ANTIHISTAMINIC, ANTICHOLINERGIC AND ANTIVIRAL ACTIVITIES OF FUCOSTEROL FROM *TURBINARIA CONOIDES* (J.AGARDH) KUTZING

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Summary

Fucosterol (Stigmasta-5,24(28)-dien-3-ol) was isolated from cyclohexane extract of *Turbinaria conoides* (J.Agardh) Kutzing. The structure was identified by comparing with the reported physical and spectral data of the compound. The antihistaminic and anticholinergic activities have been evaluated using *in vitro* standard animal models in comparison to chlorpheniramine maleate and pancuronium respectively. Evaluation of the potency (EC_{50}), affinity (pA_2) of the fucosterol and the maximal response (E_{max}) to the Histamine and acetylcholine were determined in the absence and presence of fucosterol. Antiviral activity and cytotoxicity were performed in human embryonic lung, human epithelial and Vero cells. Fucosterol showed antiviral activity against tested viruses with EC_{50} values ranging from >4 µg/mL to >20 µg/mL in the cells. Fucosterol inhibited histamine (97%) and acetylcholine (94%) induced contractions at 20 µg/mL, which were comparable to that of 10 µg/mL of chlorpheniramine maleate and pancuronium respectively. Thus Fucosterol springs up to be potent antihistaminic and anticholinergic compound.

Key words: Brown alga, *Turbinaria conoides*, fucosterol, antihistaminic, anticholinergic, antiviral.

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Introduction

Turbinaria conoides (J.Agardh) Kutzing commonly named Agar-agar Lesong is a brown alga belonging to the family Sargassaceae. It is distributed in Lakshadweep. The thallus is erect, 13 cm tall, dark brown in colour. 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].

A number of sterols have been isolated from the brown algae belonging to the genus Turbinaria. Oxygenated steroids of algae have been reported to possess cytotoxic properties [3, 4, 5, 6, 7]. The ethyl acetate extract of *Turbinaria conoides* and its oxygenated fucosterols have been reported for their cytotoxicity [8]. Traditionally, it is used to cure children's fever and as fertilizer, insect repellent and antibacterial [9]. By activity-guided fractionation of cyclohexane extract in the search for antimicrobial agents, the bioactive steroid was isolated from *Turbinaria conoides* and identified as Fucosterol [10].

Fucosterol was found to be the predominant sterol in brown seaweeds (83-97% of total sterol content; 662-2320 µg/g dry weight) [11]. Fucosterol was reported for its anti-oxidant and hepatoprotective [12], anti-diabetic [13], butyrylcholinesterase inhibitory activities [14], which was isolated from the other algal species. Regardless of the number of studies on the properties of Fucosterol, there are no data in the literature concerning antihistaminic, anticholinergic and antiviral properties in human embryonic lung, human epithelial and Vero cells. Thus the aim of the present investigation was to isolate Fucosterol from *Turbinaria conoides* and screen for its comprehensive pharmacological activity.

Materials and methods

Algal material

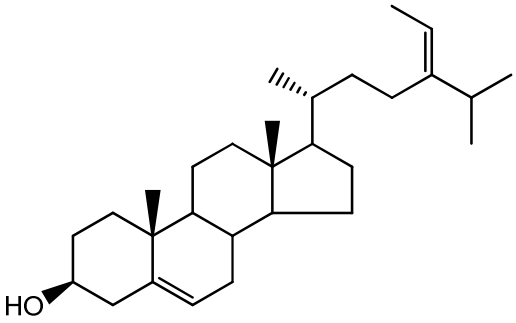
Turbinaria conoides was collected in September 2005 from Salin Munthal, Gulf of Mannar, Bay of Bengal, Ramanathapuram district, Tamil Nadu, India and voucher specimen was deposited at Marine algal research station, Mandapam camp, Tamil Nadu, South India. It was authenticated by K.Eswaran, Scientist, Marine algal research station, India.

General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on a Bruker av500 instrument in $CDCl_3$. EI-MS analyses were performed on a JEOL GCmate spectrometer. UV spectra were recorded on Systronics 2202 UV/Vis spectrophotometer. IR spectra were obtained using Jasco 4100 Fourier Transform Infrared spectrometer. All the chemicals were of analytical grade. Chromatographic separations and TLC were carried out using silica gel (Qualigens, GlaxoSmithKline Pharmaceuticals Limited, India) and silica gel 60 F_{254} precoated plates (Merck KGaA, Dermstadt, Germany).

Isolation and Identification of Fucosterol

Chromatographic fractionation of cyclohexane extract of *Turbinaria conoides* yielded Fucosterol. The compound was obtained by eluting the extract with cyclohexane: ethyl acetate (20:1) followed by re-crystallizing the crude from methanol. Spectral data, particularly ^{1}H NMR and ^{13}C NMR for the Fucosterol were in agreement with the values in the literature [15].



Fucosterol

Animal approval and drugs

The study was carried out with the prior approval from the Institutional Animal Ethical Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) [290/CPCSEA-12//12/2000/PH-07-10].

Chlorpheniramine maleate (Schering), Pancuronium (MERCK), Histamine dihydrochloride (Sigma) and Acetylcholine chloride (Sigma). All drugs were dissolved in 2% carboxy methyl cellulose. The *in vitro* antihistaminic and anticholinergic activities were carried out using isolated guinea pig ileum and frog rectus abdominus methods respectively [16, 17].

Antihistaminic activity

24 h fasted male guinea pigs weighing 250±50g were sacrificed by a blow to base of skull and cervical dislocation. 2 cm pieces of the ileum were dissected from ileum segment and placed in 30 mL baths containing aerated Tyrode solution (NaCl, 136.7; KCl, 2.68; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $CaCl_2$, 1.80; $NaHCO_3$, 11.90; Glucose, 5.55mM) at 37°C. The air was bubbled through organ bath, an initial tension of 0.5 g was applied and the preparation was allowed to equilibrate for 30 min. Chlorpheniramine maleate (10µg/mL) was used as reference antihistaminic agent. Test compound, Fucosterol was tested in the doses of 1, 2, 4, 8, 16, 20 µg/mL. Increasing concentration of histamine was added to the bath and graded concentration responses of histamine were constructed on kymograph. The heights of the contractions of histamine were determined and dose-response curves were plotted for all the recordings. The % inhibition of contraction induced by each dose of the compound was calculated. The potency (EC_{50}), affinity (pA_2) and efficacy (E_{max}) of the histamine alone and in the presence of compound were extrapolated from the graphs [18, 19].

Anticholinergic activity

Rectus abdominus muscle of the frog (*Rana Hexa Dactyla*) weighing between 175 and 190 g was dissected out humanly and divided longitudinally. Two cm long segment of

muscle was mounted in 10ml capacity tissue bath containing Ringer solution maintained at room temperature and aerated with a mixture of 5% carbon dioxide and 95% oxygen (carbogen). A preload of 1g was applied to the tissue. After equilibration for 30 min, acetylcholine was added subsequently in graded doses to the bath, from the lowest dose to determine the sensitivity of the rectus abdominus muscle. The threshold value is the concentration at which the strip of muscle recorded the first contraction, whereas the maximal response is the concentration of acetylcholine at which further increases in dose does not produce higher height of contractions. Using the dose range between the threshold value and the maximal response, contractile response of the rectus muscle to the acetylcholine was recorded by kymograph. After a dose response curve for the acetylcholine had been established, the same preparation was in turn exposed to test compound, fucosterol and predetermined dose of $1x10^{-5}$ M Pancuronium, which was used as positive control.

Statistics

The values were recorded as Mean ± S.E.M. The test of significance of the difference of the means was determined by Student's t-test [20]. The % inhibition in contractile response by the test compound and standard drug was compared.

Cells and virus

The origin of the viruses was as follows: 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 assay

Confluent cell cultures in micro titer 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 [21]. 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 compound ranging from 200 µg/ml to 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 positive controls. Antiviral activity was expressed as 50% effective concentration (EC_{50}) required to reduce virus-induced cytopathogenicity by 50%.

Cytotoxicity

Confluent cell cultures which had not been infected but were treated with various concentrations of the test extracts were incubated in parallel with the virus-infected cell cultures and examined microscopically at the same time as 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 minimum cytotoxic concentration (MCC) required to cause a microscopically detectable alteration of normal cell morphology of the confluent cell cultures that were exposed to the Fucosterol.

Results

Antihistaminic and Anticholinergic activities

Histamine and acetylcholine elicited contractile responses from the guinea pig ileum and frog rectus abdominus muscle respectively. The dose response curve obtained for the contractile effect of histamine and acetylcholine shifted to the right in the presence of test compound, Fucosterol and reference drugs. An insignificant inhibitory effect of fucosterol on the guinea pig ileum and frog rectus abdominus was obtained at 4 µg/mL and 8 µg/mL respectively; while the significant inhibitory response was attained at 20 µg/mL of fucosterol on both the muscles ($p<0.01$).

The potency of histamine and acetylcholine induced contractions of isolated smooth and skeletal muscles decreased when pretreated with fucosterol ($p<0.05$). They were exhibited by significant increase in the mean EC_{50} values of 1.28±0.04 M and 1.66±0.02 M for histamine and acetylcholine alone to 2.36±0.13 M and 2.73±0.16 M respectively in the presence of fucosterol. There were decreases from the mean pA_2 values of 0.78±0.12 M and 0.82±0.08 M obtained for histamine and acetylcholine alone to 0.33±0.10 M and 0.39±0.14 M in the presence of fucosterol, the difference of the means is statistically significant ($p<0.01$). The mean E_{max} values of histamine and acetylcholine only were 98±3.48 % and 91±2.11 %. The maximal contractile response of ileal smooth muscle to histamine was reduced significantly in the presence of fucosterol to 84±4.23 % ($p<0.01$), whereas the maximal contractile response of the frog rectus abdominus to acetylcholine was slightly reduced by fucosterol to 88±2.42 % ($p<0.05$) (Table 1) (Figures 1 and 2).

Table 1. Pharmacodynamic indices for the effect of histamine and acetylcholine alone and in the presence of Fucosterol

Parameters	Histamine only	Histamine +Fucosterol	Acetylcholine only	Acetylcholine +Fucosterol
EC_{50} (M)	1.28±0.04	2.36±0.13*	1.66±0.02	2.73±0.16*
pA_2 (M)	0.78±0.12	0.33±0.10**	0.82±0.08	0.39±0.14**
E_{max} (%)	98±3.48	84±4.23**	91±2.11	88±2.42*

Values represent the Mean±S.E.M. *$p<0.05$; **$p<0.01$.

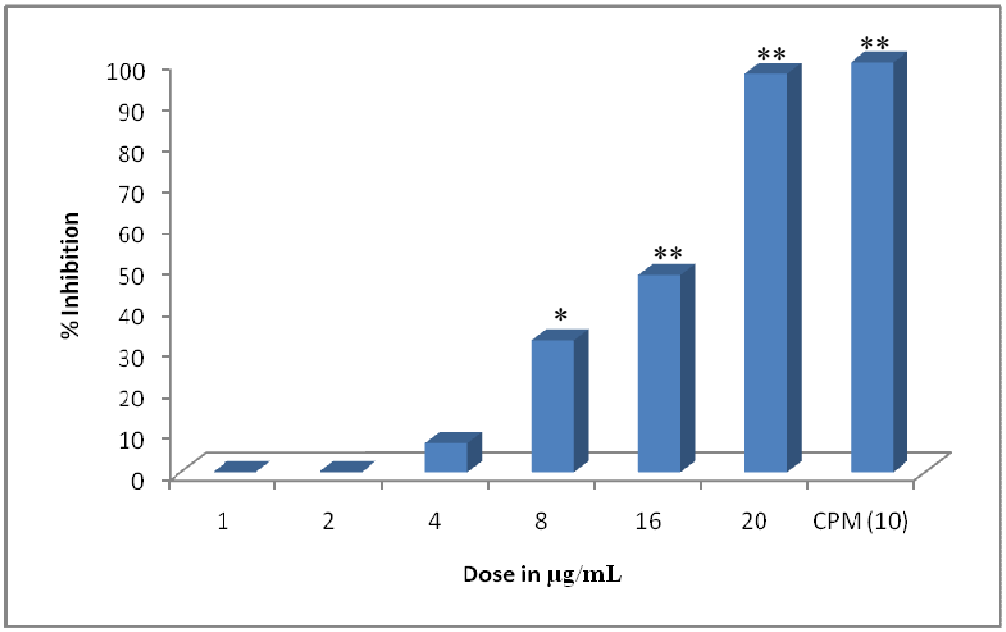


Figure 1. Inhibitory effects of Fucosterol (1, 2, 4, 8, 16 and 20 µg/mL) and Chlorpheniramine maleate (CPM) on the contraction induced by histamine. Each value shows the Mean ± S.E.M. *p<0.05; **p<0.01

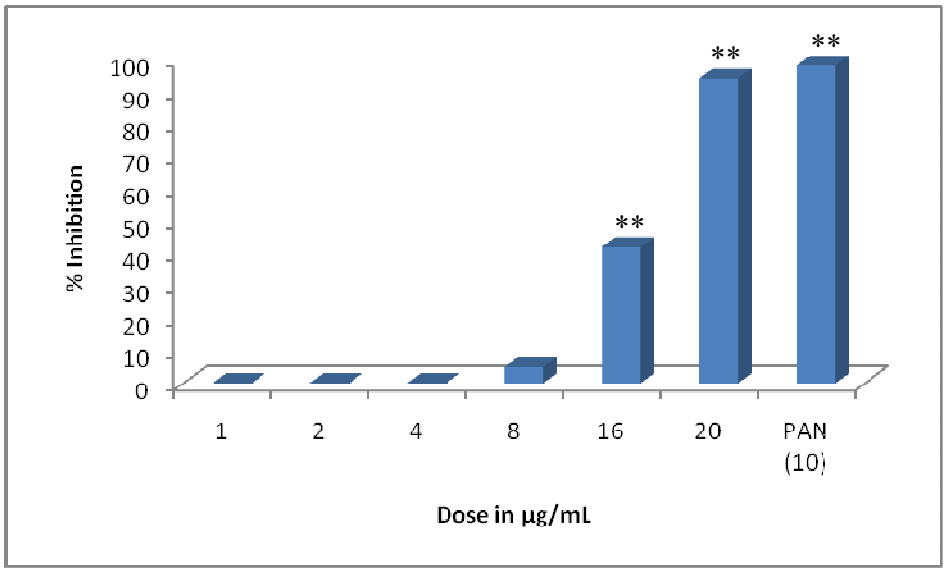


Figure 2. Inhibitory effects of Fucosterol (1, 2, 4, 8, 16 and 20 µg/mL) and Pancuronium (PAN) on the contraction induced by acetylcholine. Each value shows the Mean ± S.E.M. *p<0.05; **p<0.01

Table 2: Summaries of Antiviral activity* and Cytotoxicity of Fucosterol**

Virus (strain)	Cell	Fucosterol	BVDU[a] (µm)	Ribavirin (µm)	ACV[b] (µm)	GCV[c] (µm)
HSV-1 (KOS)	HEL	>4	0.08	>250	0.4	0.032
HSV-2 (G)	HEL	>4	50	>250	0.24	0.032
Vaccinia	HEL	>4	1.2	250	250	100
Vesicular stomatitis	HEL	>4	>250	250	>250	>100
HSV-1 (TK^- KOS ACV^r)	HEL	>4	50	>250	50	4
Cytotoxicity	HEL	20	>250	>250	>250	>100
					(S)-DHPA[d] (µm)	
Vesicular stomatitis	HeLa	>20	>250	30	150	-
Coxsackie B_4	HeLa	>20	>250	150	>250	-
Cytotoxicity	HeLa	100	>250	>250	>250	-
Parainfluenza-3	Vero	>20	>250	150	250	-
Reovirus-1	Vero	>20	>250	150	>250	-
Sindbis	Vero	>20	>250	150	>250	-
Coxsackie B_4	Vero	20	>250	>250	>250	-
Punta Toro	Vero	>20	>250	150	>250	-
Cytotoxicity	Vero	100	>250	>250	>250	-

*50% Effective 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] acyclovir [c] gancyclovir [d] (S)-9-(2, 3-dihydroxypropyl) adenine

Antiviral and cytotoxicity

The antiviral and cytotoxicity results are summarized in (Table 2). Compounds **1**, **2** and **3** showed antiviral activity against tested viruses with EC_{50} values ranging from >4 µg/mL to >20 µg/mL. Compound **2** only possessed at >4 µg/mL with MCC value of 20 µg/mL in HEL cells. Compounds **1** and **3** showed cytotoxicity with MCC value of 100 µg/mL in HEL, HeLa and Vero cells (Table 2).

Discussion

Fucosterol has been proved to possess a varied pharmacological activity, nevertheless there is no data in the literature regarding inhibitory effects on histamine and acetylcholine induced contractions on the guinea pig ileum and frog rectus abdominus muscles, antiviral effects on HEL, HeLa and Vero cells as well. In this study, it was observed that exposure of the ileum and rectus abdominus to fucosterol (1-20 µg/mL) reduced the contractile response of acetylcholine and histamine in a dose-dependent manner. Fucosterol has no specific antiviral effects (i.e. minimal antivirally effective concentration) against any of the tested viruses. The findings obtained from this study shows that spasmogens produced dose dependent contraction on the smooth and skeletal muscles. Fucosterol produced a shift to the right on the dose-response curve. There were also significant increases in the potency, affinity of fucosterol and decrease in the maximal contractile response to the spasmogens, in the presence of fucosterol. These show that histamine and acetylcholine contractions are fucosterol sensitive, thereby suggesting that the contractions are mediated by H1 histaminergic and cholinergic receptors. Antihistaminics are used in symptomatic treatment of rhinitis, sneezing, rhinorrhoea, basal itch etc. However, many of them produce drowsiness and CNS depression. H1 antagonists tend to inhibit responses to histamine (antihistaminic activity) that are mediated by muscarinic receptors. The histamine induced contraction on isolated guinea pig ileum was inhibited by fucosterol at 20 µg/mL (97%), signifying its potent antihistaminic property. It also inhibited (94%) the acetylcholine induced contraction on frog rectus abdominus muscle at the same concentration. Pancuronium combines with the cholinoceptive sites and blocks the transmitter action of the acetylcholine, therefore, mechanism of the action of the compound may be due to the action on the muscarinic receptors, thereby blocking the transmitter action of acetylcholine. In conclusion, Fucosterol shows significant antihistaminic and anticholinergic activities, which could be comparable to that of reference drugs. This potentiality demonstrates that it is a treasure house for future pharmacological research.

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