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EVALUATION OF ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF ETHANOLIC EXTRACT OF GREATER DUCKWEED, *SPIRODELA POLYRRHIZA*

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ABSTRACT

The phytochemical screening of the various extracts of *Spirodela polyrrhiza* revealed the presence of alkaloids, steroids, flavonoids and saponins. Fourteen fractions were elucidated from the ethanolic extract using the silica gel column chromatography. Antimicrobial activities of the ethanol and fractions of ethanolic extract of *S. polyrrhiza* against Gram-negative strains of *Aeromonas hydrophila* (AH1), *Psedomonas putida* (ATCC 49128), *P. aeruginosa* (ATCC 35072), *P. fluorescens* (PF1), *Vibrio parahaemolyticus* (ATCC 17802), *V. Alginolyticus* (ATCC 17749), *E. coli* (EC1) and Gram-positive strain of *Staphylococcus aureus* (ATCC 6538) and fungal pathogens, *Candida albicans* and *Saprolegnia parasitica* were done by disc diffusion methods showed that the extract possess broad spectrum of activity against the test organisms with diameter of zones of inhibition ranging from 9.0 - 16.0 mm. The minimum inhibitory concentration (MIC) of the extract against the test organisms were found to fall between 30 - 200 µg/ml.

KEY WORDS: Antimicrobial activity, alkaloid, *Candida albicans*, Minimum Inhibitory Concentration (MIC), phytochemical, *Staphylococcus aureus*

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INTRODUCTION

Antibiotics are naturally occurring or synthetic organic compounds which inhibit or destroy selective bacteria, generally at low concentrations[1]. The success of antibiotics against disease causing microbes is among modern medicines' great achievements. However, this kind of drug is beginning to lose its usefulness due to the development of resistance on the part of microbes. The increasing resistance of bacteria to antibiotics is kindled due to the misuse and over prescription of the drugs. As resistance to antibiotics spreads, the development of new antimicrobial agents has to be expedited if the problem is to be contained. Thus the search for newer sources of antibiotics is a global challenge preoccupying research institutions, pharmaceutical companies and academia[2]. One of the impact of antibiotic resistance is the emergence of microbes which are difficult to treat, which may eventually lead to increase cost of disease management and in the long run lead to increased morbidity and mortality. Widespread antibiotic resistance, the emergence of new pathogens in addition to the resurgence of old ones and the lack of effective new therapeutics exacerbate the problems[3]. Microbial infections have been reported to be the major cause of inflammation[4]. The use of medicinal plants for therapy is an ancient practice. Though much work has been done on ethno medicinal plants, there is still need to seek plants with medicinal value to combat diseases. In drug discovery, most studies have been examined on the antimicrobial potential of medicinal plants and other natural products[5]. Although active ingredients may occur in lower concentrations, plant extracts may be a better source of antimicrobial than synthetic drugs[6]. Therefore to overcome the problem various work have been done to know the different antimicrobial and phytochemical constituents of plants and their possible use in treatment of microbial infections and alternative to chemically synthetic drugs.

The present study was carried out on the phytochemical and antibacterial activity of *S. polyrhiza* (L.) Schleid is a species of duckweed popularly known as greater duckweed, common duckmeat, and duckmeal. It can be found nearly worldwide in many types of freshwater habitat. It is an aquatic perennial plant distributed in most regions of Korea, Japan and China and has long been used in Oriental medicine in these countries for treating inflammation, urticaria and skin disease[7]. Usually these are growing in dense colonies, forming a mat on the water surface. Each plant is a smooth, round, flat disc one half to one centimeter wide. It provides a high protein food source for ducks and geese, also eaten by certain fish. In Africa and Asia, giant duckweed has been harvested for cattle and pig feed. It grows quickly, especially if the water is warm and nutrient enriched and has been used to reduce nutrients in sewage effluent.

The presences of flavonoids in *S. polyrrhiza* have been reported by various workers[8, 9, 10], from the ethanolic and methanolic extracts of *S. polyrrhiza*. These flavonoids could be the major effective compounds as they showed potent antioxidant activities[11, 12]. The crude ethanol extract of a *S. polyrrhiza* species has been reported to have an inhibitory effect on preadipocyte proliferation[13]. The present study compares the phycochemical and pharmacological activities of ethanolic extracts of *S. polyrrhiza* aimed at exploring their antimicrobial activity and biomolecules of potential therapeutic interest.

MATERIALS AND METHODS

(i)Collection of S. polyrrhiza

Samples of greater duckweed, *S. polyrrhiza* were collected from ponds of Central Institute of Freshwater Aquaculture, Bhubaneswar, India in the month of October 2009. All samples were brought to the laboratory then washed with distilled water to separate

potential contaminants. The duckweed was identified as belonging to family Lemnaceae[14].

(ii) Preparation of the extracts[15]

Harvested samples were dried at room temperature and grounded in an electric grinder. Resulting powder was submitted to lipid soluble polar solvents (Hexane, Ethylacetate, Ethanol, ratio of Ethanol:Ethyl acetate for extraction, using a soxhlet extractor at 55- 60 ^{0}C. All samples were refluxed until saturation (24 h) and the respective extracts were dried in Rotary evaporator (Heidolph, Laborota 4000 efficient, East Fayban Parkway). Subsequently the residual extracts were suspended in the respective solvents to a final concentration of 20 µg µl^{-1}.

(iii) Partial purification by Silica gel column chromatography[16, 17]

The active crude ethanolic extract (0.5gm) was fractionated using silica gel (SRL, 100- 200 mesh size) column chromatography. The solvent system was fixed by a preliminary thin layer chromatographic (TLC) study. The elution was carried out successively with hexane, different ratio of ethylacetate/ hexane (5%, 15%, 30%, 50%, 70%, 90%, 100%) and 20% chloroform/ methanol and 20-60 ml of each fraction were collected. Then the fractions were reduced to 5ml by distilling. After distillation, collected fractions were mixed according to their TLC behavior. Fourteen fractions were collected after mixing. These are S(EtF1)- (5+15) %EA/Hex; S(EtF2, EtF3, EtF4)- 30%EA/Hex; S(EtF5)- (30+50) %EA/Hex; S(EtF6)- 50%EA/Hex; S(EtF7)- (50+70) %EA/Hex; S(EtF8)- (70+90+100% EA/ hex); S(EtF9, EtF10, EtF11)- (90+100) %EA/Hex; S(EtF12, EtF13)- 100%EA; S(EtF14)- (20+100) %Ch/MeOH. Final fractions were vacuum dried and stored at -4^{0}C.

(iv) Test microorganisms

Antibacterial sensitivity was tested against the pathogenic Gram-negative strains of *Aeromonas hydrophila* (CAHH1), *Psedomonas putida* (ATCC 49128), *P. aeruginosa* (ATCC 35072), *P. fluorescens* (PF1), *Vibrio parahaemolyticus* (ATCC 17802), *V. alginolyticus* (ATCC 17749), *E. coli* (EC1) and Gram-positive strain of *Staphylococcus aureus* (ATCC 6538). The fungus strains of *Candida albicans* and *Saprolegnia parasitica* were also evaluated. These pathogens maintained in the Fish Health Management Division (FHMD), CIFA, Bhubaneswar, were taken for the antimicrobial sensitivity study. Pure cultures of different bacterial strains inoculated in brain heart infusion (BHI) broth (Hi-media, Mumbai, India) except *V. parahaemolyticus* and *V. alginolyticus* which was maintained in BHI broth supplemented with 2.5% NaCl and incubated at 37 °C for 18 h and subsequently used for antimicrobial assay, while the fungal isolates were subcultured on a Potato dextrose agar (PDA) (Hi-Media) for 72 h at 25°C.

(v) Preliminary phytochemical screening of extracts[18, 19]

The phytochemical constituents of *S. polyrrhiza* was performed to verify the presence of secondary metabolites, alkaloids, flavonoids, tannin, steroids, reducing sugar and saponins by using standard methods of Sofowara (1993) and Trease and Evans (2002) with little modification.

(vi) Antibacterial activity[20,21]

Antibacterial potency of various crude and fractions of the ethanolic extract of *S. polyrrhiza* were carried out by disc diffusion technique. All bacteria were grown in BHI broth incubated at 37 °C for 24 h and plated using a sterile swab, on to petridishes containing Antibiotic Assay Medium (Hi-media) adjusting the bacterial count to 10^7 CFU mL$^-$1. Each extract of 200µg 10 µL$^-$1 concentration was applied to sterile filter paper discs (6 mm in diameter, Hi-Media). After solvent evaporation the discs were put on to inoculated plates and incubated at 37 °C. Discs with solvent (10 µL) used for dissolution were taken as control after evaporation of the solvent. Activity of the microalgae extracts against bacterial

pathogens was determined after 24 h at 37 °C by measuring the diameter of the halo around the discs (average of three experiments). The antibacterial activities of *S. polyrrhiza* extracts were compared with inhibition zones around four commercial antibacterial discs i.e. Clotrimazole (10mcg), Tetracycline (20mcg), Furazolodone (50mcg), Cephalaxin (30mcg).

(vii) Antifungal activity[21]

The sterile potato dextrose agar (PDA) plates were prepared for fungus. Then the fungal test organisms were spread over the PDA plates. At the same time, sterile discs of 6 mm diameter were embedded with 10 µl of the plant solvent extracts with four different concentrations (50µg, 100µg, 150µg & 200µg). After solvent evaporation, the discs were placed on the organism inoculated plates with equal distance control discs were also prepared. All the fungal plates were incubated at 24^{o}C for 48 h. The diameter of the minimum zone of inhibition was measured in mm. For each test, three replicates were performed. The antifungal activities of plant extracts were compared with one commercial antifungal i.e. Fluconazole (Hi Media, India).

(viii) Determination of Minimum Inhibitory Concentration (MIC)[22, 23]

The minimal inhibitory concentration (MIC) values were determined by tube dilution assay. The cultures were prepared at 24 h broth cultures of bacterial and fungal pathogens. The minimum dilution of plant extract that inhibits the growth of the organism was taken as MIC.

(ix) Statistical Analysis[24] The results were analyzed using one way analysis of variance (ANOVA) and significant difference of various fractions of ethanolic extract of *S. polyrrhiza* among different concentration was compared using Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

(i)Phytochemical analysis

Nowadays, many biological activities have been evaluated for numerous species of plants. Aquatic plants are believed to be source of medicines and research are going on exploring the medicine from the wealth of sea in a large scale. An alternative to the inhibition of bacterial growth would lie in an approach to prevent pathogens from establishing a successful infection that can be done through developing new antipathogenic drugs. The most important of these bioactive constituents of plants are alkaloids, tannin, flavonoid and phenolic compounds[25]. Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. In our study we are concentrating on ethanolic extract of *S. polyrrhiza*. From the phytochemical screening of it was depicted that the ethanolic extract of *S. polyrhiza* poses alkaloids and steroids (Table. 1). Similar type of observations was found by many workers[26,27] that the ethanolic extract of plants (*Plectranthus glandulosis*) shows the presence of tannins, alkaloids, glycosides, steroids and flavonoids. Similar types of observations were found in our experiment that ethanolic extract and a chromatographic eluent, S(EtF11, 90%+100% EA/Hex) contains alkaloids.

Table 1
Phytochemical analysis of crude and fractionated ethanolic (ETH) extract of Spirodela

Extracts	Phytochemical tests						
	Alkaloid test			Tanin	Flavonoids	Steroid	Saponins
	Dragendroff's Reagent	Wagner's Reagent	Mayer's Reagent	Ferric chloride test	(NaOH & dil. HCL)		
S:ETH	+	+	+	-	-	+	-
S(EtF3)	-	-	-	-	-	+	-
S(EtF4)	-	-	-	-	-	+	-
S(EtF5)	-	-	-	-	-	+	-
S(EtF6)	-	-	-	-	-	+	-
S(EtF11)	+	+	+	-	-	-	-

Note: S:ETH-Ethanolic extract of Spirodela; S(EtF3, EtF4)- 30%EA/Hex; S(EtF5)- (30+50) %EA/Hex; S(EtF6)- 50%EA/Hex; S(EtF11)- (90+100) %EA/Hex

(ii) Antibacterial activity

Based on the preliminary screening results, selected fractions were chosen to test the efficacy of ethanolic extracts of *S. polyrrhiza* against eight bacterial pathogens. The details of the antibacterial activity are given in the Table. 2 &3. The results showed that crude ethanolic and low as well as high polar chromatographic eluents could effectively inhibit the growth of *A. hydrophila* , three species of *Pseudomonas*, two *Vibrio* species, *S. aureus* and *E.coli*. Plant based steroids possess as antimicrobial[28] extracts. Ethanolic fractions like S(EtF5) and S(EtF6) posses steroids and showed maximum zone of inhibition (13.16±0.44 mm, 14.16±0.44 mm) against *P. flourescens* (PF1) and *Vibrio* spp. Interestingly, two eluents with unknown compounds S(EtF5) and S(EtF8) showed promising antibacterial activities against both *A. hydrophila* and *V. parahaemolyticus* ATCC 17802. Ethanol extract also inhibited gram-positive bacteria *B. cereus* (16.7mm), *B. megaterium* (17.2 mm), *B. stearothemophilus* (15.9 mm), *B. subtilis* (13.4 mm), *S. aureus* (12.9 mm), and *S. faecalis* (13.5 mm), but did not show any considerable activity against gram-negative bacteria. Phytochemical and antibacterial activity of leaves of *Alstonia scholaris* was investigated by Khyade and Vaikos[29]. The presence of alkaloids have shown as antimicrobial[30] and antioxidant[31] activity. The chromatographic eluent S(EtF11, 90+100% EA/Hex) contains alkaloids and showed highest antibacterial activity (14.33±0.647 mm, 15±0.408 mm and 14±0.408 mm) against *P. aeruginosa* ATCC 35072, *V. alginolyticus* ATCC 17749 and *V. parahaemolyticus* ATCC 17802 (Table. 3). The *Gelidium acerosa* contain large amount of valuable phytochemicals like sapononins, flavonoids and alkaloids etc., which are known for its medicinal uses[32]. Preliminary phytochemical screening of the crude extracts of *Canavalia rosea* revealed the presence of tannins, phlobatannins, saponins, flavonoids, alkaloids, cardiac glycosides and phenolics. The presence of these bioactive constituents is associated with the antimicrobial activity of the plant[33]. Subsequent experiments were conducted to determine the minimum inhibitory concentration values of *S. polyrrhiza* against five bacterial pathogens. The MIC of both fractions and crude *S. polyrrhiza* extracts were ranges from 100μg-500μg, where as comparatively lower MIC value (100μg) was noticed by all the fractions except S(EtF6) and S(EtF12) (Fig:1).

Table 2

Antibacterial activity of crude Ethanolic S(ETH) extracts and fractions of Ethanolic extract of Spirodela against A. hydrophila, Pseudomonas spp.,

Extracts	**Disc potency in µg/10µl**	**Bacteria (Zone of inhibition in mm)**			
		A. hydrophila	***Pseudomonas* spp.**		
		CAHH1	*P. aeruginosa* ATCC 35072	*P. putida* ATCC49128	*P. flourescens* PF1
S: ETH	200	**+++**	**+++**	**++**	**-**
S(EtF1)	200	**+++**	**-**	**-**	**+++**
S(EtF2)	200	**+++**	**+++**	**-**	**+++**
S(EtF4)	200	**++**	**-**	**++**	**+++**
S(EtF5)	200	**++**	**-**	**+++**	**+++**
S(EtF6)	200	**++**	**+++**	**-**	**+++**
S(EtF7)	200	**+++**	**-**	**-**	**+++**
S(EtF8)	200	**+++**	**+++**	**-**	**-**
S(EtF11)	200	**++**	**+++**	**+++**	**-**
S(EtF12)	200	**+++**	**-**	**++**	**-**
S(EtF14)	200	**+++**	**+++**	**+++**	**-**
Standard antibiotics					
Clotrimazole	10mcg	18	12	12	14
Tetracycline	20mcg	ND	19	15	19
Furazolodone	50mcg	ND	18	15	15
Cephalaxin	30mcg	15	8	9	12

Note: S:ETH-Ethanolic extract of Spirodela; S(EtF1)- (5+15) %EA/Hex; S(EtF2)- 30%EA/Hex; S(EtF4)- 30%EA/Hex; S(EtF5)- (30+50) %EA/Hex; S(EtF6)- 50%EA/Hex ; S(EtF7)- (50+70) %EA/Hex; S(EtF11)- (90+100) %EA/Hex; S(EtF12)- 100%EA; S(EtF14)- (20+100) %Ch/MeOH

(Zone size '+++' =10-20mm; '++'= 5-10mm; '- = 0mm)

Table 3

Antibacterial activity of crude Ethanolic S(ETH) extracts and fractions of Ethanolic extract of Spirodela against Vibrios, S. aureus and E. coli

Extracts	Disc potency in µg/10µl	Bacteria (Zone of inhibition in mm)			
		Vibrios		***S. aureus***	***E. coli***
		V.alginolyticus ATCC 17749	*V.parahaemolyticus* ATCC 17802	ATCC 6538	EC1
S: ETH	200	++	-	+++	++
S(EtF1)	200	++	+++	+++	+++
S(EtF2)	200	+++	+++	-	+++
S(EtF4)	200	+++	+++	-	+++
S(EtF5)	200	+++	+++	-	+++
S(EtF6)	200	+++	+++	++	-
S(EtF7)	200	+++	+++	++	-
S(EtF8)	200	+++	+++	+++	-
S(EtF11)	200	+++	+++	+++	-
S(EtF12)	200	+++	+++	-	+++
S(EtF14)	200	-	-	+++	+++

Note: S:ETH-Ethanolic extract of Spirodela; S(EtF1)- (5+15) %EA/Hex; S(EtF2)- 30%EA/Hex; S(EtF4)- 30%EA/Hex; S(EtF5)- (30+50) %EA/Hex; S(EtF6)- 50%EA/Hex ; S(EtF7)- (50+70) %EA/Hex; S(EtF11)- (90+100) %EA/Hex; S(EtF12)- 100%EA; S(EtF14)- (20+100) %Ch/MeOH
(Zone size '+++' =10-20mm; '++'= 5-10mm; '- = 0mm)

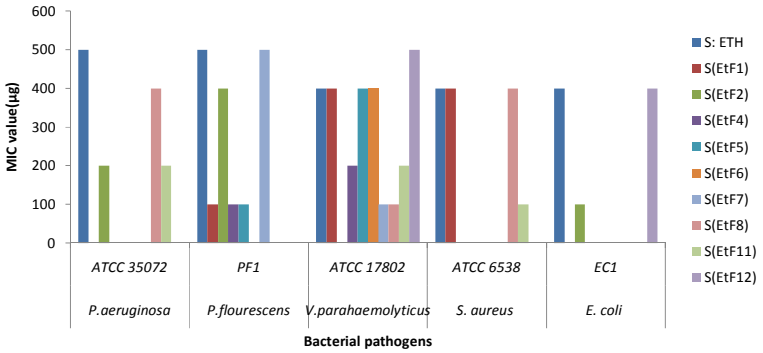


Figure 1

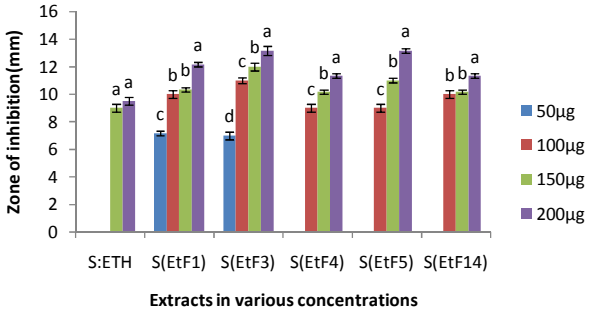
MIC values (µg) of crude ethanolic extract and fractions of Spirodela against Pseudomonas spp., Vibrios, S. aureus and E. coli

The antimicrobial sensitivity i.e. Fluconazole (as antifungal), Tetracycline, Clotrimazole and Cephalaxin (as antibacterial) are taken as control for comparing the activity of the solvent extracts of *S. polyrrhiza* to find out its efficacy and usefulness while developing a standard bioactive compound as an antimicrobial. The antimicrobial activity of crude extracts of *Ulva*

fasciata and *Chaetomorpha antennina* against ten human pathogenic bacterial strains were done and their zone of inhibition compared with standard antibiotic, tetracycline. In extracts obtained using ethanol showed a max activity against pathogen like *S. aerues* (7mm) and minimum activity against *M. luteus* (6mm), *B. cereus* (6mm), *K. pneumonia* (5mm), and no activity against the pathogen like *E. coli*, *P. aeruginos* and *B. cereus* [32] was observed.

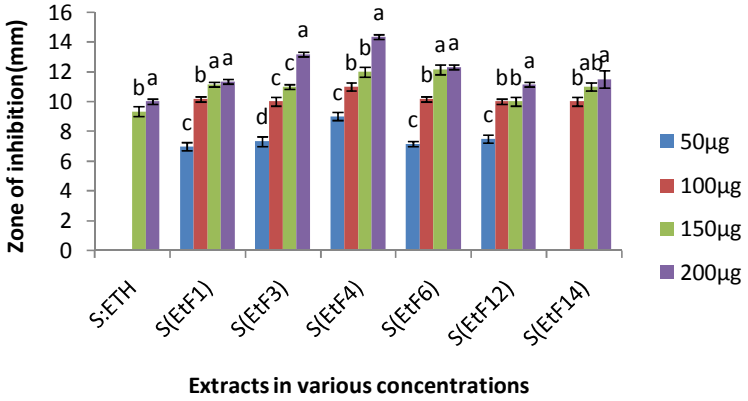
(iii) Antifungal activity

Subsequent experiments were conducted to determine the antifungal activity and MIC values of *S. polyrrhiza* extracts. The crude as well as fractionated extracts of *S. polyrhiza* were tested against two fungi, *C. albicans* and *S. parasitica.* From the detail antifungal screening it was observed that both crude ethanolic extract, S(ETH) and the steroid containing fractions, S(EtF4) and S(EtF6) exhibits maximum antifungal activity against both the selected fungi. Fractions of ethanol extract of *S. polyrrhiza* containing steroids showed maximum zone of inhibition (13.16 mm in 200µg disc potency) against *C. albicans*. Here it was noticed that the increase in concentration (50µg to 200 µg) of the extracts there is increase in zone of inhibition (Fig:2). Similar types of observations were observed by Kanwal et al. [34]. Eight steroid saponins from *Tribulus terrestris* L., were tested to investigate their properties against fluconazole-resistant yeasts often encountered clinically, especially *C. albicans* [35]. The steroidal glycosides tested in the experiment are from the same chemical class, but only two fractions exhibited anti fluconazole-resistant yeasts activity against *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. neoforman* and *C. krusei*. These results indicate that there are critical structural features that are responsible for the antifungal activity. Similarly, in fungi the ethanol extract exhibited highest zone of inhibition at 400 µg/well against *Candida albicans* [36]. In addition, the crude as well as fractions of ethanolic extract of *S. polyrrhiza* also showed good antifungal activities against *S. parasitica* with MIC values ranges from 100µg to 500µg. Fraction with steroids, S(EtF4) showed outstanding antifungal properties with significantly high zone of inhibition (14.33±0.16mm) in 200µg and had a MIC value 200µg for *S. parasitica* (Fig:3 and Fig:4).



Values represent mean±S.D.,
Values bearing common superscript are not significantly different (p<0.05)

Figure 2
Antifungal activity of crude and fractions of ethanolic extracts of Spirodela against Candida albicans (48 h culture)



Values represent mean±S.D.,
Values bearing common superscript are not significantly different (p<0.05)

Figure 3
Antifungal activity of crude and fractions of ethanolic extracts of Spirodela against Saprolegnia (48 h culture)

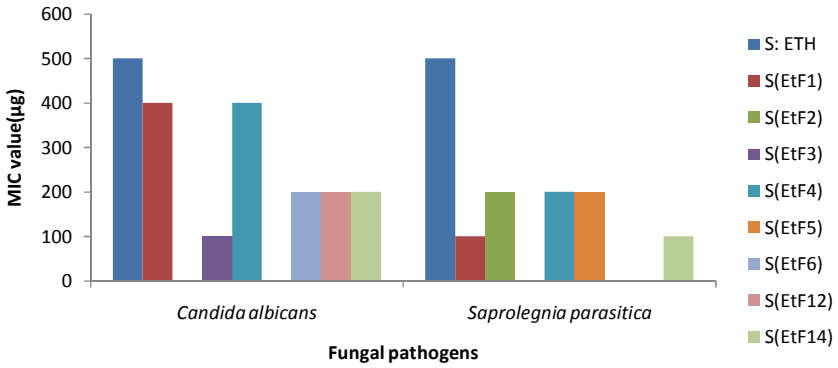


Figure 4
MIC values (µg) of crude Ethanol extract and fractions of Spirodela against C. albicans and S. parasitica

The growth of *E. coli*, *C. albicans* and *S. cerevisiae* was also distinctly inhibited by many marine alkaloids Haminol and Pulo'upone[37]. Alkaloid fractions isolated from *Strychnos potatorum* (Loganiaceae) seed were tested for their antimicrobial properties against fungi. These fractions have shown considerable antimicrobial activity against both bacteria and fungi at the tested concentrations (100 & 200 µg/mL)[38]. In this experiment alkaloids from the crude ethanolic extract, S(ETH) of *S. polyrrhiza* gives promising results against selected fungal pathogens. The zone of inhibition ranged from 9-10 mm with MIC value 500 µg against both the fungal pathogens but fraction containing steroid does not show any activity due to unknown reason. Hebsibah and DhanaRajan[32] reported that ethanol fraction of seaweeds are

found to be more active against fungi such as *A. flavus*, *A. niger*, *A. fumigatus*, *C. albicans*, *C .tropicalis.*

The ability to produce antimicrobial substances may be significant not only as a defensive instrument for the aquatic plants but also as a good source of the new bioactive compounds from a pharmaceutical point of view. A variety of solvents with different polarities were used for the extraction of this plant bioactive material. Here we are concentrating on the active phytochemicals present in the crude ethanolic as well as fractionated product of the *S. polyrrhiza*. Ethanol is considered as a safe solvent and ethanol turned out to be the most suitable solvent in extracting antioxidant components from *Spirulina* since ethanol extracts showed a high antioxidant activity together with a high extraction yield. Higher amount of chlorophyll a together with a lower content of carotenoids was present in ethanol extract opposite to petroleum ether and hexane extracts [39]. Characterization of the lipid fraction of a pressurized ethanol extract of *Spirulina* is carried out by Herrero et al. [40]. Besides the fractions containing phytochemicals, other fractions also showed good antibacterial activities, but these products are silent because of the unknown chemical nature. Hence these active extracts can be used to carry out further pharmacological evaluation. The partially purified fractions need further purifications and the details of the chemical nature by GC MS and NMR spectra.

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