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Anti-HSV-1 and hepatoprotective activities of the Soft coral *Sarcophyton acutum* from the red sea

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Abstract
Context: Herpes simplex virus type 1 (HSV-1) is one of the most common contagious viruses of the family herpesviridae. It produces infections ranging from painful skin ulcers, corneal damage to encephalitis. Previous studies showed that many isolated compounds from the genus *Sarcophyton* exhibit significant antiviral activities especially against cytomegalovirus (CMV) and influenza A virus (H_1N_1 IAV) implicating that extracts of *Sarcophyton acutum* (*S. acutum*) may have anti-HSV-1 activity. Some soft corals of the genus *Sarcophyton* have also previously reported to exert anti-tumor and hepatoprotective effects. Since maintenance of healthy liver is an important aim for overall health, we also assessed the ability of *S. acutum* extracts on protection of liver against carbon tetrachloride (CCl_4) toxicity.
Objectives: The present study deals with the evaluation of anti-HSV-1 activity of the total extract of Soft corals *S. acutum* and its different sub–fractions. The hepatoprotective activity against CCl_4 induced liver damage was also studied.
Methods: The maximum non–toxic concentration (MNTC) of the total extract and its sub–fractions on VERO cells were determined by the MTT colorimetric assay. The antiviral activity of the extracts and fractions at MNTC was assessed by measuring its inhibitory effects on HSV-1 comparing with control using MTT colorimetric assay. On the other hand, rat model with CCl_4-induced acute hepatitis was used to assess the hepatoprotective activity of the total extract and its sub–fractions.
Results: The results showed that the non-polar, dichloromethane (DCM) and *n*-hexane, fractions exert the highest HSV-1 inhibitory effect, 60.82% and 46.5%, respectively. The total extract and the methanol fraction (MeOH) exhibited moderate inhibitory effect, 22.26% and 32.45%, respectively.
The results of the hepatoprotective experiment showed that CCl_4-intoxicated rats suffered from significant elevation in ALT, AST and total bilirubin levels. Treatment of these rats with total extract, and ethyl acetate (EtOAc) fraction resulted in slight decrease in the enzymes level, while treatment with DCM fraction led to moderate decrease of their level compared with the rats treated with standard silymarin (100 mg/kg).
Conclusion: Extracts of *S. acutum* exhibit obvious anti-HSV-1 effects. They also showed weak hepatoprotective effect.

Keywords: Soft corals, *Sarcophyton acutum*, anti-HSV, MTT assay, hepatoprotective

1. Introduction
The herpes viruses belong to family herpesviridae are ubiquitous and produce infections ranging from painful skin ulcers, corneal damage to encephalitis. Among the human infectious members (HSV-1, HSV-2 HHV-6, HHV-7, HHV-8, CMV, VZV and EBV) of this family, herpes simplex virus type 1 (HSV-1) is one of the most common contagious viruses, in the form of overt or latent infections, especially among the adult population [1]. HSV-1 is known as the main cause of oral herpes including acute herpetic gingivostomatitis with fever and ulcerative lesions, mostly as primary infection, and herpes labials, recurrent or secondary infection. The ulcerative lesions involve the buccal mucosa, tongue, gums and the pharynx [2]. HSV-1 may also cause encephalitis or other systemic infections which sometimes be fatal especially in neonates and immunocompromised patients. In the USA and the developed nations, HSV-1 is the most common cause of corneal damage and blindness. Moreover, primary HSV-1 infection with neurotropic spread of the virus from peripheral sites up the olfactory bulb into the brain may also result in parenchymal brain infections [3]. Acyclovir (ACV) and its related nucleoside analogs are valuable drugs for the short term treatment of primary HSV-1 infection, and continuous therapy for overcoming recurrence of the latent infection. However, a wide range of side effects including renal failure, hepatitis, and anaphylaxis, and the development of resistance especially in immunocompromised individuals

have been reported [4, 5]. Therefore, safe and effective anti-HSV-1 alternatives are needed. Some isolated compounds from the genus *Sarcophyton* exhibit significant antiviral activities especially against CMV and H_1N_1 IAV [6]. The anti-HSV-1 activity of *S. acutum* extract is thus possible and warranting the investigation.

The soft corals of the genus *Sarcophyton* have several other biological activities such as anti-tumor and hepatoprotective activities [7, 8]. Liver has a pivotal role in the maintenance of normal physiological process through its multiple and diverse functions, such as metabolism, secretion, storage and detoxification of endogenous as well as exogenous compounds [9]. Liver diseases remain thus as one of the serious health problems [10]. Therefore, hepatoprotective activity of *S. acutum* total extract, and the DCM and the EtOAc fractions against acute hepatitis of rat caused by peritoneal injection of CCl_4 was also assessed.

2. Materials and methods

2.1. Materials

African green monkey kidney cells, known as VERO cells, and HSV-1 were kindly provided by center for viral research and studies, faculty of medicine, Al-Azhar University, Cairo, Egypt. The cells were grown in minimum essential medium (MEM, Caisson, USA) supplemented with 10% of fetal bovine serum (FBS, Caisson, USA), 100 µg/mL of streptomycin, 100 IU/mL of penicillin, and 0.25 µg/mL amphotericin B (Caisson, USA), at 37 °C and 5% CO_2. The same medium containing 2% phosphate buffer saline (PBS) was used for cytotoxicity and antiviral assays. Virus stock was prepared by infection of confluent monolayer of VERO cells in 75 cm^2 culture flask using MEM with 2% FBS, at 37 °C in 5% CO_2. Virus titer was determined by cytopathic effect (CPE) of HSV-1 in VERO cell and expressed as the 50% tissue culture infective dose ($TCID_{50}$)/mL.

Optical density data were acquired using ELISA reader (start fax-2100/ USA), Thermostar ELISA incubator (USA) and Jouan CO_2 incubator (France). Dimethyl sulfoxide [DMSO, (SDS/France)]. Trypsin-EDTA [1:250 IU, (Molekulu/UK)]. 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) [MTT, (Biobasic /Canada)], Tween-20 and silymarin (Sigma chemicals Co. St. Louis, USA). The assay kits for SGOT, SGPT and total bilirubin were procured from MDSS, GmbH, and Hannover, Germany. All other chemicals and solvents utilized were of analytical grade.

Column chromatography was carried out on silica gel (70-230, mesh, E-Merck) and Diaion Hp-20 (Sigma alderich).

2.2. Sample materials

The Soft corals *S. acutum* Tixier_Durivault 1970 (~ 2.5 Kg. wet wt.) was collected from the National Institute of Oceanography and Fisheries at Hurghada on the Egyptian Red Sea coast using SCUBA diving technique at January 2013 at a depth of 10-15 m.

The sample was identified by Dr. Montaser Aly Alhammady, lecturer of biology, National Institute of Oceanography and Fisheries, Hurghada-branch, Egypt. The sample was stored in a freezer until extraction.

2.3. Extract preparations

The frozen bodies (~2.5 Kg wet wt.) were minced and extracted by maceration in 100% MeOH till exhaustion. The MeOH extract was filtered by Whatman No. 1 filter paper (Whatman England) and concentrated under reduced pressure at 50 °C to give dry residue (60 g). The dry MeOH extract (60 g) was dissolved in distilled water (70 mL), and submitted to a diaion Hp-20 (5 × 120 cm) eluted with water (3 L), MeOH (2 L) and acetone (0.5 L). The different eluates were dried under reduced pressure to give the corresponding aqueous (12 g), MeOH (44 g) and acetone (1.5 g) fractions, respectively.

The obtained methanol eluate (total MeOH, 44 g) was subjected to fractionation by vacuum liquid chromatography (VLC) (12×500 cm) packed with 250 g silica gel (E–Merck) and eluted successively by *n* –hexane (3 L), DCM (2 L), EtOAc (2 L) and MeOH (3 L), to give the corresponding *n*–hexane (2 g), DCM (12 g), EtOAc (17 g) and MeOH (MeOH f, 7 g) fractions.

2.4. Methods

2.4.1. Cytotoxicity assay

2.4.1.1. Propagation of VERO cell line by enzyme treatment [11].

The media overlaying cells monolayer of VERO cell lines was poured off. Cells were released from tissue culture flask by treatment with about 5 mL pre-warmed trypsin–EDTA solution. The flask was rocked so that trypsin completely covers the cells monolayer. The trypsin was aspirated with a pipette, then 2 mL of trypsin were dispensed, the bottle was rocked and incubated at 37 °C. Cells were examined from time to time to avoid trypsin over action. The bottle was struck with hand to completely dislodge the cells from the bottle surface. Cells were suspended in about 8 mL of MEM supplemented with 10% of FBS, 100 µg/mL of streptomycin, 100 IU/mL of penicillin and 0.25 µg/mL amphotericin B. Cells were counted using haemocytometer and trypan blue vital stain [12]. About 10 mL of 2×10^5 VERO cell suspension were transferred to 50 cm^3 TC bottle (Falcon), tightly closed and then incubated at 37 °C. Cells were sub-cultured once weekly. For seeding of the 96-well plate, 0.1 mL (2×10^5 cells) was transferred to each flat bottomed well, and incubated at 37 °C for 24–48 h to develop a complete monolayer sheet.

2.4.1.2. Determination of sample cytotoxicity on VERO cells [13].

Growth medium was decanted from 96–well micro titer plates after confluent sheet of VERO cells was formed. Cells monolayer was washed twice with wash media, then about 1 mL of wash media was added and the plates were incubated at room temperature for 5–10 minutes. 100 mg of the *S. acutum* dried total MeOH extract and its different fractions was separately dissolved in 1 mL DMSO. Double-fold dilutions of each sample were made in MEM to give final concentrations ranging from 1×10^5 to 1.525 µg/mL. A 0.2 mL of each dilution was added to the different wells, leaving 6 wells as control and received only maintenance medium. Plate was incubated at 37 °C and examined frequently for up to 3 days. Cells were checked for any physical signs of toxicity, partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. The MNTC at which, by microscopic examination, cells showed normal morphology and cell density of the total MeOH extract and its different fractions were determined. Measured MNTC (table 1) was used for antiviral activity against HSV-1. Enough volume of MTT solution 5 mg/mL in PBS was prepared. A 20 µL MTT solution was added to each well of 96–well plate, placed on a shaking table, 150 rpm for 5 minutes, to thoroughly mix the MTT into the media. The plate was incubated at (37 °C, 5% CO_2) for 1–5 h. The media was dumped off. Formazan, MTT

metabolic product, was resuspended in 200 µL DMSO and placed on a shaking table, 150 rpm for 5 min, to thoroughly mix the formazan into the solvent. Optical density was read at 560 nm with a reference wavelength of 690 nm by an ELISA reader. Optical density should be directly correlated with cell quantity. The 50% cytotoxic dose (CD_{50}) corresponded to the concentration required to kill 50% of the VERO cells for each extract was calculated by nonlinear regression analysis using Graph Pad Prism software. The experiment was performed in triplicate, and the cell viability (%) was calculated from the following equation:

$$\%\text{of cell viability} = \frac{optical\ density(O.D)\ of\ sample\ treated\ cells}{optical\ density(O.D)\ of\ untreated\ control\ cells} x\ 100$$

2.4.2. Antiviral assay

MTT Assay [14].

VERO cells in density of 10,000 cells were plated in 200 µL media/well in a 96–well plate. A 8 wells were left empty for blank controls. The plate was incubated at (37 °C, 5% CO_2) overnight. Equal volume (1:1, *v/v*) of MNTC below the CD_{50} of each fraction and the virus suspension stock was incubated for 1 h. 100 µL from viral/sample suspension was added to each well, placed on a shaking table, 150 rpm for 5 minutes. The plate was incubated at 37 °C, 5% CO_2 for one day to allow the virus to take effect. The other steps are as discussed in the determination of sample cytotoxicity. The antiviral activity for a given sample can be calculated from the following equation:

$$Antiviral\ activity(\%) = \frac{(Optical\ density\ of\ treated\ cells - Optical\ density\ of\ virus\ control)}{(Optical\ density\ of\ cell\ control - Optical\ density\ of\ virus\ control)} x\ 100$$

2.5 Hepatoprotective assay

2.5.1. Experimental animals

Studies were carried out using Twenty four Wister albino rats of either sex, weighing 150–170 g. Rats obtained from the animal house, pharmacology department, faculty of medicine, Assiut University. They were housed in polypropylene cages and maintained under standard conditions at ambient temperature (25±2 °C), relative humidity (55 ± 5%) and 12 hr light–dark cycle. Animals had free access to standard pellet diet and water throughout the period of the experiment.

2.5.2. Experimental design

Animals were divided into six groups (4 animals each) as follow:

Group I (Control): Received distilled water orally (1 mL/kg/day) for 8 days.

Group II (Negative control): Received distilled water orally in a dose of 1 mL/kg/day for 7 days.

Group III (Positive control): Received silymarin (100 mg/kg/day) orally for 7 days.

Group IV, V and VI (Tests group): Received total MeOH extract of *S. acutum,* DCM f. and EtOAc f., respectively, (200 mg/kg/day) for each. Extracts were suspended in distilled water with the aid of a drop of tween-20, and administered orally for 7 days.

On the 7th day, animals of all groups except group I were administered CCl_4 in a dose of 2 mL/kg, I.P. (CCl_4 in olive oil 1:1 *v/v*) along with their routine treatment. On the 8th day, animals of all groups were sacrificed under ether anesthesia.

2.5.3. Estimation of hepatoprotective activity

The animals were sacrificed on 8th day under ether anesthesia and blood was collected by direct cardiac puncture and kept for 30 min without disturbing before centrifugation to avoid hemolysis. Samples were centrifuged at 3000 rpm for 15 min and the clear supernatant of each animal of all groups were estimated for SGOT, SGPT and total bilirubin [15].

2.6. Statistical analysis

Data were represented as mean percentage of antiviral activity ± SE compared with untreated control (n=3). A p-value of less than 0.05 was considered statistically significant. Statistical analysis was carried out by statistical software using one way analysis of variance and the post-hoc Tukey's test of SPSS program. For the determination of CD_{50} value, non-linear regressions of dose-cytotoxicity curves were used utilizing Graph Pad Prism software.

3. Results and discussion

3.1. Antiviral activity

The soft corals *S. acutum*, collected from red sea at a depth of 10–15 m, were extracted by maceration in MeOH. The concentrated MeOH extract was desalted using diaion HP–20, and then subjected to fractionation using VLC C.C. with *n*–hexane, DCM, EtOAc, and MeOH to gives the corresponding fractions. Cytotoxicity of each sample on VERO cells was evaluated, and the MNTC was determined. The results of our investigation (Table 1) showed that the EtOAc fraction has the highest toxic effect on VERO cells with CD_{50} value 12.2 µg/mL followed by MeOH fraction with CD_{50} value 36.46 µg/mL then DCM fraction, and the total extract with CD_{50} values ~48 µg/mL for each. The *n*–Hexane fraction showed the least toxic effect with CD_{50} value 195.31 µg/mL. The total extract and its different sub-fractions were investigated for their anti-HSV-1 activities using the predetermined MNTC. The anti-HSV-1 results (Table 1) showed that the DCM f. has the highest anti-HSV-1 effect (60.82%), followed by *n*–hexane f. (46.52%), MeOH f. (32.45%), total extract (22.46%), and finally EtOAc f (3.40%).

Noteworthy that upon relating the various anti-HSV compounds into their chemical classes, it was found that the majority (34.4%) of the anti-HSV compounds were of the terpenoid-type [5]. The Soft corals of the genus *Sarcophyton* are rich in cembranoid diterpens, ~10% of their body weight [7]. The significant anti-HSV-1 activity of DCM f. (60.84%) and *n*-hexane f. (46.5%), which usually have high content of the non-polar and semi-polar constituents, is may be due to their high content of these diterpens. Many cembranoid diterpens from the genus *Sarcophyton* have been reported to exert potent antiviral effect against CMV virus, a member of the herpisviridea family, which is agree with our findings [16].

3.2. Hepatoprotective effect

Free radicals are generated in cells by environmental factors such as ultraviolet radiation, pollutants, x-rays, as well as by normal metabolism [17]. If the *in-vivo* activity of defensive enzymes or scavengers are not adequate to neutralize these free radical, it induce an oxidative state that can lead to cellular membrane injury especially for hepatocytes causing liver damage with the consequent alteration in metabolic processes [17]. CCl_4 induced liver injury is a commonly used model for screening of hepatoprotective drugs [18]. CCl_4 is a potent hepatotoxin because it is bio-transformed by the cytochrome P_{450} in the endoplasmic reticulum system producing trichloromethyl (CCl_3*) free radicals in the body which results in oxidative damage of cell organelles and membrane. This radical in the presence of oxygen can also

form the trichloromethylperoxy radical CCl_3OO^*, which may attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical. The formation of these reactive free radicals initiates the chain reaction of lipid peroxidation. Lipid peroxidation leads to injury of the hepatocyte membrane and leakage of cytosol enzymes like ALT, AST and total bilirubin into the blood stream elevating their levels. CCl_4 induced liver damage is quantified by measuring the levels of these enzymes which considered as indicators of liver damage. Silymarin act as scavenger of free radicals, so it has the ability to inhibit lipid peroxide formation and protect hepatocytes from damage [17]. In this study, CCl_4 was used as the hepatotoxin, and silymarin (100 mg/kg) was used as the standard hepatoprotective agent to confirm the integrity of the test system and also to compare the efficacy of the test fractions. The normal control group that received distilled water only served as a baseline for all the biochemical parameters. Administration of CCl_4 to the rats in negative control group (II) resulted in a significant increase in ALT, AST and total bilirubin when compared with control group (I). Administration of silymarin 100 mg/kg in group III prevented the rise in ALT, AST and total bilirubin when compared with negative control group (I). The treatment of the intoxicated rats with total extract, and EtOAc f. (200 mg/kg/day) resulted in a week decrease in the all parameters when compared with silymarin-treated rats (group III). The treatment of intoxicated rats with DCM f. at the same dose resulted in a moderate decrease in all parameters (Table 2). The weak decrease in levels of the rats biochemical parameters, ALT, AST and total bilirubin, after treatment with the different *S. acutum* extracts compared to silymarin treated ones indicates that these fractions may don't have the ability to inhibit the formation of lipid peroxide or scavenge the free radicals.

4. Conclusion

Our results demonstrate that the diterpenes-rich DCM and *n*-hexane fractions of the MeOH extract of *S. acutum* have significant anti-HSV-1 activities. These fractions may be a potential candidate for the development of alternative natural anti-HSV-1 remedy. The previously reported anti-inflammatory effects of such diterpenes [19], could be useful for relieving inflammation associated with the viral attack. Further chromatographic purification is still needed to conclude the main active antiviral compound or group of compounds. Unfortunately, extracts of the soft corals *S. acutum* showed weak hepatoprotective effect at our experimental conditions.

5. Acknowledgment

We are grateful to Center for Viral Research and Studies, Faculty of Medicine, Al-Azhar University, Cairo, Egypt for providing VERO cell line and HSV-1.

6. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Table 1: Cytotoxicity on VERO cells, and % of viral inhibitory effects of extracts of *S. acutum* on HSV-1.

Fractions	MNTC	CD_{50}	%antiviral activity ± SE
Total MeOH	12.5 µg /mL	48.84 µg /mL	22.26 ±2.006
n-hexane f.	25 µg/mL	195.31 µg/mL	46.52 ±1.300
DCM f.	12.5 µg/mL	48.84 µgmL	60.82 ±2.34
EtOAc f.	1.5 µg/mL	12.20 µg/mL	3.40 ± 0.64
MeOH f.	7.5 µg/mL	36.46 µg/mL	32.45 ±5.67

Values are expressed as mean of % antiviral activity ± SE

Table 2: Effect of extract and fractions of *S. acutum* on the serum ALT, AST and total bilirubin in liver damaged rats.

Group	ALT (IU/L)	AST(IU/L)	Total bilirubin
I (Normal)	58 ± 3.0	125 ± 2.9	0.13 ± 0.08
II (CCL_4)	271 ± 20.2 ^{a*}	356 ± 4.4 ^{a*}	1.13 ±0.08 ^{a*}
III (silymarin)	132 ± 3.7 ^{a*}	216 ± 4.5 ^{a*}	0.70 ±0.04 ^{a*b*}
1V (total MeOH)	240 ± 5.7 ^{a*b*}	321 ± 5.8 ^{a*b*}	0.93 ± 0.02 ^{a*b*}
V (DCM f.)	171 ± 6.0 ^{a*b*}	245 ± 2.8 ^{a*b*}	0.82 ± 0.01 ^{a*}
VI (EtOAc f.)	225 ± 2.8 ^{a*b*}	327 ± 7.2 ^{a*b*}	0.87 ± 0.08 ^{a*}

Values are expressed as mean ± SE. Statistical analyses were done by one-way ANOVA followed by Tukey's multiple comparison test. $^*p<0.05$; ^{a}groups I–IV *vs* group I, ^{b}groups IV–VI *vs* group III.

7. References

1. Ficarra G, Birek C. Oral herpes simplex virus infection in pregnancy: what are the concerns. J Can Dent Assoc. 2009; 75(7):523-526.
2. Sherris J. Medical microbiology: an introduction to infectious diseases. Elsevier Biomedical Press BV. 1984
3. Qavi HB, Hessel T. The efficacy of antiviral components from plant products against herpes simplex virus type 1. Ann Virol Res. 2015; 1(1):1001.
4. Gaby AR. Natural remedies for Herpes simplex. Alternative medicine review. 2006; 11(2):93.
5. Zhong MG, Xiang YF, Qiu XX, Liu Z, Kitazato K, Wang YF. Natural products as a source of anti-herpes simplex virus agents. RSC Advances. 2013; 3(2):313-328.
6. Cheng SY, Wang SK, Chiou SF, Hsu CH, Dai CF, Chiang MY *et al*. Cembranoids from the octocoral *Sarcophyton ehrenbergi*. Journal of natural products. 2010; 73(2):197-203.
7. Phan CS, Vairappan CS. Isopropyl (ene)-type cembrane diterpene an important chemotaxonomical marker in bornean soft coral genus *Sarcophyton*. Journal of Tropical Biology and Conservation. 2015; 12:137-143.
8. Abdel-Wahhab MA, El-Nekeety AA, Hassan NS, El-Hefnawy MS, Kotb MM, El-Mekkawy SA *et al*. Hepatoprotective effect of Sarcophine isolated from soft coral *Sarcophyton glaucum* in rats. Global Vet. 2012; 8(3):244-253.
9. Ganapaty S, Ramaiah M, Yasaswini K, Nuthakki VK, Harikrishnareddy D. Quantitative phytochemical estimation and evaluation of hepatoprotective activity of methanolic extract of *Dendrobium ovatum (L.) Kraenzl* whole plant against CCl_4 induced hepatotoxicity. Journal of Pharmacognosy and Phytochemistry. 2013; 2(3):113-118
10. Dash DK, Yeligar VC, Nayak SS, Ghosh T, Rajalingam R, Sengupta P, Maity TK. Evaluation of hepatoprotective

and antioxidant activity of *Ichnocarpus frutescens (Linn.) R. Br*. on paracetamol-induced hepatotoxicity in rats. Tropical Journal of Pharmaceutical Research. 2007; 6(3):755-765.
11. Ammerman NC, Beier-Sexton M, Azad AF. Growth and maintenance of VERO cell lines. Current protocols in microbiology A–4E. 2008.
12. Patel S, Gheewala N, Suthar A, Shah A. *In-vitro* cytotoxicity activity of *Solanum nigrum* extract against Hela cell line and VERO cell line. International journal of pharmacy and pharmaceutical sciences. 2009; 1(1):38-46.
13. Duman R. Antiherpetic activity of some endemic *Hypericum species* in Turkey. African Journal of Biotechnology. 2012; 11(5):1240-1244.
14. Moradi MT, Karim A, Alidadi S, Saedi-Marghmaleki M. *In vitro* Anti-adenovirus activity of *pomegranate (Punica granatum L.)* peel extract. Advanced Herbal Medicine. 2015; 1(4):1-8.
15. Unkeshwar P, Nasiruddin M, Fayazuddin M, Khan Ra, Khan Aa, Tajuddin. Evaluation of hepatoprotective activity of *berberis aristata* against carbon tetrachloride induced hepatotoxicity in rats. International Journal of Pharmacy and Pharmaceutical Sciences. 2013; 5(4):107-110.
16. Gong KK, Tang XL, Zhang G, Cheng CL, Zhang XW, Li PL, Li GQ. Polyhydroxylated steroids from the South China Sea soft coral *Sarcophyton sp.* and their cytotoxic and antiviral activities. Marine drugs. 2013; 11(12):4788-4798.
17. Sahreen S, Khan MR, Khan RA. Hepatoprotective effects of methanol extract of *Carissa opaca* leaves on CCl_4-induced damage in rat. BMC Complementary and Alternative Medicine. 2011; 11(1):48.
18. Alagammal M, Lincy MP, Mohan VR. Hepatoprotective and Antioxidant effect of *Polygala rosmarinifolia Wight* and *Arn* against CCl_4 induced hepatotoxicity in rats. Journal of Pharmacognosy and Phytochemistry. 2013; 2(1):118-124
19. Lin WY, Su JH, Lu Y, Wen ZH, Dai CF, Kuo YH, Sheu JH. Cytotoxic and anti-inflammatory cembranoids from the Dongsha Atoll soft coral *Sarcophyton crassocaule.* Bioorganic and medicinal chemistry. 2010; 18(5):1936-1941.