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Antioxidant,Antiinflammatory and Antifungal Activity of Marine Sponge *Subergargoria suberosa*-Derived Natural Products

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Abstract: Chemical examination of the ethanol extract of the Indian ocean gorgonian coral *Subergorgia suberosa* resulted in isolation and identification of revealed compound 1 to be subergorgic acid 1 and a two bio-active analogues 2 and 7 were synthesized by the $NaBH_4$ reduction of compound 1. The present investigation aimed to carry out invitro understanding the antioxidant,anti-inflammatory and antifungal activities for understanding immunostimulatory properties subergorgic acid 1 and its two analogues compound 2 and compound7.
Keywords: Marine sponge , *Subergorgia suberosa* , subergorgic acid1,Compounds2, compound 7, Immunostimulatory activity , NMR.

Introduction and Experimental

Intensive research since four decades has proved that marine organisms are magnificient source of bioactive secondary metabolites and number of compounds originating from marine organisms have been reported to possess *in vitro* and *in vivo* immuno stimulatory activity [1-4.] Inflammation is typically a protective mechanism that is triggered in response to noxious stimuli, trauma or infection to guard the body and to hasten-up the recovery process. However, inflammation that is unchecked leads to chronic inflammatory disorders. Arachidonic Acid (AA) metabolism plays a crucial role in inflammatory process and associated diseases.. Unfortunately most of the anti-inflammatory drugs, particularly steroids and cyclooxygenase inhibitors are often associated with adverse side effects including, Gastro Intestinal irritation, ulcers, hypertension and cardiac abnormalities [5].Marine products represent a vast untapped resource of bioactive molecules, whose exploration is needed for antimicrobial compounds and anti oxidants. Bioactive compounds of marine organisms have enormous therapeutic potential. There has been growing interest in investigation of natural products from marine organisms for the discovery of immuno stimulastory activity such as antioxidant anti inflammatory and antimicrobial .

Subergorgoria suberosa (red gargorian) is a sessile colonial sandian found throughout the ocean. This gargorian is found to be rich in bioactive diterpenes such as Subergorgic acid 1. Recently it has also been claimed as a cardiotoxic marine metabolite. Subergorgic acid 1 was isolated from *S.suberosa* of the mandapam coast and few more bioactive analogs were prepared. The structures of subergorgic acid 1 and its analogous 1-8 were determined by spectroscopic methods. As a part of our continuing investigation on isolation of bioactive metabolites and study of its biological properties from the marine sources, we have isolated subergorgic acid 1 from the Indian ocean gorgonian coral. The isolation and identification of subergorgic acid 1, with unique structural features, had prompted us to investigate in detail. The in hand research aimed to highlight the isolation and structural elucidation and biological studies of Subergargoric acid 1 and it analogs compound 2 and compound 7 for their anti oxidant, anti inflammatory and anti fungal activities.

General Experimental Procedure:

IR spectra were recorded on a Perkin-Elmer 841 IR spectrometer in $CHCl_3$ solution. UV spectra were recorded on a Milton Roy Spectronic 1201 spectrometer in $CHCl_3$ solution. ^1H NMR spectra were measured on a Bruker Advance DRX 300 and JEOL JNM-EX 90 spectrometers. ^{13}C NMR spectra were measured on a JEOL JNM-EX 90 spectrometer at 22.4 MHz using $CDCl_3$ as a solvent and tetramethylsilane as an internal reference. GC-MS analysis were carried out on Shimadzu QP5050A bench-top quadrapole instrument in the EI mode under the following conditions: column: 30meter, 0.25mm O.D. DB-5 capillary column, He-flow rate : 260^{0}C at 40^{0}C/min and held at 260^{0}C for 25min, solvent: Column chromatography was performed on silica gel (100-200) mesh, TLC was carried out on silica gel G glass plates with a thickness of 1mm, (PF 254 art 7747, Merck). Solvents and reagents are purified according to standard procedure.

Marine Sponge Material:

The gorgonian coral (5 Kg) was collected from Mandapam coast (Tamilanadu) during the winter of 2007 and transported to the lab in ethanol. It was classified to be from the genus *S.Suberosa*. The voucher specimens (Code: AU71/860) have been deposited at Marine Museum of School of Chemistry, Andhra University, Visakhapatnam.

Isolation of subergorgic acid and its analog compounds:

Freshly collected gorgonian *Subergorgia suberosa* (Pallas) (5 kg) was cleaned from adhering algae and other materials, cut into small pieces and immediately immersed in ethanol (12 L). After soaking the material for 30 days, ethanol layer was separated by decantation and the material was extracted six more times with ethanol (6 x 10 L). Solvent was removed from each extract separately under reduced pressure and the resulting residues were combined to give a dark red coloured solution. This solution was extracted with EtOAc till the EtOAc layer was colorless. This combined EtOAc layer was concentrated to give a brown viscous gum (80 g).

This gum was adsorbed on SiO_2 (100 g) and the adsorbed gel was transferred on to a column of SiO_2 (130 g, 100 cm x 3 cm) set in n-hexane. Then column was eluted with solvents of increasing polarity involving hexane, and hexane EtOAc mixtures. On the basis of TLC, closely related fractions were mixed. Some of the resulting mixtures were then subjected to further purification via re chromatography and crystallization, to yield some compounds along with the desired compound Suber gargoric acd 1

Reaction of subergorgic acid with sodium borohydride in ethanol:

To an ice-cold solution of subergorgic acid 1 (500 mg) in ethanol (20 ml) was added sodium borohydride (250mg) in small portions with shaking. After addition was over, the reaction mixture was kept at room temperature for 24 h, diluted with water (20 ml) and extracted with ether (2 x 50 ml). The ether extract was washed with water (20 ml), dried over anhydrous magnesium sulphate and solvent was evaporated. A colourless crystalline mass (485 mg) resulted which on crystallization from ethanol gave the alcohols 2 as colorless shining needles (300 mg, 60%) m.p 150^0 C and compound 3 as a pale yellow gum (90 mg, 18%).

α - Alcohol 2: Colourless shiny needles, m.p.150^0. $[\alpha]_D^{27}$ - 19.8 ^{0} (c, 1.0, $CHCl_3$) υ_{max} : 3450, 1680, 1640, 1630 and 1450 cm^{-1}.GC - MS data (EI mode) : Gave a single peak with an RT of 8.60 min on DB- 5 capillary column He - flow rate : 1.5ml / min, programme : 50^{0}C (2 min) - 260 ^{0} at 40^{0}C / min and held at 260^{0}C for 25 min, solvent : *n*–hexane. Mass spectrum of the 8.60 min peak : m/z 250 (M^+ of $C_{15}H_{22}O_3$) (35), 232 (M –H_2O) (50), 217 (30), 91 (100) , 41 (95%) . ^{1}HNMR (90 MHz, $CDCl_3$, TMS) : δ 4.20 t, 6.45 s , 3.15 q, J=7Hz, 1.15 d, J=7Hz, 1.25 s, 1.40 d, J=7Hz. ^{13}CNMR (22.5 MHz $CDCl_3$, TMS): δ 66.2, 77.4, 43.5, 39.7, 62.6, 31.4, 37.7, 57.8, 152.3, 138.9, 49.4, 20.7, 22.6,169.7,19.6.

β - Alcohol 3: As a pale yellow gum. $[\alpha]_D^{27}$ - 19.2 ^{0} (c, 1.0, $CHCl_3$) υ_{max} : 3450, 1680, 1640, 1630 and 1450 cm^{-1}.GC - MS data (EI mode) : Gave a single peak with an RT of 8.44 min on DB- 5 capillary column He - flow rate : 1.5ml / min, programme : 50^{0}C (2 min)

- 260 ^{0} at 40^{0}C / min and held at 260^{0}C for 25 min, solvent : n–hexane. Mass spectrum of the 8.60 min peak : m/z 250 (M^+ of $C_{15}H_{22}O_3$) (35), 232 (M –H_2O) (50), 217 (30), 91 (100) , 41 (95%) . ^{1}HNMR (90 MHz, $CDCl_3$, TMS) : δ 4.38 dd, 6.42 s , 2.68 q, J=8Hz, 1.05 d, J=7Hz, 1.40 s, 1.15 d, J=7Hz. ^{13}CNMR (22.5 MHz $CDCl_3$, TMS): δ 68.2, 76.0, 43.5, 45.7, 39.7, 63.7, 30.4, 40.1, 59.4,156.3, 136.2, 50.4, 20.5, 22.3,168.7,17.5.

Reaction of subergorgic acid methyl ester (6) with sodium borohydride in methanol:

Sodium borohydride (100 mg) was added to a solution of methyl ester 6 (200 mg) in methanol (5 ml) at room temperature. After keeping it overnight at room temperature, the reaction mixture was diluted with water (10 ml) and extracted with ether (2 x 20 ml). The combined ether extract was dried over anhydrous magnesium sulphate and solvent was evaporated. The resulting brownish gum was chromatographed on a small column of silica gel (30g: 10 cm x 2.5 cm) by eluting with *n*-hexane and mixtures of *n*-hexane and EtOAc. Elution with 8.5% EtOAc in hexane furnished compound 7 (85 mg, 42.5%) and compound 4 (55 mg, 27.5%).

Compound 7 : Colorless gum, $[\alpha]_D^{27}$ - 112.8^0 (c, 1.0, $CHCl_3$), $\upsilon_{max}^{CHCl_3}$: 3450, 1730, 1630, 1450 and 1250 cm^{-1}. GC – MS data: Showed a single peak with an RT of 8.17 min. Mass spectrum of the 8.17 min peak: m/z 264 (M^+ of $C_{16}H_{24}O_3$) (45), 232 (M – CH_3OH) (50), 90 (100) and 41 (95%). ^{1}H – NMR data : δ 4.15 t (H – 2), 6.35 s (H – 9), 3.12 q, J = 7Hz (H -11) , 1.20 d, J=7 Hz (H -12), 1.25 s (H – 13),1.35 s (H -15), 3.75 s (OCH_3) . ^{13}C - NMR data (C – 1 to C – 15 and OCH_3) : δ 66.3, 77.5, 43.7, 40.2, 62.6, 31.5, 37.7, 57.7, 150.0, 139.2, 49.7, 20.9, 22.8, 165.4, 19.8, 51.3.

Compound 4: Colourless oil, $[\alpha]_D^{27}$ + 44.8^0 (c, 1.0, $CHCl_3$) $\upsilon_{max}^{CHCl_3}$: 3450, 1640 and 1450 cm^{-1}.GC - MS data (EI mode): Showed a single peak with an RT of 7.84 min on DB- 5 capillary column He - flow rate : 1.5ml / min, programme : 50^{0}C (2 min) - 260 ^{0} at 40^{0}C / min and held at 260^{0}C for 25 min, solvent : n – hexane. Mass spectrum of the 7.84 min peak: m/z 236 (M^+ of $C_{15}H_{24}O_2$); (traces), 218 (M –H_2O) (25), 205 (60), 147 (95), 91 (100), 41 (95). ^{1}H NMR (90 MHz, $CDCl_3$, TMS): δ 4.18 m, 5.14 s, 2.76 q, J=7Hz, 1.13 d, J=6.6 Hz, 1.15 s, 4.18 m, 1.30 d, J=7Hz. ^{13}C - NMR data (C – 1 to C – 15) : δ 66.6, 77.9, 43.7, 39.7, 63.60 31.5, 38.0, 56.7, 147.0, 132.9, 50.7, 22.9, 22.8, 60.5, 19.2.

Free radical scavenging activity (FRSA)

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure phenolic compounds were measured from the bleaching of the purple-colored methanol solution of 2, 2-0-diphenylpicrylhydrazyl (DPPH).This spectrophoto metric assay uses the stable radical DPPH as a reagent [15.16] . 50 µl of subergargoric acid1 , compound 2 and compound 7 were added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Drug activity was expressed as the 50% inhibitory concentration(IC50). The reaction mixture contained 1 x 10-4 M methanolic solution of DPPH and various concentrations of the test substances. Percentage inhibition was determined by comparing the absorbance values of test and control tubes. IC50 values are obtained from the best fit line drawn concentration (µg) vs. percentage inhibition.

***In vitro* 5-Lipoxygenase inhibition:**

5-LOX enzyme inhibitory activity of Subergargoric acid analogues were measured using the method of Reddenna *et al*.[15] modified by Ulusu *et al*.[16]. The assay mixture contained 80 µM linoleic acid and 10 µl potato 5-LOX in 50 mM phosphate buffer (pH 6.3). The reaction was initiated by the addition of enzyme buffer mix to linoleic acid and the enzyme activity was monitored as the increase in absorbance at 234 nm. The reaction was monitored for 120 sec and the inhibitory potential of the test substances was measured by incubating various concentrations of test substances for two minutes before addition of linoleic acid. All assays were performed in triplicate. Percentage inhibition was calculated by comparing slope of test substances with that of enzyme activity.

Antifungal activity:

Aspergilus niger, Pencillium notatum and Fusarium moniliformis cultures are collected from the *Microbiology Department, ANU A.P India.* The fungal growth was harvested and washed with sterile normal saline and finally suspended in (100ml) of sterile normal saline and the suspension was stored in refrigerator till used. The agar diffusion method was adopted according to[5] .To assess the antifungal activity of the prepared extracts. 0.6 ml of standardized fungal stock suspensions was thoroughly mixed with 60ml of sterile Saboraud dextrose agar. 20 ml of the inoculated Saboraud dextrose agar containing 0.1ml of each compound were distributed into sterile Petri dishes . The Saboraud dextrose agar was left to set The plates were then incubated in the upright position at 25^{o}C for 48 hours. Two replicates were carried out for each extract against the test organism.. After incubation the diameters of the results and growth inhibition zones were measured.

Table 1.: Antifungal activites of Subergorgoric acid 1 and its analogous compounds-compound2 and compound7

Name of the compound	*Zone Of Inhibition(diameter in mm)*		
	Aspergillus niger	*Pencillium notatum*	*Fusarium monoliformis*
Gresiofulvin*	25.6	14.6	14.6
Compound (1)	12.0	10.2	12.5
Compound (2)	17.5	15.5	42.5
Compound (7)	12.0	12.5	36.6

*Positive control ** Average of two replicate

Figure 1. Antioxidant activity of subergargoric acid 1, compound 2 and compound 7 [IC50µg/ml]

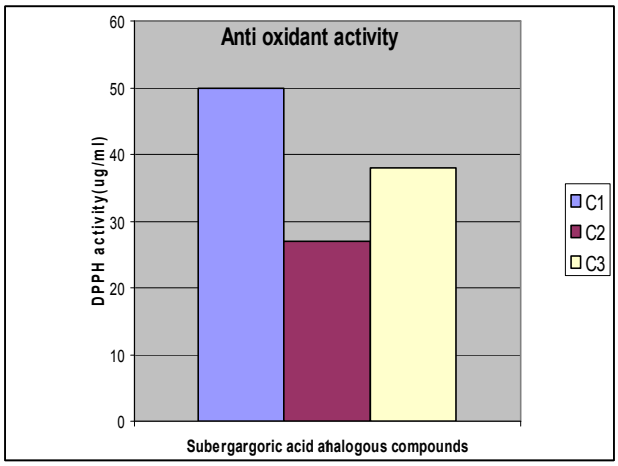
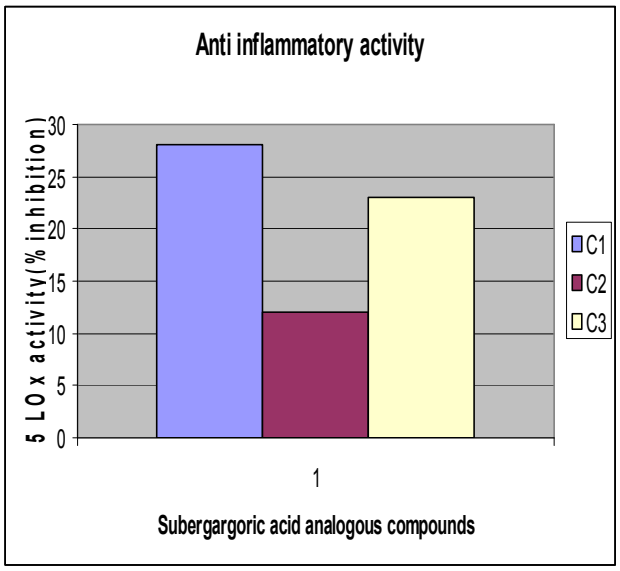


Figure 2. 5-Lox Activity of subergargoric acid1 and analogous Compounds 2 and 7.



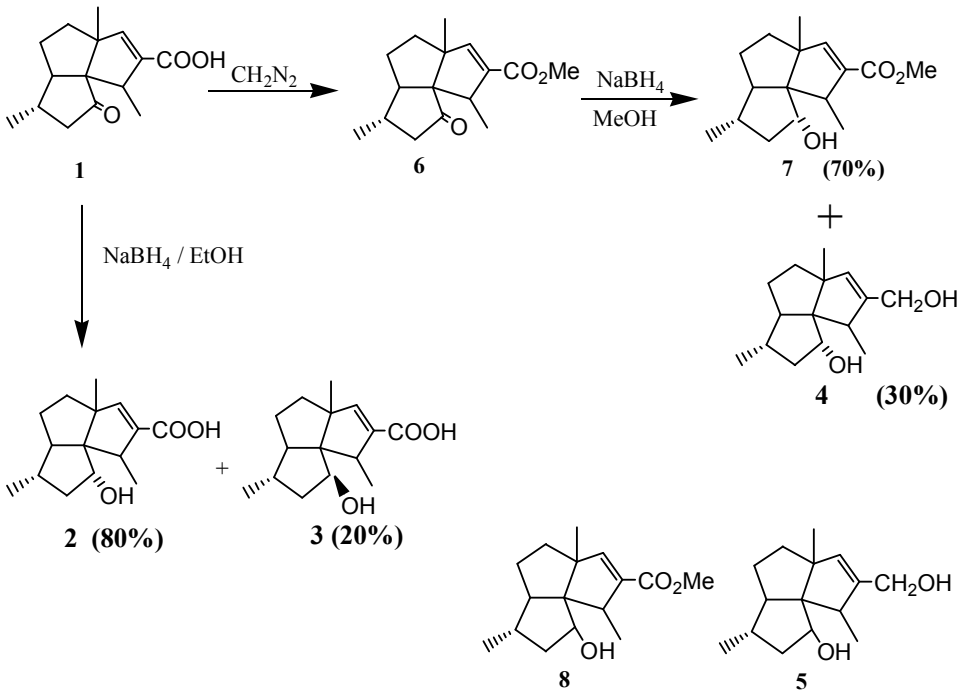
Results and Discussion :

Subergorgic acid 1 on reaction at room temperature with $NaBH_4$ in ethanol (in methanol the reduction did not take place) furnished the epimeric alcohols 2 and 3 in the ratio 4: 1, respectively .The major product, the α - isomer 2 was obtained by crystallization of the reaction mixture. The NMR spectra pounds 4 and 5, the $LiAlH_4$ reduction products of 1[1].In the α – isomer 4, the H -15 was observed at a deshielded position, while in the β – isomer 5, the H – 13 was observed at a deshielded position. Moreover, the H – 11 in 4 was observed at a lower field (δ 2.76q) than in 5 (2.36q). The ^{1}H – NMR spectrum of the major product of the $NaBH_4$ reduction showed the H – 11 and H -15 protons at deshielded positions compared to the spectrum of 3, indicating that it is the α – isomer 2, a new derivative of 1.

Subergorgic acid 1 was converted into its methyl ester 6 by reaction with ethereal diazomethane. The methyl ester 6 on reaction with $NaBH_4$ in methanol furnished two products, products A and B in 7:3 ratio, respectively. Product A was identified as the alcoholic ester 7 and product B as the diol 4 on the basis of their spectral data. Product A showed the molecular ion at m/z 264 (two mass units higher than that of the methyl ester 6) and its NMR spectra showed the presence of a carbinol (δ 4.15 t, H – 2; 77.5 d) and a methyl ester group (3.75 s, 165.2 s, 51.3 q). $NaBH_4$ reduction of 6 can yielded the epimeric alcohols 2 and 3. Compound 8 was isolated from S.*suberosa* by Parameswaran et al[3].Like compounds 2,3 & 4,5 the ^{1} H – NMR spectra of compound 7 and 8 also differ at H – 11, H -13 and H – 15 show similar trends. The ^{1}H – NMR spectrum of the major product of the present reaction 7 showed the H – 15 and H – 11 signals at deshielded positions indicating that it was the α – isomer.

The NMR spectra of product – 4 did not show the methyl ester group, indicating that the ester group was also reduced. It is not uncommon for the reduction of α, β – unsaturated esters into alcohols in $NaBH_4$ reductions. The ^{1}H – NMR spectrum of product – 4 showed the presence of a shifted olefinic protons (δ 5.15 s), a broad singlet at 4.18 for the H – 14 and H – 2 protons and deshielded H -15 and H – 11 protons.

Antioxidant refers to any substance that hinders the reaction of a substance with dioxygen or any substance that inhibits free radical reaction[6]. Nowadays Antioxidants have gained more importance on account of their positive effects, as health promoters in the treatment of cardiovascular problems, atherosclerosis, many forms of cancer, the ageing process, etc. many antioxidant compounds which are naturally occurring in plant sources have been identified as free radical scavengers [7,8,9]. In present study, invitro antioxidant activity (FRSA) of three compounds subergargoric acid1,compound2 and compound 3 show potential free radical scavenging activities expressed in IC_{50} value 50 µg/ml .27 µg/ml and 38 µg/ml respectively.



Scheme-1

Results revealed that subergargoric acid 1 has highest free radical scavemging activity ,among derived compounds compound 7 has greater antioxidant property than compound 2(*fig.1*).5-Lox activities of subergargoric acid 1 and its analogous bioactive Compounds 2 and 7 are shown in *Fig*2. Anti inflammatory activities of these compounds 1, 2 and 7 are 28%,15% and 23% respectively. The results suggest that subergargoric acid 1 has highest anti inflammatory activity and also reveals compound 7 has greater antioxidant and anti inflammatory activity than compound2. These results suggest that subergorgic acid1as well as its analogous derivatives compound 2 and compound7 have great potential as a source of antioxidant and anti inflammatory material due to their free radical scavenging property.

Chemical compounds produced biosynthetically that could destroy or usefully suppress the metabolism of pathogenic microbes are referred as antibiotics which are extensively studied in various organisms in recent times [5].Fungi like Asprgillus, Fusarium and pencillium spp cause serious diseases in economically important species of fish shrimp and crustaceans [10,11,12]. Fungal species, a serious opportunistic pathogens cause skin lesions in tilapia fish and catfish. Also cause ulceration of skin and gills of tilapia mossambica [13,14] . However to our knowledge a little is known about antimicrobial activity of bioactive compounds isolated from marine gargorians. The results of antifungal assay against *A.niger P.notatum and F.moniliformis* are summarized in Table-1. These three different types of fungi which cause infection in common Tilapia fish are tested with subergorgic acid 1 and its analogous compound 2 and 7. Bioactive compound 2 has shown greater inhibition on *F.moniliformis* than *P.notatum* and *A.niger*. Results also showed that compound 2 has highest anti fungal activity than compound1 and 7 . The results of the anti fungal assay for Subergorgic acid 1 and its analogous 2 and 7 suggest that they have great potential as a source of fungicide.

However the results of the present investigation on Subergorgic acid 1 and its analogous compound 2 and 7 have potential bioactive properties. To our Knowledge the results describe antioxidant and anti-inflammatory activity of Subergorgic acid compound and its bioactive analogous compounds2 and7 are promising derived marine products fot the development of drug.

Conclusions

The life saving drugs are mainly found abundantly in microorganisms ,algae and in vertebrates while they are scarce invertebrates .This investigation reveals a detailed Schematic extraction of subergorgoric acid 1 derivatives from subergargoria and also attempted standard assays for immunostimulatory activities of the derived natural products .Hence this information may help to develop potential purified bioactive compounds as fungicides, analgesics and to treat inflammation in pharmacological studies.

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