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Full Length Research Paper

Cytolytic and antinociceptive activities of starfish *Protoreaster linckii* (Blainvilli, 1893)

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Echinoderms are exclusively marine animals. Starfish extracts have drawn attention, because of their wide spectrum of biological effects associated with antifungal, cytotoxic, hemolytic and immunomodulatory activities. The study was carried out to investigate the hemolytic and antinociceptive effect of the methanolic extract of *Protoreaster linckii* (MEPL) using male albino mice models of chemicals and thermal antinociception. The extract was injected intraperitoneally at doses of 5, 10, 15 and 20 mg/kg. The hemolytic activity was tested against human, chicken, sheep and goat blood red blood cells (RBCs). The percentage of protein was estimated in the crude extract as 4.14%. Among the ten human pathogens tested, *Proteus mirabilis* showed the most sensitive activity against 0.75 g/ml (9 mm) of sample compared with positive control (15 mm) and *Streptococcus pyogenes* showed activity at 0.75 g/ml concentration and no activities was noticed at 0.25 and 0.50 g/ml concentration. The hemolytic activity was high in human blood (128 HU). The crude extract shows dose dependent analgesic effect in male albino mice. The maximum mortality was noticed at 100% concentration, that is, 93.6±1.2 and minimum amount of mortality was noticed at 20% concentration, that is, 22.0±2.1 in brine shrimp cytotoxicity assay. The results from the present study appear to support the steroids belief in the medicinal properties of *P. linckii* against pain in which the central antinociception activity was found and it has also showed good cytolytic properties against blood RBCs.

Key words: Echinoderm, hemolytic, antinociception, mortality.

INTRODUCTION

Marine organisms represent excellent source for bioactive compounds (Bickmeyer et al., 2005). Approximately 7000 marine natural products, 25% of which are from algae, 33% from sponges,18% from coelenterates, 24% from representatives of other invertebrate phyla such as ascidians, opisthobranches, mollusks, echinoderms and byrozoans (Anake, 2004). The phylum Echinodermata includes a diverse group of typically slow-moving and non-aggressive marine animals, including three venomous classes, namely the crown-of-thorns starfish, *Acanthaster planci* is being venomous animal for the human envenomations (Monico et al., 2007). The huge potential of echinoderms as a far fairly untapped source of bioactive molecules is described. Examples are presented that show the usefulness of echinoderm-derived molecules for therapeutic application in selected fields of cancer research, in the control of bacterial growth as substances with new antibiotic properties, and finally in the context of technical applications such as antifouling substances.

Asteroidea, Echinoidea and Holothuroidea. Among them, the sea stars have variety of chemical constituents such as steroids, glycosides consisting of astrosaponins,

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steroids cyclic glycosides, steroid monoglycosides and di-glycosides, saponins, astrosaponins and carotenoids. Moreover, the echinoderm tissue extracts have demonstrated cytotoxic (Wang et al., 2003), hemolytic (Ivanchina et al., 2006), antifungal (Chludil and Maier, 2005), antimicrobial (Haug et al., 2002), feeding deterrent (McClintock et al*.,* 2003), antifouling (Iorizzi et al*.,* 1995; Bryan et al*.,* 1996; Greer et al*.,* 2006) activities. Hence, the present investigation was made from sea star *Protoreaster linckii* demonstrated the hemolytic, cytotoxic and antinociceptive properties.

MATERIALS AND METHODS

Collection

The starfish *P. linckii* was collected from landing centre of Mandapam, Southeast coast of Tamilnadu (Ramanadhapuram district) and brought to the laboratory with seawater in fresh condition and immediately air dried for further analysis.

Extraction

Crude extract from *P. linckii* was extracted following the method of Braekman et al*.* (1992) with certain modifications. For methanolic extraction, starfish was dried in air for 10 days and completely dried specimen was put in methanol, covered and kept standing for 7 days. The solvent was then removed after squeezing of starfish and the extract was filtered using Whatman No. 1 filter paper (pore size 0.4 µm). The solvent was removed at low pressure using rotary evaporator (VC 100A, Lark Innovative, India) at 30°C. The resultant compound was finally dried in vacuum desiccators and stored at 4°C in a refrigerator for future use as crude methanol extracts.

Purification

A properly cleaned glass column (29×2.3 cm) was mounted vertically on to a stand. The methanol extract was dissolved in water and passed through an Amberlite-XAD-2 column, washed with water and eluted using methanol (Chludil et al., 2000). The methanol fraction was further purified using silica gel column and eluted using methanol and distilled water (2:1) and distilled water. The aqueous fraction was lyophilised for further analysis.

Biochemical assays

The percentage of protein, carbohydrate and lipid in crude extract of *P. linckii* was estimated.

Protein estimation

The concentration of protein was estimated by the method of Raymond and Howard, (1976). 200 mg of crude sample was dissolved in 1 ml of distilled water, 4 ml of biurett reagent was added and incubated at room temperature for 30 min and read at 540 nm spectrophotometrically.

Carbohydrate estimation

The concentration of carbohydrate was estimated followed by the method of Dubois et al. (1956). 5 mg of crude sample was dissolved in 1ml of distilled water followed by 1ml of 5% phenol and 5 ml of concentrated sulphuric acid was added and incubated for 15 minutes at room temperature and measured at 490 nm spectrophotometrically.

Lipid estimation

Total percentage of lipid was estimated using the methodology of Folch et al. (1956). For this 400 mg of sample was taken in a test tube, 5 ml of chloroform: methanol (2:1) was added and incubated at room temperature for overnight. After incubation the mixture was filtered using Whatman No.1 filter paper. The filtrate was collected in a 10 ml pre-weighed beaker, which was then kept on a hot plate. The beaker with the residue at the bottom was weighed after the chloroform: phenol mixture gets evaporated and the weight of the empty beaker was subtracted from this to know the weight of the lipid present in the sample.

Antibacterial assay

Antibacterial activity was carried out by using standard disc diffusion method (Dulger and Gonuz, 2004; Parekh and Chanda, 2007; Laouer et al., 2009). The antibacterial activity of crude sample of *P. linckii* was tested against 10 human pathogens. Various concentration of crude sample dissolved in methanol (0.25, 0.50, and 0.75 g each in 1ml of solvent) and used for assay. The extracts were applied to 6 mm sterile discs in aliquots of 30 µl of solvent, allowed to dry at room temperature and placed on agar plates seeded with microorganisms. The bacteria were maintained on nutrient agar plates and incubated at 37°C for 24 h. Zones of growth inhibition were measured following incubation.

Hemolytic study

Hemolytic assay microtitre plate

The crude extract of *P. linckii* was assayed on chicken, sheep, goat and human erythrocytes followed by the method of Prasad and Venkateshvaran (1997). The chicken, goat and sheep bloods were obtained from nearby slaughter house in Parangipettai, while clinically healthy human blood was obtained from local hospital using 2.7% ethylene diamine tetra acetic acid (EDTA) solution as an anticoagulant at 5% of the blood volume and brought to the laboratory. The blood samples were centrifuged thrice at 5,000 rpm for 5 min. 1% erythrocyte suspensions was prepared for hemolytic study.

Hemolytic assay on blood agar plate

The hemolytic activity was assayed using blood agar plates by following the method of Lemes-Marques and Yano (2004). Chicken and goat blood agar plates were prepared by adding 5 ml of blood to 95 ml of sterile blood agar aseptically, with the result poured immediately onto the Petri dishes. After solidification, wells were cut into the agar plate-using a corkscrew borer (8 mm diameter). Wells were loaded with 50 µl (1 mg/ml) of samples. The plates were observed for hemolysis after overnight incubation at room temperature.

Cytotoxicity using brine shrimp lethality assay

The toxicity effects of the crude extract on *Artemia salina* (brine

Table 1. Hemolytic activity of chicken and goat blood samples (Blood agar plates)

Sample	Zone of inhibition (mm)	
	Chicken	Goat
Crude	8.3±0.4	4.3±0.4
Fraction 1 (Methanol)	4.6±1.2	2.0±0.8
Fraction 2 (Aqueous)	4.6±0.4	1.6±0.9

shrimp) were determined using the methodology of Meyer et al. (1982). The extracts were dissolved in 0.01 ml of DMSO and incorporated into 5 ml of sea water (pH= 8.8 and salinity = 28%) containing ten *A. salina*. Each concentration (10, 20, 40, 60, 80 and 100 µg/ml) was tested thrice, and a control DMSO was done each time. The vials were maintained under illumination. Survivors were counted after 24 h and the percentage of deaths at each dose and control (DMSO and saline). The LC_{50} values of brine shrimp were obtained from counts using the prohibit analysis method described by Litchfield and Wilcoxon (1949).

Antinociceptive activity

The crude extract of *P. linckii* was tested for anti-nociceptive activity by two models, namely, acetic acid induced writhing response (chemical method) and tail flick assay (thermal methods). The albino mice were employed to study the anti-nociceptive effect followed by Turner and Hebban, (1984) method.

Chemical method

Acetic acid induced writhing test

Writhing test was performed by Whittle (1964). Various amount of sample (5 mg/ml), that is, 0.125, 0.250, 0.375 and 0.50 ml was administered orally to different healthy male albino mice. After 15 min, 1 ml of 0.6% acetic acid was injected intraperitoneally. Antinociception was recorded by counting the number of writhes after the injection of acetic acid for a period of 20 min. A writhe is indicated by abdominal constriction and full extension of hind limb.

Thermal method

Tail flick test

Analgesic activity was measured according to the method described by Jansen et al. (1963) using tail flick analgesia meter INCO. During the testing period, the mice were restrained in a plastic tube, to which they had been previously adapted twice (10 min) a day for three days. The tail flick latency was recorded as the time onset of stimulation to the withdrawal of the tail. The crude extract of *P. linckii* was dissolved in DMSO at the dose of 5, 10, 15 and 20 mg/kg of body weight and then injected intraperitoneally (i.p) to mice. Mice without administration of any toxin or known painkiller was used as control while those injected i.p with paracetamol (crocin ® at 0.25 ml/170±2 g) will serve as reference standards. The mice were tested 30 min after injection. Analgesic activity was expressed as a ratio between the difference in reaction time of envenomated mice and control since analgesic potential is proportional to the difference in tail flick latency between the toxin and control.

Tail immersion test

The procedure is based on the observation that morphine like drugs selectively prolongs the reaction time of the typical tail withdrawal reflex in mice (Toma et al., 2003). The animals were treated as discussed earlier. Above 1 to 2 cm of the tail of mice was immersed in warm water kept constant at 55°C. The reaction time of the mice to deflect their tails were noted. The first reading was discarded and the reaction time was recorded as a mean of the next three readings. A latency period of 20 s was defined as complete analgesia and the measurement was then stopped to avoid injury to mice. The latent period of the tail-flick response was determined 30, 60 and 90 min after the administration of drugs.

Fourier transform infrared (FTIR) spectroscopy

Chemical characterization of sample was performed using FTIR spectroscopy (Abu et al., 1991).

RESULTS

Biochemical assays

The percentage of protein was estimated in the crude extract shown as 4.14%. The amount of lipid and carbohydrate was estimated at 3.63 and 17.5%, respectively.

Antibacterial activity

Ten human pathogens were used for the antibacterial activity of starfish extracts (Figure 1). Among the ten human pathogens tested, *Proteus mirabilis* showed the most sensitive activity against 0.75 g/ml (9 mm) of sample compared with positive control (15 mm) followed by *Klebsiella oxytoca* (8 mm), *Vibrio parahaemolyticus* (8 mm), *Salmonella paratyphi, Salmonella typhi* (6 mm) at 0.75 g/ml concentration. *Streptococcus pyogenes* showed activity at 0.75 g/ml concentration and zone of inhibition was noticed at 0.25 and 0.50 g/ml concentration. The one way analysis of variance (ANOVA) of antibacterial activity showed 0.05% level significance at between the groups ($p>0.05$).

Hemolytic activity

The hemolytic activity was high in human blood (128 HU). The activity on human blood is very high compared with other bloods (Figure 2). The hemolytic activity was further confirmed using blood agar plates with chicken and goat blood. The zone of inhibition was measured and the results were presented in Table 1.

Antinociceptive activity

Chemical method: Acetic acid induced writhing test

In writhing test, the number of writhing was decreased with increase in concentration of the extract and the results

Table 2. Writhing test of starfish extracts against male albino mice (mg/kg).

Treatment	Dose (mg/kg)	Route of injection administration	No. of writhing	Inhibition of writhing response (%)
Control	5	I.P	48	-
Aspirin	10	I.P	10	75.6
Group I	5	I.P	42	12.5
Group II	10	I.P	19	60.4
Group III	15	I.P	13	73.0
Group IV	20	I.P	11	77.1

Table 3. Tail flick analysis of starfish extracts against male albino mice (mg/kg).

Treatment	Dose (mg/kg)	Response after extract injection (s) time in min			
		30	60	90	120
Control (Saline)	5	12	10	8	7
Group I	5	9	10	7	6
Group II	10	16	18	14	7
Group III	15	20	22	16	14
Group IV	20	33	34	30	20
Standard	-	5	6	3	4

Table 4. Tail immersion test of starfish extracts against male albino mice (mg/kg).

Group/Treatment	Dose (mg/kg)	Mean reaction time before administration	Mean reaction time after administration (min)		
			30	60	90
Group I (Control)	5	3.20	-	-	-
Group II	5	3.53	6	3	4
Group III	10	3.67	11	5	3
Group IV	15	3.82	15	3	3

results were tabulated (Table 2).

Tail flick method

The heat sensitivity of the mice injected with crude extract was calculated using tail flick analgesia meter. The tail flick latency was assessed by the analgesiometer (Inco, India). No adverse effect or mortality was detected in albino mice up to 1 g/kg, i.p administration of extracts during 24 h observation period (Table 3).

Tail immersion test

The analgesic studies revealed that the methanolic extract of *P. linckii* exhibited potent analgesic (central analgesic activity) effect against thermal noxious stimuli (Vogel, 2002) and also revealed that the extract shows dose dependent analgesic effect (Table 4).

Cytotoxic assay

In the brine shrimp lethality assay, the degree of inhibition observed was directly related to the concentration of the active *P. linckii* crude extract. The concentration level was 20 to 100%. The maximum mortality was noticed at 100% concentration (93.6±1.2) and minimum amount of mortality was noticed at 20% concentration (Figure 3). The regression analysis showed the LC_{50} value of 65.3% (Figure 4). The regression analysis showed R^2=0.989.

Fourier transform infrared spectroscopy

The FTIR spectrum of methanolic extract of starfish produced by *P. linckii* (Figure 5) exhibited many peaks and absorption at 3411.36 to 2958.90 cm^{-1} are assigned to N-H stretches (primary amine groups) and C-H stretches (alkyl group). Stretches 1216.51 to 1047.42 cm^{-1}

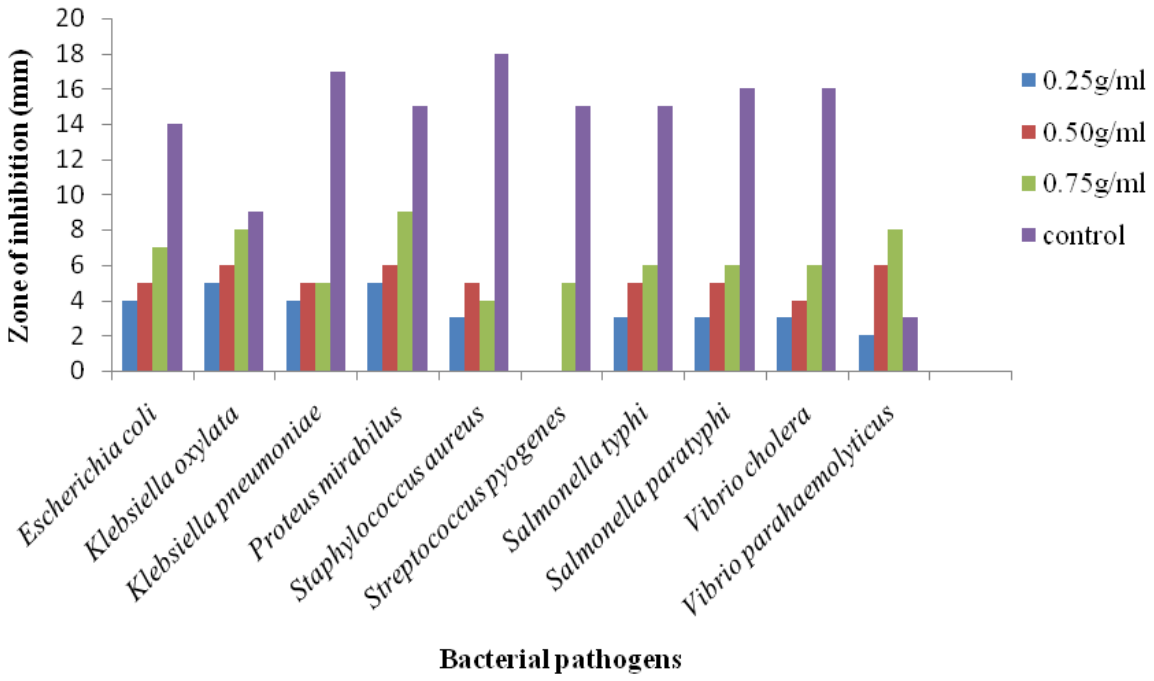

Figure 1. Antibacterial activity of starfish extracts against human pathogens.

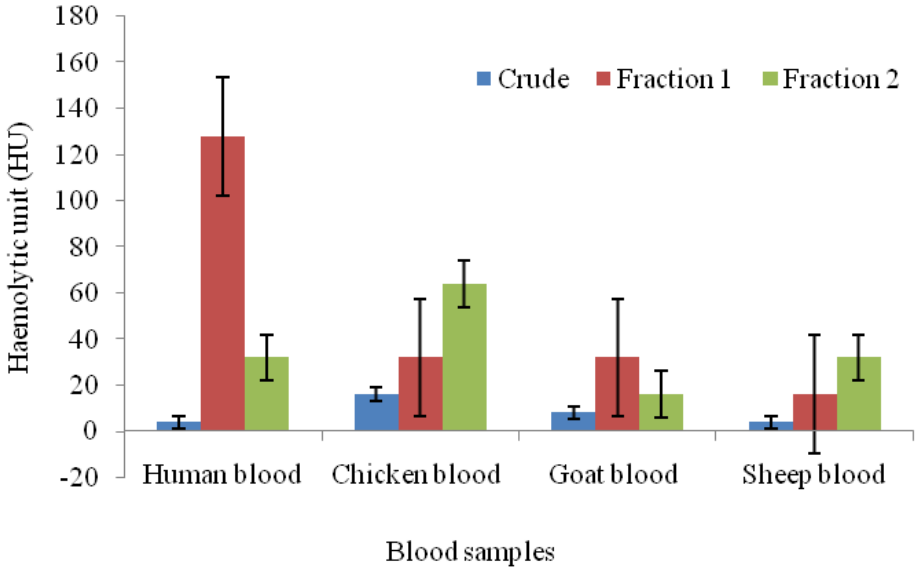

Figure 2. Hemolytic activity of starfish extracts against various blood samples.

were assigned to C-N stretch (aliphatic amine groups). The spectrum 1084 to 1044 cm^{-1} is assigned to C-O strengthening in the COH groups. This spectrum showed that the aromatic C-H stretches are to the left of 3000 cm^{-1} and the alkyl C-H stretches are right of 3000 cm^{-1}.

DISCUSSION

Echinodermata is one of the most distinct phyla among the marine invertebrates, which contain antibacterial, antifungal, antiviral, antitumor, anticoagulant, cytotoxic, hemolytic, antithrombotic and even anti-HIV agents. In this present investigation, crude extract have been isolated from *P. linckii* using methanol solvent which is a universal solvent could extract even the basic proteins. Rio et al. (1965) demonstrated some biochemical and physiological properties from Asteroidea species, with water soluble fractions. Sunahara et al. (1987) extracted two toxins from the body extracts of *Anthopleura*

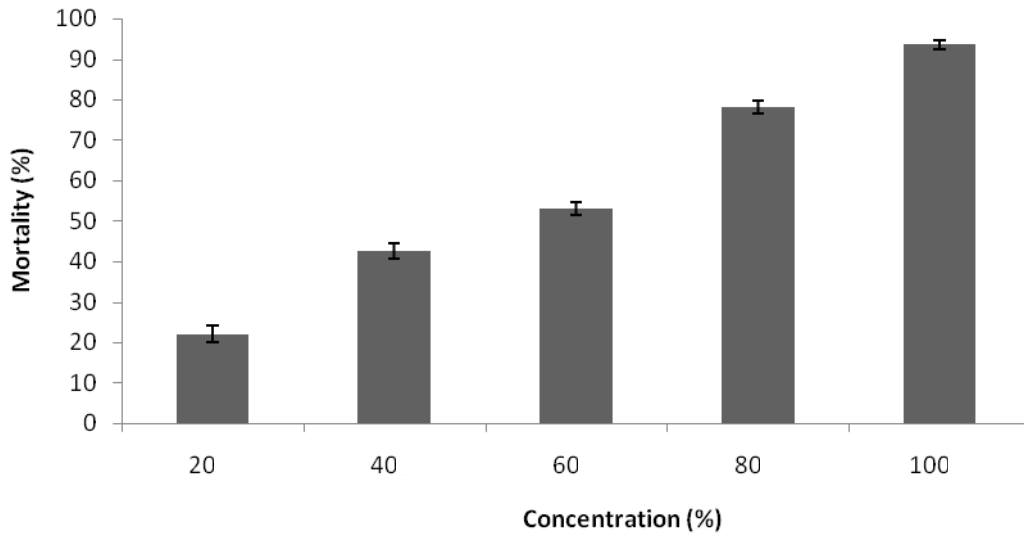

Figure 3. Cytotoxicity of *P. linckii* extract against *Artemia salina*.

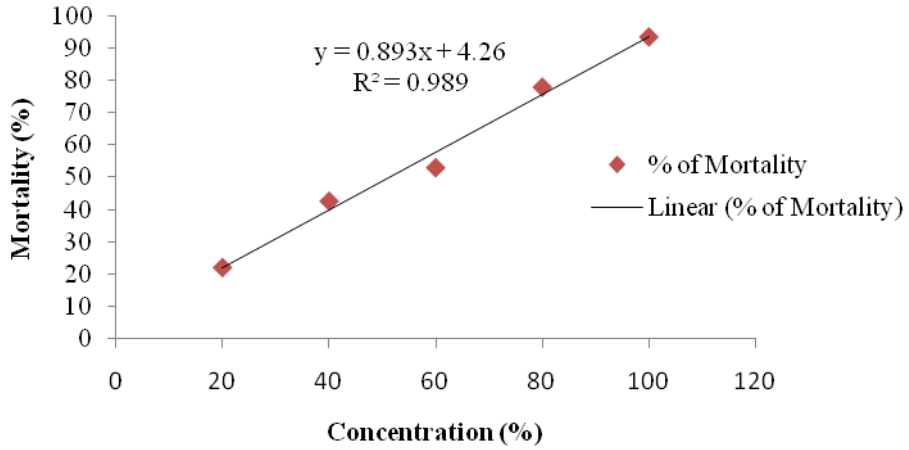

Figure 4. Regression analysis of brine shrimp lethality test.

fuscoviridis using methanol as extraction medium. Kanagarajan et al. (2008) reported the protein content of the crude toxin/fractions was 531 µg/ml, while the amount of protein in the purified fractions varied between 29 and 68 µg/ml in *Stellaster equestris*. The present investigation also coincides with the previous study to the extraction of crude proteins (4.14, 3.63 and 17.5%) from the body of *P. linckii.* Karasudani et al. (1996) purified an anticoagulant factor from the spine venom of the crown-of-thorns starfish, *A. planci*. Antiadhesive mucintype glycoproteins were characterized from the mucus secretions of starfish *Marthasterias glacialis* and *Porania pulvillus*, and the brittle star *Ophiocomina nigra* (Bavington et al., 2004).

The alcoholic extracts of holothurians were found to be inhibitory to human pathogens like *Aeromonas hydrophilia*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* and the green sea urchin *Strongylocentrotus droebachiensis*, the common sea star *A. rubens* and the sea cucumber *Cucumaria frondosa* studied for antibacterial activity (Abraham et al., 2002; Haug et al., 2002). The crude extract of *Holothurians atra* was non-inhibitory to *E. coli* and *S. typhi* at low concentrations. In our study, the crude extract shows good activity against the same, but in higher concentration, that is, 0.75 g/ml and moderate activity in lower concentration, that is, 0.25 g/ml. Similar results were found by SriKumaran et al. (2011) in antimicrobial activity against crude extracts of starfishes *P. linckii* and *P. regulus.* Sri Kumaran et al. (2012) reported the antimicrobial activity of ascidian *Lissoclinum fragile* against human bacterial pathogens and biofouling bacteria.

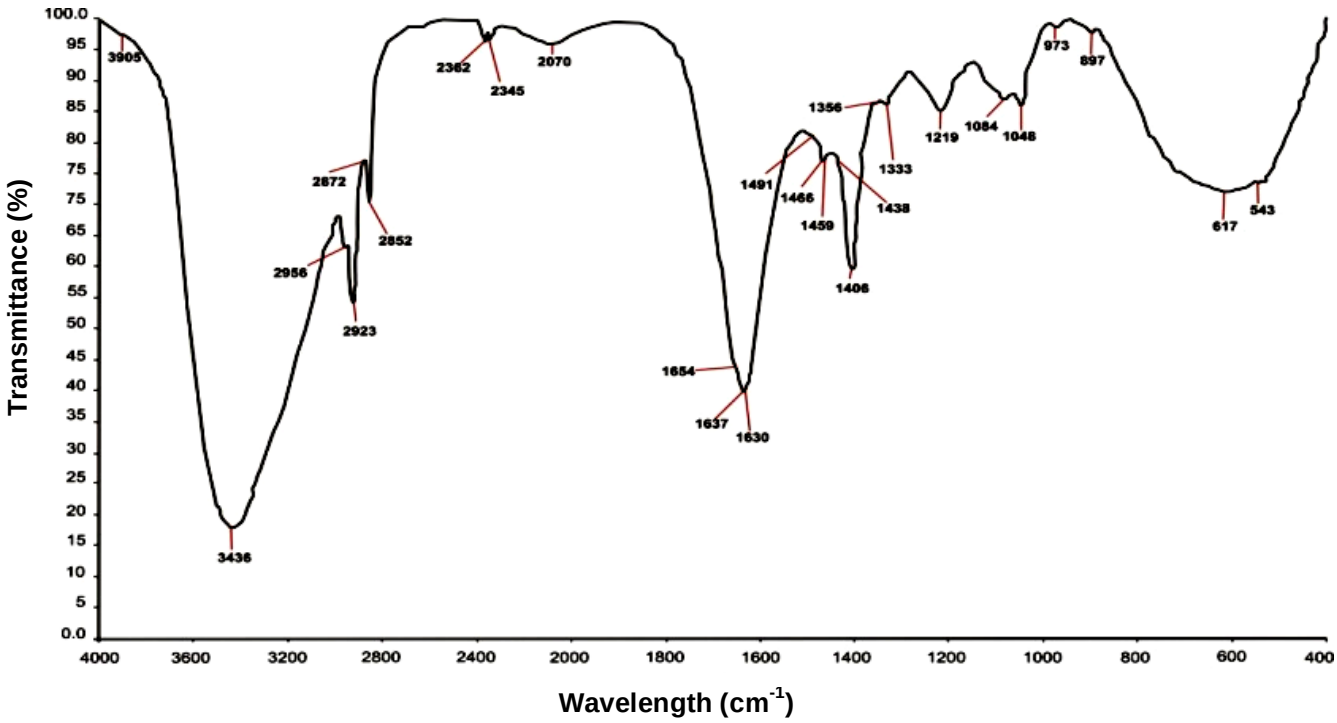

Figure 5. FTIR analysis of starfish *P. linckii* crude extract.

Several drug discovery projects have screened echinoderms for antibiotic activities. Rinehart et al. (1981) showed that the presence of antimicrobial activity in 43% of 83 unidentified species of echinoderms and 58% of 36 unidentified Caribbean species displayed antimicrobial activities. In the Northern Gulf of Mexico, 80% of 22 echinoderm species showed antimicrobial activity (Bryan et al., 1994). Body wall extracts of echinoderms displayed activity against marine bacteria, but did also inhibit settlement of marine larvae (Bryan et al*.,* 1996). The previous studies done about the anti-microbial properties of echinoderm extracts were mainly focus on their glycosides component, particularly the saponin (triterpene glycosides) and saponins-like components that are present on the polar fraction of echinoderm samples (Ivanchina et al*.,* 2000; Avilov et al*.,* 2000; Maier et al*.,* 2001). These substances exhibit anti-microbial activities against fungi, yeast and viruses, however, majority of them do not act against Gram (+) and Gram (-) bacteria.

The capacity of crude extract to lyses red blood cells was found by performing hemolytic assay on microtitre plates and blood agar plates. In the present study, more haemolysis has occurred in human blood. In chicken, sheep and goat blood, moderate haemolytic activity was observed. The metabolites from echinoderms havetendency to cause cell lysis (Carte, 1996) and Rao et al*.* (1991) found out that echinoderms are toxic to both fish and mice. It is evident from the present study that the starfish species have potent hemolytic activity against various red blood cells.

Shiomi et al*.* (1988) studied the properties of starfish venom and found out the amount of protein. In the present study, the amount of protein in crude sample was found out as 4.14%. This will be comparable to previous results. Saponins are the major compound found in starfish and it may be the reason for the pharmacological activities of starfish. The toxin from soft coral *Sarcophyton trocheliophorum* shows significant inhibitory effect on blood agar plate (Karthikayalu et al*.,* 2010) and fish epidermal mucous shows significant hemolytic effect (Bragadeeswaran et al., 2011). The elimination of hemolytic activity by the serum is probably due to the antagonistic effects of serum proteins.

The results of anti-nociceptive assays reveal that the crude extract of *P. linckii* provides the heat tolerance capacity up to 55˚C to the mice. This indicates the central nervous system (CNS) depressant activity. The result of writhing test shows that our extract has the capacity to cure stomach irritation as the writhing response decreases when the sample was injected to mice. The present result exhibited preliminary idea to study the analgesic activity of starfish as a potent drug which may be used as analgesic. Monastyrnaya et al*.* (2002) have reported the cytolysin from *Radianthus* species to be thermolabile and its haemolytic activity decreased linearly with increasing temperature. Kanagarajan et al*.* (2008) reported that the Paw edema in mice was caused by the crude toxin and all fractions of the starfish *Stellaster*

equestris. Sudharsan et al. (2013) screened for hemolytic activity, mice bioassay, analgesic activity and neuromodulatory activity of crude methanolic extract (CME) of sea anemone *Stichodactyla mertensii.*

Recently, novel cytotoxic triterpene glycosides have been isolated from sea cucumbers *Pentamera calcigera* (Avilov et al., 2000), *Staurocucumis liouvillei* (Maier et al., 2001), *Hemoiedema spectabilis* (Chludil et al., 2002) and *Mensamaria intercedens* (Zou et al., 2003). The crude extract of starfish *P. linckii* has been exposed at the cytotoxicity of brine shrimp lethality assay at different concentration. The larval mortality has been increased with increasing concentration. The regression analysis shows that the significance between larval mortality and concentration at 0.01% level (R^2=0.989). The LC_{50} value of the larval mortality has been shown to be LC_{50}=62%. Previously, Carballo et al*.* (2002) used two brine shrimp assays to identify potential cytotoxic substances useful in cancer therapy. The whole body extracts from three echinoderms (*Holothuria impatiens*, *Pseudoconus californica* and *Pharia pyramidata*) that showed a strong cytostatic (growth inhibition) and cytotoxic effect against two human cell lines, lung carcinoma A-549 and colon carcinoma HT-29. Palagiano et al*.* (1996) isolated up to 20 steroid glycosides from the starfish *Henricia downeyae* that caused growth inhibition in bacteria and fungi.

Conclusion

In this work, it is remarkable that the biological activity originally identified in methanolic extracts was related to single compounds whose molecular structures were even identified. The current results suggest that the crude and fractionated extract of *P. linckii* had antimicrobial, haemolytic, antinociceptive and cytotoxic activities.

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