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Original Research Paper

Isolation and Characterization of Angiogenic Active Compound From Sea Urchin, Temnopleurus Alexandri (Bell, 1884)

Zoology

KEYWORDS : Sea urchin, structural elucidation, Angiogenic compound

R.Parvathavarthini — Research and Development Centre, Department of Zoology, Bharathiar University, Coimbatore, Tamil Nadu, India.

*** Dr.B.Uma** — Department of Zoology, Bharathi Women's College, Chennai,Tamil Nadu, India.

ABSTRACT

Sea is a source of novel organic bioactive molecules that have much importance in medicine, physiology, pharmacology and biochemistry. In the present study, a biologically important active compound which promote angiogenesis ex vivo and in vitro have been isolated from sea urchin, Temnopleurus alexandri. The compound was isolated from hexane crude extract and was tested for its purity with Thin layer chromatography. It was further subjected to Characterization and structure elucidation by IR, NMR, MASS and GC-MS. The compound isolated was penta decanoic acid 14-methyl- methyl ester.

Introduction

Since ancient times, plants and animals have formed the basis of traditional medical systems, such as Indian, Chinese and African ones. In recent years, the interest in folk medicine from different cultures, also known as traditional medicine, has increased significant in industrialized countries, due to the fact that many prescription drugs worldwide have originated from the fauna and flora (Nelson-Harrison et al., 2002).

Plants and animals have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. Traditional knowledge and historic literature on medicine play an important role in the discovery of novel leads from medicinal plants. Recently, the search for novel pharmacologically important medicines has focused on invertebrate animals because of their efficacy in human clinical trials and the minimal side effects of drugs derived from animals. In view of the increasing prevalence, there is a growing need to develop integrated approaches towards the management and prevention of diseases by exploring the potential of traditional healers (Tag et al., 2012).

The animal kingdom is a potential source of new drugs. The marine organisms represent excellent source for bioactive compounds (Bickmeyer et al., 2005). The secondary metabolites have various functions. It is likely that some of them may be pharmacologically active on humans and useful as medicines (Briskin, 2000). A majority of pharmacologically active secondary metabolites have been isolated from Echinoderms (Carballeria et al., 1996).

The present study is aimed at identifying an angiogenic active compound from T. alexnadri.

Materials and Methods

Extraction:

Sea urchin, T.alexandri were collected from bycatch from fish landing center, Chennai coast, which were thrown as waste. Authentication of the echinoid was done with Zoological Survey of India (ZSI), Chennai(India). Shade dried specimens were immersed in hexane (1:3 w/v). Extract was obtained by cold percolation and concentrated under reduced pressure using rotary evaporator at 40º C. Finally, crude extract was obtained. The crude extract was stored at 4º C until further use.

Chick chorio allantoic membrane (CAM) assay:

Angiogenic activity of sub fraction 2 of hexane crude extract was determined (Uma & Parvathavarthini, 2010) using the chick embryo chorio allantoic membrane assay (Indap & Pathares, 2003; Scanlon et al., 2013). In brief, a small window of 1cm2 size was made in 5 days old embryonated eggs obtained from Tamil Nadu Veterinary University, Chennai, under aseptic conditions to observe CAM. Sterile empty disc (Sigma-Aldrich) of 6mm size was loaded with 10µl of known concentrations (10, 50,100 ng) dissolved in 2% DMSO. Each disc was then placed on the CAM of one egg, away from central blood vessel. The windows were resealed with adhesive tape and the eggs were returned to the incubator. Eggs were further incubated for 48 hours and the number of blood vessels were counted and tabulated. Embroys treated with VEGF (10 ng) and DMSO (2%; Janice, et al., 2013) was used as positive and solvent control, respectively. Experiments were repeated five times for each dose of all extracts and mean value calculated.

Purification:

The crude hexane extract that was found active in the preliminary CAM assays was subjected for further isolation of the main bioactive compound. The hexane extract (25g) was separated on silica gel (65G) using stepwise gradient elution with hexane, chloroform, ethyl acetate and methanol to yield 16 fractions. Fraction 4 was further chromatographed on silica gel plates (35G F254) eluted with ethyl acetate: methanol (10:90) to yield 3 sub-fractions. The bioactive sub-fraction 2 was separated and purified using thin layer chromatography on a silica gel GF 254 (Kieselgel 60G Merck) using the solvent system ethyl acetate: formic acid (70:30). The eluent was separated from the adsorbent by centrifuging at 2500 rpm for 10 minutes and evaporated to dryness using a hot air oven at the temperature of 400C.

Structure elucidation of active sub fraction

The boiling point of the compound was determined by micro boiling point apparatus. Effective sub fraction was further analyzed IR, MASS, NMR and GC-MS for identification of compound.

IR Spectroscopy

Solvent used for IR spectra was carbon tetra chloride. Purified sample was ground with potassium bromide, pellets were made and spectra taken. The IR spectrum was taken on a Perkin-Elmer Spectrum RX-I FT-IR spectrophotometer in the range of 4000 - 4500 cm.

Nuclear Magnetic Resonance

The information about the structure of a compound based on NMR is by measuring the magnetic moments of its hydrogen atom. For measuring or plotting NMR spectra, a standard substance is used, whose peak is taken as ref-

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erence. The standard used is Tetra Methyl Silane (TMS), which shows chemical shift value at zero on the δ scale. NMR is used as analytical tool for predicting the structure of the molecule based on the different environments of hydrogen atoms in that molecule. Purified sample was subjected to NMR studies. 1H NMR was run at either 300 or 400 MHz and 13C NMR at 75 MHz using the solvent signal as reference. NMR studies were performed in AL-300 MHz, JEOL spectrometer.

Mass Spectroscopy:
With Mass Spectroscopy the molecular mass of a compound and its elemental composition can be easily determined. Further this method involves very little amount of the test sample, which will give molecular weights accurately. High Resolution Electron Impact Mass Spectroscopy (EI-MS) was performed. Purified sample was subjected to mass spectroscopy studies and a mass spectrum was taken on JoEL GC-MS spectrophotometer. The melting point of the isolated compound was taken on a melting block apparatus.

Gas chromatography – Mass spectrometry (GC-MS)
The hexane crude extract showing bioactivity was subjected to gas chromatography (GC-MS shimadzu) equipped with a DB-5 MS column (inner diameter 0.25mm, length 30.0m, film thickness 0.25 μm); mass spectrometer (ion source 200OC, RI 70ev) programmed at 40-650OC with a rate of 4O C/min. Injector temperature was 280OC; carrier gas was helium (20 psi), column flow rate was 1-4ml/min. and injection mode-split.

Statistical analysis
The significance of treatments was found out by one-way Analysis of Variance (ANOVA) and Mann Whitney test. Differences with P values of less than 0.05 ($P<0.05$) were considered statistically significant.

RESULTS
In the present study, sub fraction 2 of fraction 4 of hexane crude extract of T.alexandri was isolated..To confirm the purity of sub fraction 2, it was subjected to thin layer chromatography with the suitable mobile phase (ethyl acetate: methanol ::10:90). The spot was visualized either by exposing to Iodine vapours, UV light and anesaldihyde sulphuric acid. It showed single spot on TLC over the silica gel with trace impurity (Plate 1). The impurities were removed by washing with methanol.

Selection of active sub fraction by CAM assay
To find the exact compound responsible for angiogenic activity, the 3 sub-fractions were tested by CAM assay. Of the 3 sub-fractions, the sub-fraction 2 was positive for CAM assay as can be seen from Table 1, Figure 1 and Plate 2. The blood vessel count in CAM assay for different concentrations of sub fraction 2 (of fraction 4) of hexane crude extract of T.alexandri, are 116±12.852 at 10ng, 209±17.263 at 50ng and 269±4.536 and all the values for all the concentrations were statistically significant.

Nature of compound:
The isolated compound was found to be semi solid and waxy in nature. It was brown in colour.

Boiling point of the compound
The boiling point was found to be 313 degree at 760 mm Hg.

FT-IR spectrum
The FT-IR spectrum of the compound isolated from hexane extract of T. alexandri is shown in Figure 2. The peak at 3428 cm-1 is due to keto-enol tautomerism hydrogen bonding formed in the carboxylic group. The peaks at 2924 cm-1 and 2852 cm-1 are due to symmetrical C-H and asymmetrical C-H of CH2 groups [(CH2)12], respectively. The C=O stretching of saturated ester is observed at 1623 cm-1 (S-Strong) and 1778 cm-1 (W-Weak). The peak at 1384 cm-1 (S) show the presence of C-H symmetrical bending of CH3 group and 1232 cm-1 (S) indicates the C-C vibrational frequency of C-C bond in alkyl CH2 groups in chain. Also the peak at finger print region, 550 cm-1 (S) indicates the presence of longest CH2 chain. The peaks at 1623 and 1047 cm-1 (S) supports the presence of C=O stretching frequencies of ester. These observations confirm all the characteristic groups in pentadecanoic acid 14- methyl- methyl ester.

The 1H NMR spectrum
1H NMR spectrum of the isolated compound is shown in Figure 3. The peaks observed at 0.879 to 0.913 ppm represents methyl group at carbon 14th position; peak at 2.319 ppm represents methine proton; peak at 1.299 ppm represents the longest CH2 groups and the peak at 1.652 represents OCH3 protons.

The 13C NMR spectrum
The 13C NMR spectrum of isolated compound is shown in Figure 4. The characteristic peak assignment (δ ppm) seen are as follows:

Peak at δ 77.0 denotes solvent: CDCl3; peak at δ 174.37 denotes C=O (carbonyl carbon) of COO- ester; peak at δ 51.43 denotes HC= (methine carbon); peaks at δ 24.27-34.11 denote carbon of longest carbon chain and peak at δ 14.12 denotes methyl carbon of CH3 group.

Mass spectrum
The mass spectrum of the isolated compound is shown in Figure 5. The molecular mass of the compound is 270 m/e and it confirms the isolated compound as pentadecanoicacid, 14- methyl- methyl ester. The base peak observed at 74.0 m/e shows the 100% abundance of the fragmented CH2-CO-OCH3 group from the compound. The loss of iso propyl group (43 m/e) and the remaining groups are indicated by 227 m/e value. The peak at 143.0 m/e scission of eleven CH2 groups as single fragments.

GC-MS Spectrum
GC-MS of the active compound showed single peak (100%). The compound was identified as pentadecanoic acid 14-methyl- methyl ester (Figure 6) by comparison of the mass spectrum with that of the reference compound in the GC-MS library (SI=91%).

structure of the compound is as given below:

and the molecular formula of pentadecanoic acid 14-methyl- methyl ester is C17H34O2

Discussion:
In the present study, Pentadecanoic acid 14- methyl- methyl ester was isolated and identified from hexane extract of T.alexandri

Pentadecanoic acid 14- methyl- methyl ester is commonly known as isopalmitate or palmitic acid methyl ester. The

other chemical names for the isolated compound are methyl 14-methyl pentadecanote, 14 methyl penta decanoic acid methyl ester, methyl iso hexa deconate. It has a molecular weight of 270.4507 and the retention time of the compound is 35.405. The boiling point of penta decanoic acid 14 methyl methyl ester is 312 degree at 760 mm Hg and the vapour pressure is 0.001000 mm Hg at 20.00 degree.

Pentadecanoic acid 14- methyl- methyl ester angiogenically active as evidenced from CAM assay done in the present study. Similarly, a compound fucosylated chondroitin (FucCs) isolated from an echinoderm sea cucumber increased wound healing property and was proved to be pro-angiogenic (Tapon et al., 2008)

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been developed from natural sources. Pentadecanoic acid-14-methyl-methyl ester (8.2%) constituent had been isolated from Leucaena leucocephala plant (Salem et al., 2011). Pentadecanoic acid, 14-methyl-, methyl ester, (3.47 %) was extracted from Andrographis paniculata 70% methanol solvent extract resulted good effect with antioxidant properties (Wei1et al., 2011). It also had been reported to have antimicrobials (Bashir et al., 2012). Penta decanoic acid, 14 methyl methyl ester was previously found to have anti-microbial and anti-oxidant property (Vijisaral & Arumugam 2014).

The compound penta decanoic acid 14 methyl methyl ester was previously identified and isolated from the hexane extract of Azadirachta indica and they also found the compound to have anti-oxidant property (Akupuaka et al, 2013). The anti fungal and anti-microbial activity of penta-decanoic acid 14 methyl methyl ester was tested and proved by Bashir et al. (2012) from the hexane extract of Acacia modesta leaf. The compound has been previously isolated from the earthworm, Allolobophora caliginosa Savigry and Pheretima hawayana Rosa.

Plate 1: TLC of sub-fraction 2 (of fraction 4 of hexane extract of T.alexandri)

Table 1: Angiogenic activity (blood vessel count) of subfraction 2 (of fraction 4) of hexane crude extract of T.alexandri (Mean of 5 values)

Untreated	DMSO	VEGF (10ng)	Sample Concentrations (ng)		
			10	50	100
52.80± 6.301	51.60± 5.941	521.20± 1.125	*116± 12.852	*209± 17.263	*269± 4.536

***values significant at <0.05 level**

Figure 1: Angiogenic activity (blood vessel count) of subfraction 2 (of fraction 4) of hexane crude extract of T.alexandri (Mean of 5 values)

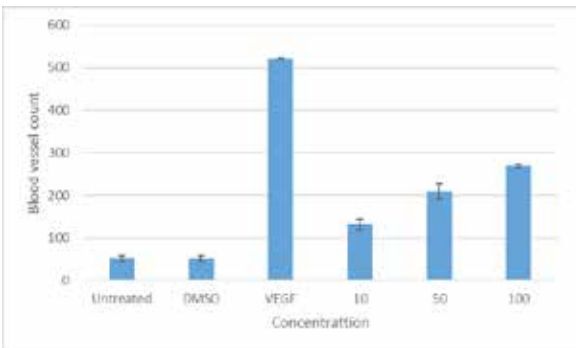

Plate 2: Angiogenic activity of subfraction 2 (of fraction 4) of hexane crude extract of T.alexandri (mean of 5 values)

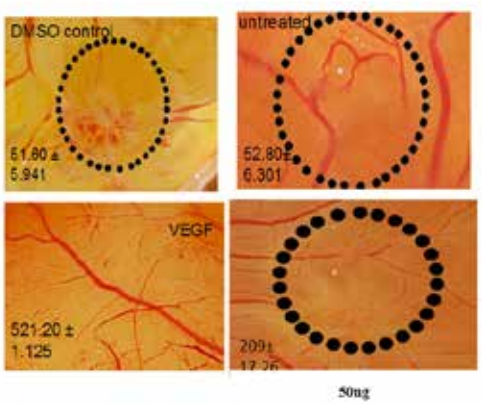

Figure 2: IR spectrum of the compound

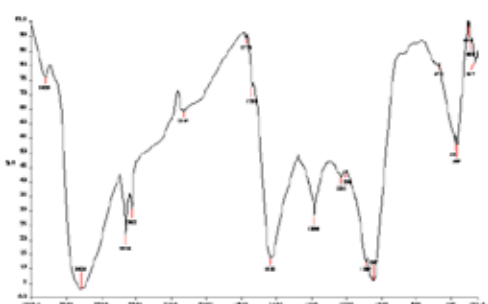

Figure 3: 1H NMR of the compound

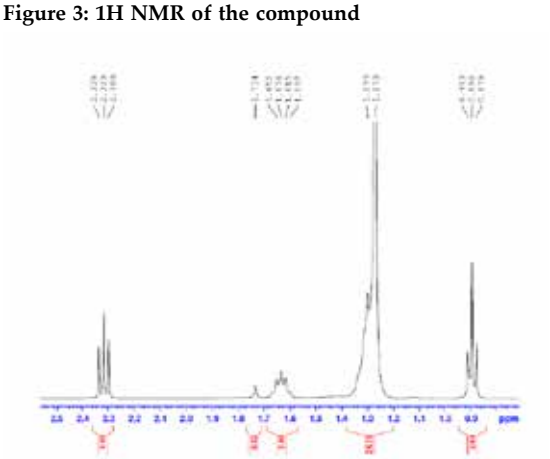

Figure 4: 13C –NMR of the compound

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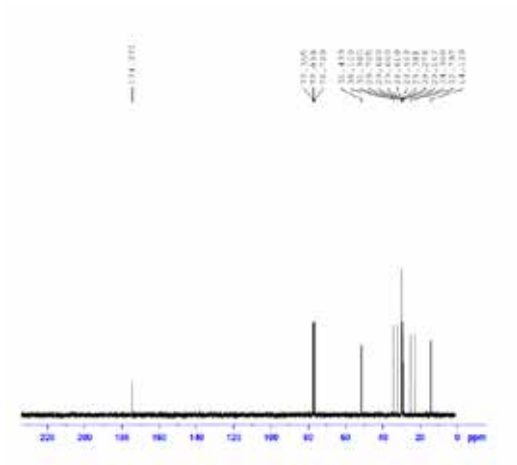


Figure 5: MASS Spectrum of Pentadecanoic acid, 14-methyl-, methyl ester

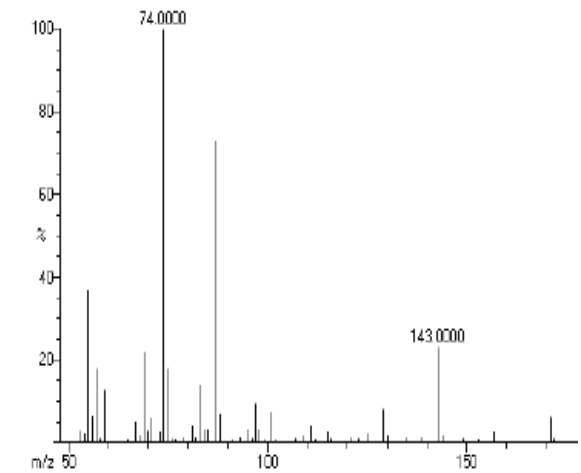
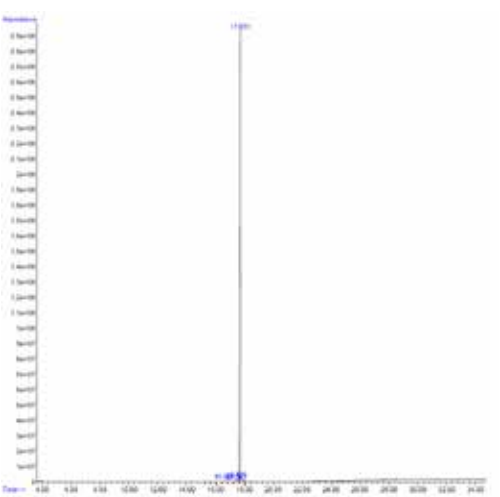


Figure 6: GC- MS spectra of the compound



Peak No	Retention Time	Compound name	Area/ composition (%)
1	16.609	Pentadecanoic acid, methyl ester	0.17
2	17.382	9-Hexadecenoic acid, methyl ester	0.06
3	17.426	9-Hexadecenoic acid, methyl ester	0.38
4	17.694	Pentadecanoic acid, 14-methyl-, methyl ester	99.39

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