Iranian Journal of Pharmaceutical Research (2010), 9 (4): 411-416
Received: March 2008
Accepted: January 2009

Original Article

Antimicrobial and Cytotoxic Activities of *Turbinaria conoides* (J.Agardh) Kuetz

Sadish Kumar Shanmugam[a*], Yatendra Kumar[a], Khan Mohammad Sardar Yar[b], Vivek Gupta[c] and Erik De Clercq[d]

[a]Department of Pharmaceutical Chemistry, I.T.S.Paramedical College (Pharmacy), Ghaziabad, Uttar Pradesh, India. [b]Department of Pharmaceutical Chemistry, Jamia Hamdard, New Delhi, India. [c]Department of Pharmaceutics, I.T.S.Paramedical College (Pharmacy), Ghaziabad, Uttar Pradesh, India. [d]Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000, Leuven, Belgium.

Abstract

Brown alga, *Turbinaria conoides* was successively extracted with *n*-hexane, cyclohexane, methanol and ethanol:water (1:1). The extracts were evaluated for antibacterial and antifungal activities by disc diffusion method. Minimal inhibitory concentration was determined for active extracts by broth dilution method. The antiviral activity and cytotoxicity of the extracts were tested in human embryonic lung (HEL) cells (herpes simplex virus-1, herpes simplex virus-2, vaccinia virus, vesicular stomatitis virus and herpes simplex virus-1 TK^- KOS ACV^r), human epithelial (HeLa) cells (vesicular stomatitis virus and coxsackie virus B4) and Vero cells (parainfluenza-3 virus, reovirus-1, sindbis virus coxsackie virus B4 and punta toro virus). The results revealed that extracts exhibited cytotoxicity ranged from 20 to >100 µg/mL. Moderate activity was demonstrated by *n*-hexane and cyclohexane extracts against viruses, whereas methanol and ethanol: water (1:1) extracts were not active. Ethanol: water (1:1) presented neither antibacterial nor antifungal activity against tested organisms. Cyclohexane extract possessed a broad array of antibacterial activity and exhibited remarkable antifungal property. It is noteworthy that minimal inhibitory concentration of cyclohexane extract against *Aspergillus niger* is comparable with that of clotrimazole. This potentiality demonstrates that it could be used to treat bacterial and fungal infections.

Keywords: Brown alga; *Turbinaria conoides*; Antibacterial; Antifungal; Antiviral; Cytotoxicity.

Introduction

Marine Brown alga, *Turbinaria conoides* belongs to the Sargassaceae family. Turbinaria and other members of the family Sargassaceae are inedible, due to the concentration of polyphenolic substances based upon the polymerization of phloroglucinol (1, 2). Oxygenated steroids of algae have been shown to exhibit cytotoxic properties (3-8). The ethyl acetate extract of *Turbinaria conoides* and its oxygenated fucosterols has been reported for their cytotoxicity (9). Traditionally, *Turbinaria conoides* has been used to cure children's fever, as fertilizer, insect repellent, pesticide and antibacterial (10). Phytochemical investigation of Brown alga revealed the presence of steroids,

* Corresponding author:
E-mail: jesisjes@yahoo.co.in

flavonoids and reducing sugars (11). The present investigation was carried out to explore upon antibacterial, antifungal, antiviral and cytotoxic activities with various extract viz. *n*-hexane, cyclohexane, methanol and ethanol:water (1:1) of Brown alga.

Experimental

Algal material

Turbinaria conoides was collected in September 2005 from Salin Munthal, Gulf of Mannar, Bay of Bengal, Ramanathapuram district, Tamil Nadu, India and voucher specimen was deposited at Marine algal research station, Mandapam camp, Tamil Nadu, South India. It was also authenticated by K.Eswaran, Scientist, Marine algal research station, India. Brown alga was air-dried for 4 weeks at room temperature. The dried algal material was coarsely powdered and stored in a polyethylene bag under refrigeration.

Extraction

The powdered *Turbinaria conoides* (1kg) was successively extracted with 2.5 L of *n*-hexane, Cyclohexane, methanol and ethanol: water (1:1), each by maceration with occasional shaking at room temperature for 72 h. The *n*-hexane 1, cyclohexane 2, methanol 3 and ethanol:water (1:1) 4 extracts were concentrated under reduced pressure and kept in desiccator for further investigation. The yields of 1, 2, 3 and 4 were 0.21%, 0.22%, 8.68% and 10.31% w/w, respectively. The chemicals were obtained from Qualigens (GlaxoSmithkline Pharmaceuticals Ltd.), Mumbai, India and Rankem (Ranbaxy Pharmaceuticals), New Delhi, India.

Bacteria and fungi

The extracts were screened against a panel of microorganisms, including *Staphylococcus aureus* subsp. *aureus* (MTCC 737), *Staphylococcus epidermidis* (MTCC 3615), *Escherichia coli* (MTCC 1687), *Psuedomonas aeruginosa* (MTCC 424), *Aspergillus niger* (MTCC 228) and *Candida albicans* (MTCC 183). The investigated microbial strains were procured from the Institute of Microbial Technology, Chandigarh, India.

Preparation of inoculum

Active cultures for screening were prepared by transferring a loopful of cells from the stock to test tubes of nutrient broth for bacteria, yeast peptone dextrose broth for *Candida albicans* and Czapek yeast extract broth for *Aspergillus niger*. Moreover, they were incubated without agitation for 24 h at 37 ºC, 48 h at 30 ºC and 7 days at 30 ºC, respectively as per the guidelines specified by Institute of Microbial Technology, Chandigarh, India. The cultures were diluted with fresh broths to achieve optical densities corresponding to 10^6 colony-forming units (cfu/mL) for bacteria and 10^5 spores/mL for fungal strains.

Antibacterial and antifungal studies

Extracts 1, 2, 3 and 4 were dissolved in 100% dimethylsulphoxide (DMSO) at a concentration of 1 mg/mL and used as working stocks. Ampicillin (25 µg) for bacteria, and Clotrimazole (30 µg) for fungi were used as reference agents. Susceptibility test was determined by disc diffusion method (12- 14). The nutrient agar plates were prepared by pouring 15 mL of molten media into sterile petriplates. The plates were allowed to solidify for 5 min, 0.1% inoculum suspension was swabbed uniformly, and the inoculum was allowed to dry for 5 min. The extracts 1, 2, 3 and 4 were loaded on 6 mm discs. The loaded discs were placed on the surface of medium and the extracts were allowed to diffuse for 5 min and the plates were kept for incubation at 37 °C for 24 h for bacteria and 30 °C for 48 h for fungi with yeast peptone dextrose agar and Czepak yeast extract agar media. At the end of incubation, inhibition zones formed around the discs were measured with transparent ruler in millimeters.

Determination of minimal inhibitory concentration (MIC)

A broth dilution susceptibility assay was used for the determination of the MIC (15). Briefly, bacterial strains were cultured overnight at 37 °C in nutrient agar; *Candida albicans* and *Aspergillus niger* were cultured overnight at 30 °C in yeast peptone dextrose agar and Czepak yeast extract agar, respectively. Bacterial and fungal strains were suspended in their corresponding broths to

give a final density of 10^6 and 10^5 organism/mL respectively. Dilutions of extracts 1, 2, 3 and 4 ranged from 1000 µg/mL to 0.05 µg/mL were prepared in capped tubes. A control was also served; 20 µL from each of the test organisms was used to inoculate the tubes. The tubes were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h for fungi. Tubes containing broth (2 mL) were inoculated with organisms and kept at +4 °C in a refrigerator overnight to be used as standards. The MIC was recorded as the lowest concentration at which no microbial growth was observed.

Viruses and cells

The origin of the viruses was as the following: herpes simplex virus-1 (strain KOS), herpes simplex virus-2 (strain G), vaccinia virus, vesicular stomatitis virus, herpes simplex virus-1 TK^- KOS ACV^r , coxsackie virus B-4, sindbis virus, punta toro virus, reovirus-1 (ATCC VR-230) and parainfluenza virus-3 (ATCC VR-93) (American Type Culture Collection, Rockville, Md.). The virus stocks were grown in human embryonic lung (HEL) cells (herpes simplex virus-1, herpes simplex virus-2, vaccinia virus, vesicular stomatitis virus and herpes simplex virus-1 TK^- KOS ACV^r), human epithelial (HeLa) cells (vesicular stomatitis virus and coxsackie virus B4) and Vero cells (parainfluenza-3 virus, reovirus-1, sindbis virus, coxsackie virus B4, and punta toro virus).

Antiviral assays

Confluent cell cultures in microtiter trays were inoculated with 100 $CCID_{50}$ (1 $CCID_{50}$ corresponding to the virus stock dilution that proved infective for 50% of the cell cultures). After 1 h of virus adsorption to the cells, residual virus was removed and replaced by cell culture medium (eagle minimal essential medium) containing 3% fetal calf serum and various concentrations of the test extracts (200, 100, 40, 20, 10, 4 µg/mL). Viral cytopathogenicity was recorded as soon as it reached completion in the untreated virus-infected cell cultures, *i.e*., at 1 to 2 days for vesicular stomatitis; at 2 days for coxsackie; at 2 to 3 days for herpes simplex types 1 and 2, and vaccinia; and at 6 to 7 days for reo and parainfluenza viruses. brivudin, ribavirin, acyclovir, gancyclovir and (S)-9-(2, 3-dihydroxypropyl) adenine were used as reference agents. Antiviral activity was expressed as minimal inhibitory concentration (MIC_{50}) required to reduce virus induced cytopathogenicity by 50% (within the micro tray well) (16).

Cytotoxicity

Although confluent cell cultures had not been infected, they were treated with various concentrations of the test extracts, which were incubated in parallel with the virus-infected cell cultures and examined microscopically at the same time as the viral cytopathogenicity was recorded for the virus-infected cell cultures. A disruption of the cell monolayer, *e.g*. rounding up or detachment of the cells, was considered as evidence for cytotoxicity. Cytotoxicity was expressed as minimal cytotoxic concentration (MCC) required causing a microscopically detectable alteration of normal cell morphology of the confluent cell cultures that were exposed to the test extracts.

Results and Discussion

Extracts 2 and 3 were found to be effective against both Gram-positive and Gram-negative organisms. They exhibited a broad array of antibacterial activity at 1mg/mL concentration. The MIC ranges of extracts 2 and 3 were found to be between 27 and 2 µg/mL and 105 and 2.1 µg/mL, respectively. Extract 1 showed activity only against *Escherichia coli* with MIC of 3.4 µg/mL. The result is presented in Table 1.

In the antifungal activity, extracts 1 and 2 were effective against both the screened fungi. Extract 1 had MIC of 4.6 and 5.1 µg/mL against *Aspergillus niger* and *Candida albicans*, respectively, whereas extract 2 possessed 0.09 and 3.9 µg/mL. Extract 3 showed activity only against *Aspergillus niger* with MIC of 0.55 µg/mL. The MICs of extracts against tested bacterial and fungal strains are depicted in Table 2.

As prerequisite for antiviral tests, the cytotoxicity of the extracts against virus cells was investigated. A moderate cytotoxicity was observed for extract 2 in HEL cells with MCC at ≥ 20 µg/mL. However, it was found to be non-toxic

Table 1. Antimicrobial activity of extracts 1, 2, 3 and 4.

Microorganisms	Zone of inhibition(mm)*						
	1	2	3	4	Ampicillin (25µg)	Clotrimazole (30µg)	DMSO
Staphylococcus aureus subsp. *aureus*	Nl	10	10	Nl	25	ND	Nl
Staphylococcus epidermidis	Nl	8	8	Nl	8	ND	Nl
Escherichia coli	12	12	9	Nl	16	ND	Nl
Psuedomonas aeruginosa	Nl	10	9	Nl	7	ND	Nl
Aspergillus niger	16	23	12	Nl	ND	25	Nl
Candida albicans	15	17	Nl	Nl	ND	17	Nl

*: values indicate sterile disc diameter (6 mm) and are mean of three replicates
DMSO : Dimethyl sulphoxide
ND : Not determined
Nl : No inhibition

to HeLa and Vero cells at 100 µg/mL. Extracts 1, 3 and 4 were also non-toxic to all the tested cell lines. Moderate activity (≥ 20 µg/mL) was shown by extracts 1 and 2 against all the viruses tested while rest of the extracts were not active (> 100 µg/mL). The results are shown in Table 3.

Cyclohexane and *n*-hexane extracts show moderate antiviral activity. Among them, cyclohexane extract possesses moderate cytotoxicity in HEL cells. The extracts only exhibited medium activity, because the bioactive compounds may be present in too low concentrations to show effective antiviral activity. Furthermore, the amount of active constituents present in the Brown alga depends on the geographical distribution, season of collection, climate, and ecological condition at the collection site.

n-hexane extract exhibits inhibitory activity only against *Escherichia coli* and Ethanol: Water (1:1) extract, thus it is ineffective against all the tested organisms. However, cyclohexane and methanolic extracts present a broad spectrum of antibacterial activity, while they are effective against *Staphylococcus aureus* subsp. *aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Psuedomonas aeruginosa*.

Cyclohexane and *n*-hexane extracts show an appreciable antifungal property. Among these two extracts, cyclohexane is more active than *n*-hexane with zone of inhibition of 23 mm and 17 mm against *Aspergillus niger* and *Candida*

Table 2. Minimum inhibitory concentration (µg/mL) of extracts 1, 2, 3 and 4.

Microorganisms	1	2	3	4	Ampicillin	Clotrimazole
Staphylococcus aureus subsp. *aureus*	ND	2.0	2.1	ND	0.15	ND
Staphylococcus epidermidis	ND	10.3	105	ND	5.0	ND
Escherichia coli	3.4	3.0	3.2	ND	0.2	ND
Psuedomonas aeruginosa	ND	27.0	13	ND	1.3	ND
Aspergillus niger	4.6	0.09	0.55	ND	ND	0.05
Candida albicans	5.1	3.9	ND	ND	ND	0.06

ND: Not determined

Table 3. Antiviral* and Cytotoxicity** of extracts 1, 2, 3 and 4.

Virus (strain)	Cell	1	2	3	4	BVDU[a] (µm)	Ribavirin (µm)	ACV[b] (µm)	GCV[c] (µm)
HSV-2 (G)	HEL	>20	>20	>100	>100	50	>250	0.4	0.032
Vaccinia	HEL	>20	>20	>100	>100	0.4	150	250	>100
Vesicular stomatitis	HEL	>20	>20	>100	>100	>250	>250	>250	>100
HSV-1 (TK-KOS ACVr)	HEL	>20	>20	>100	>100	10	>250	50	4
Cytotoxicity	HEL	100	≥20	>100	>100	>250	>250	>250	>100
								(S)-DHPA[d] (µm)	
Vesicular stomatitis	HeLa	>20	>20	>100	>100	>250	30	250	
Coxsackie B_4	HeLa	>20	>20	>100	>100	>250	150	>250	
Cytotoxicity	HeLa	100	100	>100	>100	>250	>250	>250	
Parainfluenza-3	Vero	>20	>20	>100	>100	>250	250	150	
Reovirus-1	Vero	>20	>20	>100	>100	>250	>250	250	
Sindbis	Vero	>20	>20	>100	>100	>250	>250	>250	
Coxsackie B4	Vero	>20	20	>100	>100	>250	>250	>250	
Punta Toro	Vero	>20	>20	>100	>100	>250	150	>250	
Cytotoxicity	Vero	100	100	>100	>100	>250	>250	>250	

*Minimum inhibitory concentration (µg/mL) required to reduce virus-induced cytopathogenicity by 50%.
**Minimum cytotoxic concentration (µg/mL) required to reduce to cause a microscopically detectable alteration of normal cell morphology.
Cell lines used: human embryonic lung (HEL), human epithelial (HeLa) and Vero cells.
[a] Brivudin [b] gancyclovir [c] acyclovir [d] (S)-9-(2, 3-dihydroxypropyl) adenine.

albicans, respectively.

Our results indicate that cyclohexane extract of *Turbinaria conoides* has moderate antiviral activity, cytotoxicity and broad spectrum of antibacterial property. This potentiality would seem to support the traditional claim as an antibacterial. It can also be concluded that cyclohexane extract (0.09 µg/mL) is as active as clotrimazole (0.05 µg/mL) against *Aspergillus niger.* The phytochemical characterization of the cyclohexane extract, the identification of the responsible bioactive compounds and the elucidation of the mode of action are necessary.

Acknowledgements

We would like to thank Dr.K.Eswaran for authentifying the Brown alga and are grateful to Professor Erik De Clercq for his support in antiviral research. We also thank I.T.S.Paramedical College (Pharmacy), Ghaziabad, U.P. for providing necessary facilities to carry out this research work.

References

(1) Hay ME. Predictable spatial escapes from herbivory: How do these affect the evolution of herbivore resistance in tropic marine communities? *Oecologia* (1984) 64: 396-407.
(2) Norris JN and Fenical W. Chemical defenses in tropical marine algae. *Smithsonian Cont. Mar. Sci.* (1982) 12: 417-431.
(3) Heltzel CE, Gunatilaka AA and Kingston DGI. Synthesis and structure-activity relationships of cytotoxic 7-hydroxysterols. *J. Nat. Prod.* (1994) 57:

620-628.

(4) Schroeder G, Rohmer M, Beck JP and Anton R. 7-oxo-, 7α-hydroxy- and 7β-hydroxy sterols form *Euphorbia fischeriana. Phytochem.* (1980) 19: 2213-2215.

(5) Sheu JH, Huang SY and Duh CY. Cytotoxic oxygenated desmosterols of the red algae, *Galaxaura marginata*. *J. Nat. Prod.* (1996) 59: 23-26.

(6) Sheu JH, Huang SY, Wang GH and Duh CY. Study on cytotoxic oxygenated desmosterols isolated from the red algae, *Galaxaura marginata*. *J. Nat. Prod.* (1997) 60: 900-903.

(7) Sheu JH, Liaw CC and Duh CY. Oxygenated clerosterols isolated from the marine algae, *Codium arabicum*. *J. Nat. Prod.* (1995) 58: 1521-1526.

(8) Sheu JH, Wang GH, Sung PJ, Chiu YH and Duh CY. Cytotoxic sterols from the formosan Brown alga, *Turbinaria ornata*. *Planta Med.* (1997) 63: 571-572.

(9) Sheu JH, Wang GH, Sung J and Duh CY. New cytotoxic oxygenated fucosterols from the Brown alga, *Turbinaria conoides*. *J. Nat. Prod.* (1999) 62: 224-227.

(10) Erdmann AM and Bason D. List of algal species in Komodo national park. In: Erdmann AM. (ed.) *A Natural History Guide to Komodo National Park.* The Nature Conservancy, Indonesia (2004) 207.

(11) Khandelwal KR. *Practical Pharmacognosy*. Nirali Prakashan, Pune (2004) 149-153.

(12) Bauer RW, Kirby MDK, Sherris JC and Turck M. Antibiotic susceptibility testing by standard single disc diffusion method. *Am. J. Clin. Pathol.* (1966) 45: 493-496.

(13) Eftehkar F, Nariman F, Habibi Z and Mohammadi N. Antibacterial activity of the aerial extracts from *Xanthium brasilicum. Iranian J. Pharm. Res.* (2007) 6: 65-8.

(14) Ankur P, Gokul T, Jitendra P and Manda S. Synthesis and antimicrobial activity of some new istatin derivatives. *Iranian J. Pharm. Res.* (2006) 4: 249-254.

(15) Lalitha MK. Methods of Antimicrobial Susceptibility Testing. *Manual Antimicrobial Susceptibility Testing.* 2004 Sep [cited 2007 Dec 10], 15 [47 screens]. Available from: URL: http://www.ijmm.org/document/Antimicrobial. doc.

(16) De Clercq E. Antiviral and antimetabolite activities of neplanocins. *Antimicrob. Agents Chemother.* (1985) 28: 84-89.

This article is available online at http://www.ijpr-online.com