In vitro and *in silico* studies on the anticancer and apoptosis-inducing activities of the sterols identified from the soft coral, *subergorgia reticulata*

Kuniyil Byju, Vattoni Anuradha, Gopalakrishnapai Vasundhara, S. Muraleedharan Nair, N. Chandramohana Kumar

Inter University Centre for Development of Marine Biotechnology, Cochin University of Science and Technology, Cochin, India

Submitted: 13-04-2013 Revised: 18-04-2013 Published: 21-02-2014

ABSTRACT

Background: Gorgonians and other octocorals are known to possess a huge array of secondary metabolites in which sterols are the major group of secondary metabolites apart from sesquiterpenes and diterpenes, and the bioactive metabolites could show marked biomedical potential for future drug discovery. **Objective**: This study was intended for the isolation and identification of sterols from the octocoral *Subergorgia reticulata* and to evaluate the anticancer and apoptosis-inducing activities of the identified sterols through *in vitro* and *in silico* approach. **Materials and Methods**: The organism was collected from Lakshadweep Island. The isolated sterols were identified using Gas chromatography-mass spectrometry (GC-MS). The structure was confirmed by using comparison of their spectra those in National Institute of Standard Technology (NIST) library. The apoptosis inducing effect of identified sterols were determined by PASS online prediction. *In vitro* cytotoxity studies were carried out using Dalton's lymphoma ascites cells (DLA) and the cell viability was determined by trypan blue exclusion method. **Results**: Six sterols were identified from the soft coral *S. reticulata.* They are Cholesta-5,22-diene-3ol (3β), Ergosta-5-22-dien-3ol (3β,22E 24S), Cholesterol, 26,26-Dimethyl-5,24(28)-ergostadien-3β-ol. β-sitosterol, and Fucosterol. *In silico* predictions showed that the identified sterols exhibited remarkable apoptosis agonist activity. The probability of apoptosis agonist activity were found maximum for 26,26-Dimethyl-5,24 (28)-*S. reticulata* sterol fractions isolated were found to be having anticancer activity. **Conclusions**: These findings suggest that *S. reticulata* contained biologically active sterol compounds that may be useful in the treatment of cancer.

Key words: Apoptosis, cytotoxicity, Prediction of activity spectra for substances PASS, sterols, *Subergorgia reticulata*

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.127345

Quick Response Code:

INTRODUCTION

The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural and chemical features not found in terrestrial natural products.[1] There are more than 6,100 species all over the world, in which corals of the class Anthozoa in the phylum of Coelenterata are the most dominant benthic invertebrates living mainly in tropical seas.[2] Cnidaria, formerly known as Coelenterata, is one of the largest among the phyla.[1] The phylum Cnidaria possesses an array of secondary metabolites, mainly terpenes, and the soft coral group possesses more than 80% of all cnidarian compounds.[3,4] In the marine environment, the success of defense used by soft corals and gorgonians against consumers and competitors has been attributed to their production of secondary metabolites, many of which show predator deterrence and allelopathic activities.[5]

Sterols are the major group of secondary metabolites characterizing corals next to sesquiterpenes and diterpenes.[6] Sterols advocate their dietary inclusion as an important strategy in prevention and treatment of cancer.[7] The "usual" sterols have a 3β-hydroxy-Δ^5- (or Δ^0-) cholestane nucleus and a C_8-C_{10} side chain. There are over 200 such sterols, occurring in marine organisms as complex inseparable mixtures, and their identification is usually done by Gas chromatography-mass spectrometry (GC-MS).

Address for correspondence:
Prof. S. Muraleedharan Nair, Inter University Centre for Development of Marine Biotechnology, Cochin University of Science and Technology, Cochin - 682 016, India.
E-mail: unnimurali@yahoo.com

Mounting evidence suggests that phytosterols possess anti-cancer effects[8] against cancer of the lung,[9] stomach,[10] ovary[11] and estrogen-dependent human breast cancer.[12] It has been speculated that phytosterols inhibit the production of carcinogens, cancer-cell growth, invasion and metastasis, and promote apoptosis of cancerous cells.[13]

Among the genus *Subergorgia*, very limited studies were reported on the chemical constituents of *S. reticulata*. Presence of few known polyhydroxylated steroids, among which some exhibited cytotoxic activity, were reported from *S. reticulata*[14] Yang *et al.*, (2006)[15] isolated nine compounds including three sterols and some alkaloids from *S. reticulata*. Apart from this, some diterpenoids and sesquiterpenoid compounds were also reported from *S. reticulata*.[15-17] The present study was aimed for the isolation and identification of steroids from the *S. reticulata* collected from Lakshadweep Island, Kavaratti and also for the tracing of the anticancer and apoptosis-inducing activities of the isolated steroids using *in vitro* and *in silico* methods.

MATERIALS AND METHODS

Collection of coral

The soft corals were collected from Lakshadweep Islands during December 2011. The collection was carried out by Scuba diving in the region at 25 m depth. The soft coral were collected in a glass bottle and brought to the shore, washed with fresh water free from detectable other adhering organisms like algae, cut into thin slices, and preserved in methanol in a glass bottle. A large specimen with clear morphological features was separately preserved in ethanol in small glass container for identification purpose. The organisms [Figure 1] were brought to the laboratory for processing.

Identification

The soft corals were identified as *S. reticulata*, by Dr. P.A. Thomas, Emeritus Scientist (ICAR). The voucher specimen (IUCDMB.R.No. 3) of the soft corals was preserved in the Inter University Centre for Development of Marine Biotechnology, Cochin University of Science and Technology. Taxonomical Details-Kingdom: Animalia; Phylum: Cnidaria; Class: Anthozoa; Subclass: Octacorallia; Order: Alcyonacea; Sub order: Scleraxonia; Family: Subergorgiidae; Genus: *Subergorgia*.

Extraction, isolation and purification

The extraction of the fragmented organism, *S. reticulata* (400 gm), was carried out at room temperature with methanol for 4 days. The process of extraction was repeated until, it left negligible residue on removal of the solvent. The solvent was removed under reduced pressure.

Figure 1: The soft coral *Subergorgia reticulata*

The residue was dissolved in minimum quantity of aqueous methanol. In an effort to further characterize the chemical constituents in the original sample, we fractionated the aqueous methanol sample by solvent partitioning with hexane. The hexane extracts were combined, concentrated under vacuum and the hexane soluble portion was washed with water, dried over anhydrous $MgSO_4$ and the solvents removed under reduced pressure. Column chromatography of the residue collected from hexane soluble fraction was done using silica gel with hexane: ethyl acetate on varying polarity. The fractions were collected and analyzed by Thin Layer Chromatography (TLC) and GC-MS. The fraction of 5% ethyl acetate was a mixture of six sterols.

GC-MS analysis

Gas chromatographic analysis was done on Perkin Elmer Clarus 680 GC-MS equipped with headspace sampler. Helium was employed as carrier gas and the ionizing voltage was 70 eV. Oven temperature was programmed from 60°C to 290°C at 10°C min^{-1}, where it remained constant for 15 min. Injector and detector temperature were kept constant at 280°C and 290°C, respectively. The column used was Elite 5MS having 30 m length and 250 μm id. Mass spectrum of each peak in the total ion chromatogram was resolved, and it was compared with National Institute of Standards and Technology (NIST) library spectra for the identification of the compounds.

Apoptosis inducing effect of identified sterols

Apoptosis inducing effect of the identified sterols from *S. reticulata* was studied using Prediction Activity Spectra of Substances (PASS). PASS is a software product designed as a tool for evaluating the general biological potential of an organic drug-like molecule. PASS provides simultaneous predictions of many types of biological activity based on the structure of organic compounds. Thus, PASS can be used to estimate the biological activity profiles for virtual molecules, prior to their biological testing.[18] It was found

that the apoptosis inducing effect of identified sterols is prominent compared to other biological activity predicted by the PASS software.

In vitro cytotoxicity study

In vitro cytotoxicity studies were carried out using Dalton's lymphoma ascites cells (DLA). The tumor cells aspirated from the peritoneal cavity of tumor bearing mice were washed thrice with phosphate buffered saline. Cell viability was determined by trypan blue exclusion method. Viable cell suspension (1×10^6 cells in 0.1 ml) in phosphate buffered saline (PBS) was used. Control tube contained only cell suspension. These assay mixtures were incubated for 3 hours at 37°C. Further, cell suspension was mixed with 0.1 ml of 1% trypan blue and kept for 2-3 min and loaded on a hemocytometer. Dead cells take up the blue color of trypan blue while live cells do not take up the dye. The numbers of stained and unstained cells were counted separately. Five different concentrations of the sterol fraction were prepared, respectively, as 10 μg ml^{-1}, 20 μg ml^{-1}, 50 μg ml^{-1}, 100 μg ml^{-1}, and 200 μg ml^{-1}.

$$\text{Percentage of dead cell} = \frac{\text{Number of dead cells}}{\text{Number of viable cells} + \text{Number of dead cells}} \times 100$$

RESULTS

Silica gel chromatography of the crude residue (4 g) obtained from hexane soluble fraction was done using hexane: ethyl acetate solvent composition. GC-MS analyses of the fractions collected were carried out. From the 5% ethyl acetate fraction, we identified six sterols by comparison of their spectra with those in National Institute of Standard Technology (NIST) library (version 2.2). The total ion chromatogram obtained was shown in Figure 2 and the respective mass spectra of the isolated sterols are shown in Figure 3. The sterols are 26,26-dimethyl-5,24 (28)-ergostadien-3β-ol, β-sitosterol, cholesta-5,22-diene-3ol (3β), cholesterol, ergosta-5-22-dien 3βol (3 β,22E 24S), and fucosterol. Cholesta-5,22-diene-3ol (3β) was the maximum yielded compound by area percentage followed by ergosta-5-22-dien 3βol (3 β,22E 24S), cholesterol, 26,26-dimethyl-5,24 (28)-ergostadien-3β-ol, β-sitosterol, and fucosterol. The structures of the sterols were shown in Figure 4.

The probability of apoptosis inducing effect of identified sterols from *S. reticulata* was shown in Figure 5. The apoptosis agonist activity was found to be maximum for 26,26-Dimethyl-5, 24 (28)-ergostadien-3β-ol (80.3) followed by Cholesta-5, 22-diene-3-ol (3β), Cholesterol, Ergosta-5-22-dien 3-ol (3β,22E 24S), Fucosterol, and β-sitosterol. Cholesta-5, 22-diene-3ol (3β) is prominent among the five sterols identified, and the apoptosis agonist activity was found to be 0.773.

The 5% ethyl acetate (steroid) fraction collected after the column chromatography of crude methanolic extract residue was concentracted under vacuum and the white residue obtained was weighed, five different concentrations were prepared, respectively, as 10 μg ml^{-1}, 20 μg ml^{-1}, 50 μg ml^{-1}, 100 μg ml^{-1}, and 200 μg ml^{-1} in Dimethyl sulfoxide (DMSO). The sterol mixture was studied for short term *in vitro* cytotoxicity. The graph showing the variation of cell death of tumor cells on increase in concentrations of the sterol fraction is represented in Figure 6. The study resulted in significant cell death, it was very clear that the number of dead cells was increased when the concentration of the sterol fraction was increased. Ninety percent cell death was obseverd at 200 μg ml^{-1} concentration of the sterol fraction. The results are summarized in Table 1.

DISCUSSION

This is the first time reporting the four sterols cholesta-5, 22-diene-3ol (3β), ergosta-5-22-dien 3ol (3β,22E 24S), 26,26-dimethyl-5,24 (28)-ergostadien-3β-ol, and β-sitosterol

Table 1: Anticancer activity of sterol fraction from *Subergorgia reticulata*

Concentration of sterol fraction in μg ml^{-1}	Cell death (DLA) %
200	90
100	84
50	65
20	42
10	32

DLA: Dalton's lymphoma ascites

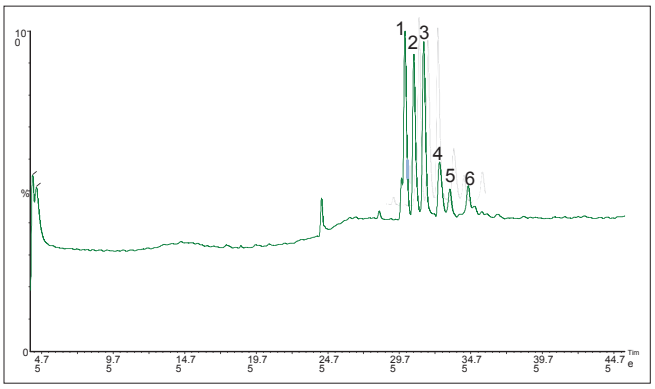

Figure 2: Total ion chromatogram of the sterol fraction isolated from *Subergorgia Reticulata* (1. Cholesta-5, 22-diene-3ol (3β), 2. Cholesterol, 3. Ergosta-5-22-dien-3-ol (3β,22E 24S), 4. 26,26-Dimethyl-5,24 (28)-ergostadien-3β-ol, 5. β-sitosterol, and 6. Fucosterol)

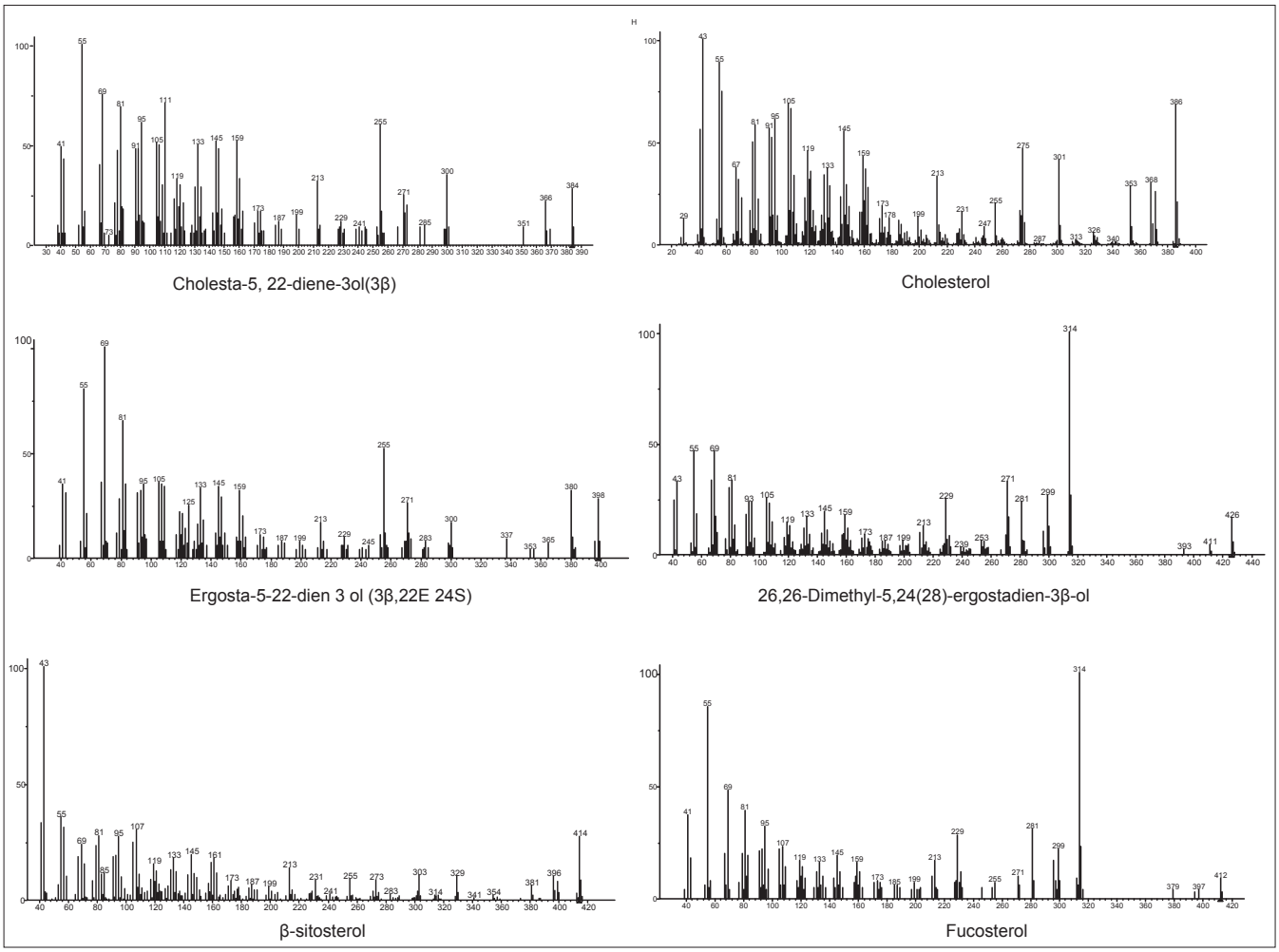

Figure 3: Mass spectrum of sterols isolated from *Subergorgia reticulata*

from the soft coral *S. reticulata*. From the *in vitro* studies, it is evidenced that almost a saturation value (>85%) was attained at slightly above 100 μg ml^{-1} concentration of the sterol fraction. The anticancer activities of sterol fraction isolated from *S. reticulata* can be due to a natural mixture of its components, and a single constituent may not have an activity greater than that of the mixture. Individually, cholesterol and β-sitostrol were reported for their potential for apoptosis agonist. β-sitosterol is generally considered as a phytosterol with chemical structure similar to that of cholesterol and is the most common sterol in human diet. Studies have indicated that β-sitosterol can inhibit the growth of various cultured cancer, and the simulation of apoptotic cell death.[19-21] From the PASS prediction, it is inferred that though β-sitosterol exhibited apoptosis agonist activity as a natural component in cancer prevention agreeing with findings of Award *et al.*, (1996)[22] it is found to be the lowest among the sterols studied from *S. reticulata*.

The apoptosis agonist activity was maximum for 26,26-Dimethyl-5,24 (28)-ergostadien-3 β-ol and the probability of apoptosis inducing effect of all other sterols studies were found to be greater than 70%. There is no proven report for supporting 26,26-Dimethyl-5,24 (28)-ergostadien-3 β-ol, ergosta-5-22-dien 3-ol (3β,22E 24S), fucosterol, and Cholesta-5, 22-diene-3-ol (3β) that these are apoptosis agonist. It has been reported that intracellular cholesterol accumulation induces apoptosis of pancreatic cells,[23] it also support the results with our findings that inducing effect of cholesterol is prominent with a probability of 0.77. Fucosterol is the most abundant phytosterol in brown algae and it was proved that fucosterol containing fraction of marine algae responsible for cytotoxic effect against breast and colon carcinoma cell lines.[24] There is no evidence to prove fucosterol itself can induce apoptosis, but the PASS study conducted here have supported the findings of Khanavi *et al.*,(2012)[24] by predicting the probability of apoptosis inducing effect of fucosterol as 0.763.

Yang *et al.*, (2006)[15] reported the presence of cholesterol and isofucosterol from the South China Sea gorgonian

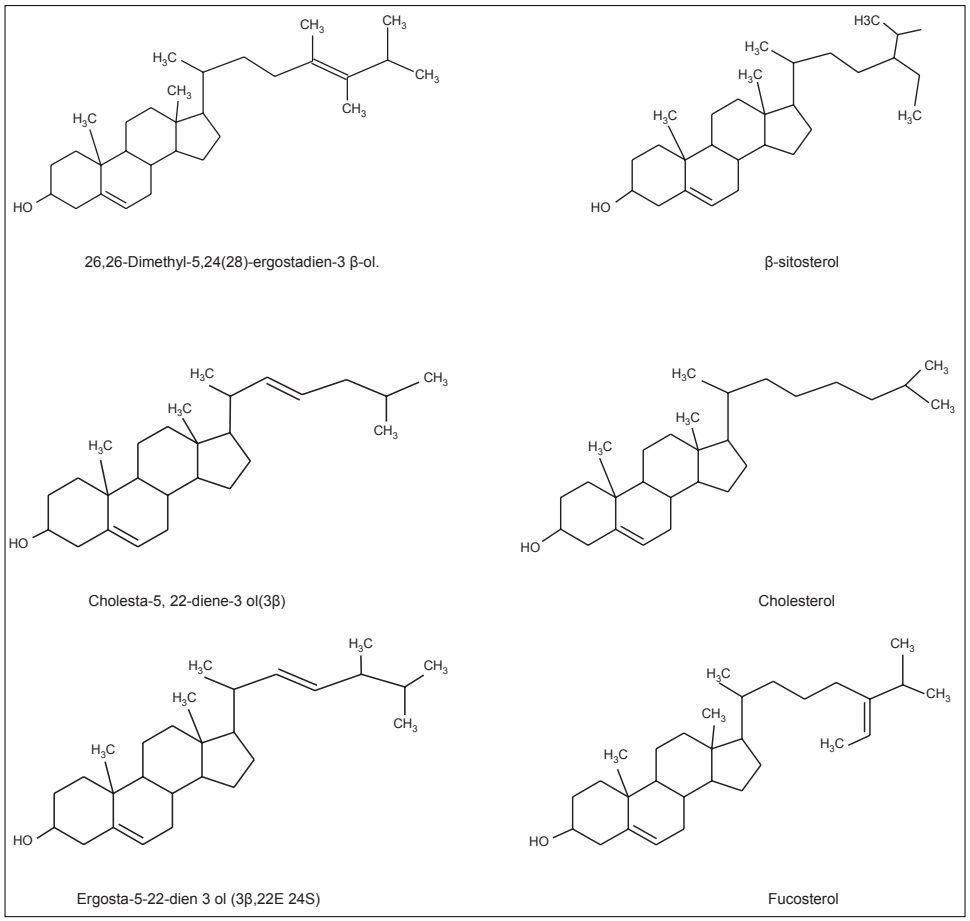

Figure 4: The structures of identified sterols from *Subergorgia reticulata*

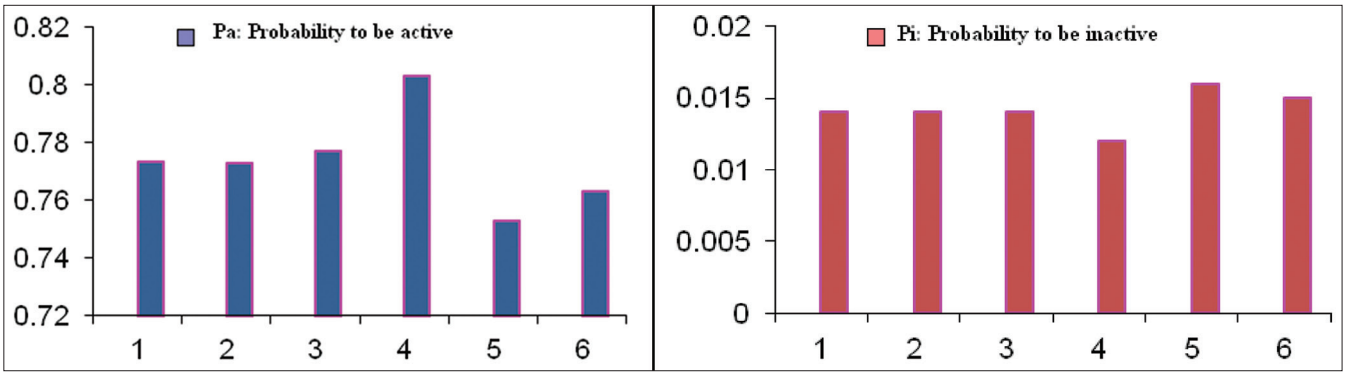

Figure 5: Apoptosis inducing effect of the six identified sterols (1. Cholesta-5, 22-diene-3-ol (3β), 2. Ergosta-5-22-dien 3-ol (3β,22E 24S), 3. Cholesterol, 4. 26, 26-Dimethyl-5, 24 (28)-ergostadien-3β-ol, 5. β-sitosterol, and 6. Fucosterol)

coral *S. reticulata* with seven other compounds. Li *et al.*,(2005)[25] and Hsu *et al.*,(2005)[26] investigated that anticancer activity of some therapeutic substances is involved in the induction of apoptosis which can be used for cancer control. This is in accordance with our findings that apoptosis induction of these sterol molecules found to be anticancer agents. Induction of apoptosis in cancer cells is one useful strategy for anticancer drug development.[27]

It has been realized well in case of octocorals that *de novo* biosynthesis is an important mechanism.[28] However, the alternative possibility must be considered that they are able to absorb sterols from food which may well comprise of largely plankton and small crustacean species that are rich in sterols.[29,30] Since soft corals are associated with plankton and micro organisms, the contribution of sterols from their part cannot be omitted. Biosynthesis of sterol from this soft coral, *S. reticulata* is yet to be investigated[31] and it

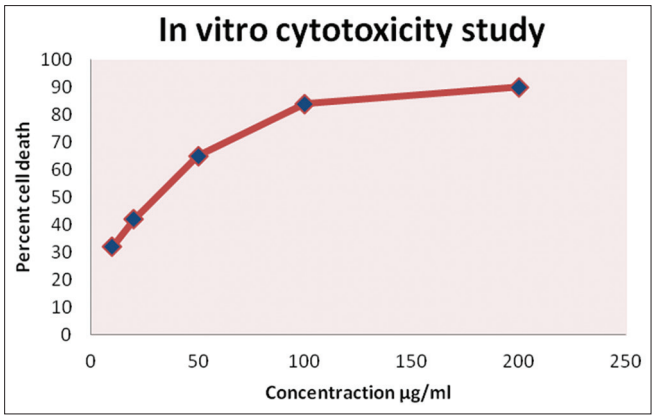

Figure 6: Effect of the application of the sterol fraction from S. reticulata on the tumor cells. Cell viability was determined by trypan blue exclusion method

could be beneficial to know the sterol pathway to provide additional clues to understand its role in reproduction, chemical signaling or as defensive metabolite.

CONCLUSIONS

The apoptosis inducing effects and *in vitro* cytotoxicity studies of identified sterols from the soft coral *S. reticulata* were investigated. A significant apoptosis inducing effect was observed for each compound and it is the primary screening of pharmacological potential of sterols that we had identified. However, the determination of apoptosis by *in silico* may be considered as an indirect method, comparing to those performed in tissues. The cytotoxic results of this sterol compounds mixture confirm it as a natural potent chemopreventive and chemotherapeutic agent. In contrast, this apoptosis inducing effect was apparent when measured using PASS, online prediction assay give us an idea about the possibility for the development of an anticancer drug, and still more *in vivo* studies are required to prove the potentiality for an anticancer drug from the marine soft coral *S. reticulata*.

REFERENCES

1. Williams G, Cairns SD. Systematic List of Valid Octocoral Genera, [serial on the Internet]. 2013. Available from: http://www.calacademy.org/research/izg/OCTOCLASS.htm [Last accessed on 2013 Feb 23].
2. Zhang W, Guo YW, Ernestoto M, Guido C. Chemical studies on sesquiterpenes in soft coral *Lobophytum* sp. from the South China Sea. Nat Prod Res Dev 2005;17:740-3.
3. Harper MK, Bugni TS, Copp BR, James RD, Lindsay BS, Richardson AD, *et al*. Introduction to the chemical ecology of marine natural products. In: Mcclintock JB, Baker BJ, editors. Mar Chem Ecol, Boca Raton, FL: C.R.C. Press; 2001. p. 3-69.
4. Blunt TW, Copp BR, Hu WP, Munro MH, Northcotec PT, Prinsepd MR. Marine natural products. Nat Prod Rep 2007;24:31-86.
5. Changyun W, Haiyan L, Changlun S, Yanan W, Liang L, Huashi G. Chemical defensive substances of soft corals and gorgonians. Acta Ecol Sin 2008;28:2320-8.
6. Coll JC. The chemistry and chemical ecology of octocorals (Coelenterata, Anthozoa, Octocorallia). Chem Rev 1992;92:613-31.
7. Woyengo TA, Ramprasath VR, Jones PJ. Anticancer effects of phytosterols. Eur J Clin Nutr 2009;63:813-20.
8. Choi JM, Lee EO, Lee HJ, Kim KH, Ahn KS, Shim BS, *et al.* Identification of campesterol from Chrysanthemum coronarium L and its antiangiogenic activities. Phytother Res 2007;21:954-9.
9. Mendilaharsu M, De Stefani E, Dene Pellegrini H, Carzoglio J, Ronco A. Phytosterols and risk of lung cancer: A case-control study in Uruguay. Lung Cancer 1998;21:37-45.
10. De Stefani E, Boffetta P, Ronco AL, Brennan P, Deneo-Pellegrini H, Carzoglio JC, *et al.* Plant sterols and risk of stomach cancer: A case-control study in Uruguay. Nutr Cancer 2000;37:140-4.
11. McCann SE, Freudenheim JL, Marshall JR, Graham S. Risk of human ovarian cancer is related to dietary intake of selected nutrients, phytochemicals and food groups. J Nutr 2003;133:1937-42.
12. Ju YH, Clausen LM, Allred KF, Almada AL, Helferich WG. β-Sitosterol, β-sitosterol glucoside, and a mixture of β-sitosterol and β-sitosterol glucoside modulate the growth of estrogenresponsive breast cancer cells *in vitro* and ovariectomized athymic mice. J Nutr 2004;134:1145-51.
13. Méric JB, Rottey S, Olaussen K, Soria JC, Khayat D, Rixe O, *et al.* Cyclooxygenase-2 as a target for anticancer drug development. Crit Rev Oncol Hematol 2006; 59:51-64.
14. Guo Y. Proceedings of the 227th ACS National Meeting. Anaheim, United States: American Chemical Society; 2004.
15. Yang J, Qi SH, Zhang S, Li QX. Chemical constituents from South China Sea gorgonian coral *Subergorgia reticulata.* Zhong Yao Cai 2006;29:555-7.
16. Parameswaran PS, Naik CG, Kamat SY, Puar MS, Das P, Hegde VR. Studies on the secondary metabolites from the Indian gorgonian Subergorgia suberosa: Isolation and characterization of four analogues of the cardiotoxin subergorgic acid. J Nat Prod 1998;61:832-4.
17. Limna Mol VP, Raveendran TV, Naik BG, Kunnath RJ, Parameswaran PS. Calamenenes-aromatic bicyclic sesquiterpenes-from the Indian gorgonian Subergorgia reticulata (Ellis and Solander, 1786). Nat Prod Res 2011;25:169-74.
18. Lagunin A, Filimonov D, Poroikov V. Multi-targeted natural products evaluation based on biological activity prediction with PASS. Curr Pharm Des 2010;16:1703-17.
19. Raicht RF, Cohen BI, Fazzini EP, Sarwal AN, Takahashi M. Protective effect of plant sterols against chemically induced colon tumors in rats. Cancer Res 1980;40:403-5.
20. Choi YH, Kong KR, Kim YA, Jung KO, Kil JH, Rhee SH, *et al.* Induction of Bax and activation of caspases during beta-sitosterol-mediated apoptosis in human colon cancer cells. Int J Oncol 2003;23:1657-62.
21. Awad AB, Roy R, Fink CS. β-sitosterol, a plant sterol, induces apoptosis and activates key caspases in MDA-MB-231 human breast cancer cells. Oncol Rep 2003;10:497-500.
22. Awad AB, Chen, YC, Fink CS, Hennesey T. β-Sitosterol inhibits HT-29 human colon cancer cell growth and alters membrane lipids. Anticancer Res 1996;16:2797-804.
23. Lu X, Liu J, Hou F, Liu Z, Cao X, Seo H, *et al.* Cholesterol induces pancreatic β cell apoptosis through oxidative stress pathway. Cell Stress Chaperones 2011;16:539-48.

24. Khanavi M, Gheidarloo R, Sadati N, Ardekani MR, Nabavi SM, Tavajohi S, *et al.* Cytotoxicity of fucosterol containing fraction of marine algae against breast and colon carcinoma cell line. Pharmacogn Mag 2012;8:60-4.
25. Li Y, Ahmed F, Ali S, Philip PA, Kucuk O, Sarkar FH. Inactivation of nuclear factor kappaB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agent in human cancer cells. Cancer Res 2005;65:6934-42.
26. Hsu YL, Kuo PL, Lin LT, Lin CC. Asiatic acid, a triterpene, induces apoptosis and cell cycle arrest throught activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways in human breast cancer cells. J Pharmacol Exp Ther 2005;313:333-44.
27. Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway. Lancet Oncol 2003;4:721-9.
28. Garson MJ. Biosynthetic studies on marine natural products. Nat Prod Rep 1989;6:143-70.
29. Shimizu Y, Alam M, Kobayashi A. Letter: Dinosterol, the major sterol with a unique side chain in the toxic dinoflagellate, *Gonyaulax tamarensis*. J Am Chem Soc 1976;98:1059-60.
30. Withers NW, Tuttle RC, Holz GG, Beach DH, Goad J, Goodwin TW. Dehydrodinosterol, dinosterone and related sterols of a non-photosynthetic dinoflagellate, *Crypthecodinium cohnii*. Phytochemistry 1978;17:1987-9.
31. Dewick PM. The biosynthesis of C_5-C_{25} terpenoid compounds. Nat Prod Rep 1999;16: 97-130.

Cite this article as: Byju K, Anuradha V, Vasundhara G, Nair SM, Kumar NC. *In vitro* and *in silico* studies on the anticancer and apoptosis-inducing activities of the sterols identified from the soft coral, *subergorgia reticulata*. Phcog Mag 2014;10:S65-71.

Source of Support: Nil, **Conflict of Interest:** None declared.