Chiang Mai J. Sci. 2012; 39(1) : 76-83
http://it.science.cmu.ac.th/
Contributed Paper

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Antifungal and Cytotox *Everniastrum cirrhatum* (Fr.) Hale

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Received: 7 January 2011
Accepted: 25 July 2011

ABSTRACT

Everniastrum cirrhatum (Fr.) Hale (Parmeliaceae) is a foliose macrolichen which usually grows on the barks of trees in temperate regions. In the present study, we report for the first time antifungal and cytotoxic activity of *E. cirrhatum*. The dried lichen material was powdered and extracted with methanol using by soxhlet apparatus. Antifungal activity of extract (10-100 mg/ml) was tested against two clinical fungal isolates namely *Candida albicans* and *Cryptococcus neoformans* by well-in-agar method. Cytotoxic activity of extract (0 - 1,000 µg/ml) was assessed against the brine shrimp *Artemia nauplii*. Antifungal activity of methanol extract revealed that the growth of the fungi was affected in a dose dependent manner. The susceptibility to methanol extract was higher in case of *C. neoformans*. In cytotoxic study, the degree of lethality of extract was found to be concentration dependent. Highest mortality of shrimps (100%) was observed in case of extract concentration 1,000 µg/ml. LC_{50} value for extract was found to be 474.06 µg/ml and thus the extract is toxic. Preliminary phytochemical analysis of methanol extract by chemical tests revealed the presence of alkaloids, saponins, tannins and terpenoids. In our study, the extract was potent as it produced marked antifungal and cytotoxic activity which may be attributed to the presence of secondary metabolites. In suitable form, the lichen could be used for the treatment fungal infections and cancer. Further, detailed investigations on active principles in the lichen extract and their bio-efficacy could provide leads to interesting antifungal and cytotoxic or anti-tumor drugs.

Keywords: *Everniastrum cirrhatum* (Fr.) hale, well-in-agar method, cytotoxicity, brine shrimp lethality, *Artemia nauplii*, LC_{50}.

1. INTRODUCTION

Lichens and lichen products have been used in traditional medicines for centuries and still hold considerable interest as alternative treatments in various parts of the world. They produce characteristic secondary metabolites that are unique with respect to those of higher plants. India is a rich center of lichen diversity, contributing nearly 15% of the 13,500 species of lichens so far recorded in the world. Until now, several hundred secondary metabolites including depsides, depsidones, naphthoquinones, anthraquinones, pulvinates, chromones and dibenzofurans have been detected in lichens. Slow growth and often harsh living conditions, make production of protective metabolites, a necessity to lichens, and many secondary constituents are believed to serve as antigrowth, antimicrobial or antiherbivore agents [1, 2]. In our continuing research on the biological activities of tropical lichens, we have investigated a foliose type lichen belonging to the family Parmeliaceae, *Everniastrum cirrhatum* (Fr.) Hale is a foliose lichen in which phycobiont is a chlorolichen alga. It usually grows on the barks of trees in temperate regions [3, 4]. It grows luxuriantly in tropical Himalayas, central India and higher altitudes of southern India. It is characterized by linear, laciniate lobes which are grey in color. Thallus is tapering apically, 2-6mm wide and 10cm long. Thallus is loosely attached to the substratum and pendulous in nature. Apothecia are laminal, margins are inflexed, cilia are black in color, and spores are large [5]. In Ayurveda, it is mentioned as astringent, resolvent, laxative, carminative and aphrodisiac. It is also useful in bleeding piles, leprosy and excessive salivation. It is used as spices in Madhya Pradesh. Whole boiled material used as vegetable in Nepal and north Sikkim [6, 7]. It is traditionally used as antiseptic, to heal wound and bronchitis in Sikkim [8]. It has been used as material for sacrificial fire by Gaddi tribe of Kangra valley. It is used as a spice and flavoring agent for meat and vegetables by Bhaiga, Bhil, Bhilala, Gond, Korka, Muria of Madhya Pradesh. It is used as vegetables in Lepchas and Nepalese of Sakyong valley, North Sikkim. In Uttaranchal, Uttar Pradesh and Sikkim, it is commercially sold as spice [9]. The mycobiont (lichen forming fungus) of *E. cirrhatum* was shown to cause mycelial growth inhibition of hot pepper anthracnose pathogen, *Colletortichum acutatum* [10]. Ethanol extract of *E. cirrhatum* has shown antimycobacteral properties against *Mycobacterium tuberculosis* H37Rv and H37Ra strains [4]. In a study, it was reported that the whole thallus of *E. cirrhatum* yielded a red brown dye [11]. Although *E. cirrhatum* is used as traditional medicine in Indian subcontinent, literature survey revealed that many bioactivities including the antifungal and cytotoxic activity of this lichen has not been yet documented. In present study, we have investigated antifungal activity against *Candida albicans* and *Cryptococcus neoformans*, which cause Candidiasis and Cryptococcosis in human beings and cytotoxic activity in terms of Brine shrimp lethality of methanol extract of *E. cirrhatum* collected from Bhadra wildlife sanctuary.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Lichen

The lichen *E. cirrhatum*, growing on barks of trees, was collected from the Bhadra wildlife sanctuary, Karnataka, India, during August 2010. The lichen specimen was identified by morphological, anatomical, chemical tests [12]. The voucher specimen of the lichen (Voucher no. KU00703) was deposited in the University herbaria, Department of PG Studies and Research

in Botany, Shankaraghatta-577451, Karnataka, India for future reference.

2.2 Preparation of Extract Using Methanol

The lichen was shade dried at room temperature under shade. After air-drying, the lichen material was ground to fine powder and extracted by soxhlet apparatus using methanol as solvent. The extract was filtered using Whatman filter paper no. 1 and concentrated at 40°C under reduced pressure and percentage of yield was calculated. The extract was stored at 4°C until use. Preliminary phytochemical screening for the various secondary metabolites in methanol extract was qualitatively tested using standard procedures [13-15]. Presence of alkaloids was tested by using Dragendorff's and Mayer's reagents. Tannins were determined using ferric chloride test. Presence of saponins (frothing test and hemolysis test), glycosides (Salkawski test and Keller- Kiliani test), sterols (Burchard test), flavonoids (Shinoda test) and terpenoids (Salkowski test) were determined.

2.3 Antifungal Activity of Methanol Extract of *E. cirrhatum*

The antifungal activity of methanol extract was tested against two clinical fungal isolates namely *Candida albicans* and *Cryptococcus neoformans*. The fungi were inoculated into Sabouraud dextrose broth (Peptone 10 g; Dextrose 40 g; Distilled water 1,000 ml) and incubated for 24 hours at room temperature. The screening for antifungal activity was done using the well-in-agar method [16]. Sabouraud dextrose broth culture of *C. albicans* and *C. neoformans* was aseptically swabbed on the sterile Sabouraud dextrose agar medium to get uniform distribution of fungus. Using a sterile cork borer, wells of 0.5 cm diameter were made in the inoculated medium and 0.2 ml of different concentrations of extract (10-100 mg/ml of DMSO) and standard (Fluconazole (1mg/ml) in sterile distilled water) were filled into the respectively labeled wells, and the plates were kept in room temperature for an hour to allow spread of extract and standard in the medium. Later the agar plates were incubated for 5 days at 25 ± 2°C. The presence of zones of inhibition around the wells was observed and interpreted as an indication of antifungal activity. The experiment was carried in triplicate for calculation of standard deviation.

2.4 Cytotoxic Activity of Methanol Extract of *E. cirrhatum*

Brine shrimp lethality assay was performed according to the method of McLaughlin, [17]. The different concentrations of positive control i.e., potassium dichromate (10-50 μg/ml) and methanol extract (10-1,000 μg/ml) were prepared in methanol. Eggs of brine shrimp *Artemia nauplii* (Nihon Animal Pharmaceutical Inc., Tokyo, Japan) were hatched in a container filled with air-bubbled artificial sea water which was prepared using 10 g of a commercial salt mixture (GEX Inc., Osaka, Japan) and 500 ml of distilled water. After 36-48 h, the phototropic shrimps were collected by pipette and used for bioassay. The different concentrations of positive control (potassium dichromate) and methanol extract were tested in vials containing 5ml of brine and 25 shrimp in each of three replicates. Twenty five shrimps were transferred to each vial, artificial sea water was added to make up to 5 ml, incubated at 25°C and surviving shrimps were counted microscopically in the stem of the pipette against a lightened background after 24 hours. The LC_{50} values of extracts greater than1,000 μg/ml were considered inactive (non-toxic).

2.5 Statistical Analysis

All experiments were repeated three times and data were recorded as mean ± SD (Standard Deviation). LC_{50} values were determined by linear regression using Origin 6.0 software

3. RESULTS AND DISCUSSION

E. cirrhatum was successfully extracted with methanol and its yield was found to be 6.21±0.91 %. Taking into consideration of the industrial requirements for extraction, both yield and economic parameters were primarily emphasized before detailed study [18]. The curative properties of natural products are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols etc. Preliminary phytochemical analysis of methanol extract revealed the presence of alkaloids, saponins, tannins and terpenoids. Thus the preliminary screening tests may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development. Further, these tests facilitate their quantitative estimation and qualitative separation of pharmacologically active chemical compounds.

Antifungal activity of different concentrations of methanol extract against *C. albicans* and *C. neoformans* were represented

Table 1. Antifungal and cytotoxic activity of methanol extract of *E. cirrhatum*.

Antifungal activity of methanol extract of *E. cirrhatum*			
Treatment	**Concentration**	**Zone of inhibition (mm)**	
		C. albicans	***C. neoformans***
DMSO	10%	0.0±0.0	0.0±0.0
Fluconazole	1mg/ml	3.8±0.51	3.9±0.43
Methanol extract	100mg/ml	2.5±0.19	2.6±00.24
	50mg/ml	1.8±0.13	1.9±0.07
	25mg/ml	1.4±0.09	1.5±0.12
	10mg/ml	0.8±0.12	0.9±0.06
	5mg/ml	0.0±0.0	0.6±0.04
Cytotoxic activity of methanol extract of *E. cirrhatum*			
Treatment	**Concentration(μg/ml)**	**Mortality (%)**	**LC_{50} (μg/ml)**
Methanol extract	0	0.0±0.0	474.06
	10	0.0±0.0	
	100	19.67±1.53	
	1000	100±0.0	
Potassium Dichromate	0	0.0±0.0	32.77
	10	0.0±0.0	
	30	57±2.64	
	50	77.67±3.51	

in the Table 1 and its data revealed that the growth of the fungi was affected in a dose dependent manner. Highest inhibition of test fungi was observed at extract concentration 100 mg/ml while the lowest concentration of extract tested (5 mg/ml) did not produced any inhibition of the *C. albicans*. Overall, the susceptibility to methanol extract and standard was higher in case of *C. neoformans*. Inhibition caused by standard (Fluconazole) was marked when compared to extract. The reason for higher inhibition by the standard antibiotic is the purity of the drug while the bioactive component in extract would be less in concentration due to the crude nature of the extract. There was no inhibition of test fungi observed in case of control (DMSO). Infectious diseases particularly of fungal origin are major health problem all over the world and in some cases they cause premature deaths, i.e. almost 50,000 people per day and they have increased markedly during the last decade [19-21]. Candidiasis is a common opportunistic fungal disease in the entire world, and vulvovaginal candidiasis is also one of the most frequent infections in female genital tract with a high incidence [22,23]. Amphotericin B and fluconazole, two important agents against human pathogenic fungi, have side effects as well as toxic effects. Thus there is a need for better, novel antifungal agents against infections by fungi, especially *Candida* species [24, 25]. A marked dose dependent inhibition of test fungi was observed in this study and the antifungal effect of the extract may be due to the presence of secondary metabolites in the lichen extract.

The result of cytotoxic activity of methanol extract of *E. cirrhatum* and positive control potassium dichromate in terms of mortality of brine shrimps were presented in Table 1. The degree of lethality was found to be directly proportional to the concentration of the extract. The mortality of shrimps caused by the extract was lesser when compared with the reference control. Highest mortality (100%) was observed in case of extract concentration 1,000 μg/ml. LC_{50} value for extract was found to be 474.06 μg/ml and thus extract found to be toxic. Mortality caused by potassium dichromate (positive control) was more marked (LC_{50} = 32.77 μg/ml) than methanol extract.

Bioactive compounds are almost always toxic in high doses. Thus *in vivo* lethality in a simple zoologic organism can be used as a convenient monitor for screening and fractionation in the discovery and monitoring bioactive natural compounds [26]. The brine shrimp lethality test is considered to be very useful in determining various biological activities such as cytotoxic, phototoxic, pesticidal, trypanocidal, enzyme inhibition, and ion regulation activities [27-21]. Recently, there has been interest in the brine shrimp lethality assay as a means of detecting ion regulation or ion-channel activity such as that involving Na^+-K^+-ATPase or calcium channels [32, 33]. It can also be extrapolated for cell-line toxicity and antitumor activity [27]. The brine shrimp assay is very useful for the isolation of biogenic compounds from plant extracts [34]. This is a rapid method utilizing only 24 hours, inexpensive and needs no special equipment. It is so simplethat no aseptic technique is required. It utilizes a large number of organisms for validation and a relatively small amount of sample. It does not require animal serum as needed for other methods of cytotoxicity testing [35]. This bioassay has been employed to determine cytotoxic activity of several extracts. In our study, the extract showed a marked dose dependent cytotoxic activity in terms of brine shrimp lethal effect. The lethality of methanol extract of *E. cirrhatum*

might be due to the presence of secondary metabolites.

4. CONCLUSION

In our study, the methanol extract of *E. cirrhatum* was shown to be potent as it exhibited a marked antifungal and cytotoxic activity which may be attributed to the presence of secondary metabolites. In suitable form, the lichen could be used for the treatment of opportunistic mycotic infections and cancer. Even though, the present study on the crude extract is an addition to the scientific literature, further detailed investigations on pharmacological activities and active ingredients in the lichen extract could provide leads to interesting antifungal, cytotoxic or anti-tumor drugs.

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