1:Nuclear Magnetic Resonance of the extract of *Colpomenia peregrina*

Fig. 2:Efficacy of methanolic extract from *Colpomenia peregrina* towards opportunistic fungal pathogens

Fig. 3: Methanolic extract of *Colpomenia peregrina* and standard fluconazole efficacy towards opportunistic fungal pathogens