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Pharmacological and Biochemical studies on *Telescopium telescopium* – a marine mollusk from the Mangrove regions

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SUMMARY

The tissue extract (TTE) of a marine snail *Telescopium telescopium,* collected from the coastal regions of West Bengal, India, was extensively screened for pharmacological and biochemical properties. *Telescopium telescopium* (TTE) produced significant lysis of washed rat erythrocytes (both direct and indirect), produced haemorrhagic lesions in the skin and also released haemoglobin (*in vitro* tissue damage) from different tissue samples. TTE was found to produce pro-inflammatory effects when injected into the rat hind paw and also increased peritoneal vascular permeability. Furthermore, intravenous administration of TTE produced a decrease in blood pressure (hypotensive effect) in anaesthetized rats. The extract produced potent esterase activity, as was evident from the breakdown of FDA with subsequent release of fluorescein (*in vitro*). TTE also demonstrated prominent cholinesterase, phospholipase, phosphatase and protease activities.

Key words: Mollusk; *Telescopium telescopium;* Haemolysis; Oedema; Phospholipase; Cholinesterase

INTRODUCTION

Biologically active chemical compounds isolated from cone snails (belonging to the Phylum Mollusca) have found application in medicine and also in research. More than 2600 scientific studies have been carried out over the past 20 years on different species of this phylum. Apart from conotoxins (α, ω and μ; Myers *et al*., 1993), kahalide F, a depsipeptide present in the marine mollusk *Elysia rufescens*, has been reported to posses anti-cancer as well as anti-HIV activities (Hamann *et al*., 1996).

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Telescopium telescopium, a marine multicellular organism, belonging to the phyllum Mollusca, is widely found in the Indian subcontinent, particularly in the eastern coastline and also in the Sunderban Mangroves. Survey of literature revealed the presence of potent anti-microbial (Pakrashi *et al*., 2000) and immunocontraceptive (Pakrashi *et al*., 1992) substances in the spermathecal gland of *Telescopium telescopium*. Moreover, two endo-(1 linked to3)-beta-D-glucanases (Cutler and Yellowlees, 1979) have also been purified from this mollusk.

However, till date there is an acute dearth of information related to the pharmacological and biochemical properties of *Telescopium telescopium*. Accordingly, in the present study, an attempt has

been made to explore the pharmacological and biochemical properties of the tissue extract with particular reference to the enzymatic activities.

MATERIAL AND METHODS

Collection and Identification

Live molluskan species of *Telescopium telescopium* were collected from the creeks of the river Matla, in the Jharkhali area of Sundarbans, West Bengal (India). The Zoological Survey of India (ZSI) New Alipore, Kolkata, India, carried out the identification of the sample.

Preparation of extract

The outer hard shell was removed; the soft tissue portion was homogenized with three volumes of 20 mM phosphate buffer (pH 7.2; 5 min), thereafter the sample was sonicated for 2 min and centrifuged (r.p.m. 10000). The pooled extract was defatted with dichloromethane (DCM) and concentrated under reduced pressure. The DCM cut extract (TTE) was subsequently freeze-dried and stored at -20 °C.

Protein Estimation

Protein concentration was estimated following the method of Bradford (1976), using standard Bradford Kit (Gene) and the concentrations of the extract (unless otherwise specified) was expressed in terms of protein equivalent.

Animals Used

The pharmacological experiments were conducted using adult Swiss albino mice (18 - 22 gm) and rats of Charles Foster strain (120 - 180 gm). The animals were used after an acclimatization period of at least 10 days in the laboratory environment and were housed in standard plastic cages with food and water *ad libitum.* Control vehicle or test materials were administered intraperitoneally unless otherwise specified. All the experiments were performed according to the guidelines of the Institutional Animal Ethical Committee (constituted under the guidelines Committee for the Purpose of Control and Supervision of Experiments on Animals, India).

Acute Toxicity Study - Determination of LD_{50}

For testing of lethality, predetermined doses of the extracts were administered (i.p.) to male mice (18 - 22 gm) and the animals were observed (24 h) for signs and symptoms of toxicity and the mortality in each group (n = 20) was recorded. LD_{50} was determined according to the method of Litchfield and Wilcoxon (1949).

Haemolytic activity

Haemolytic activity of animal toxins can be categorized into two groups: i) those, which produce direct haemolysis of washed erythrocytes and ii) indirect action (requiring the presence of lysolecithin) for inducing haemolysis (Rosenfeld *et al.*, 1962).

Direct Haemolytic activity

Direct haemolytic activity on washed rat erythrocytes was determined according to the method of Boman and Kaletta (1957), with some modifications. Washed erythrocyte suspension (final concentration of 1%) was incubated (at 37 °C for 45 min) with different concentrations (in terms of protein equivalent) of TTE, with occasional shaking. Two control samples were included, one without TTE with saline and another with distilled water (for producing 100% lysis of the RBC suspension). The reaction was terminated by rapid cooling, with ice-cold normal saline. The tubes were then centrifuged for 5 min and the absorbance was recorded at 540 nm.

Indirect Haemolytic activity

Indirect haemolytic activity was assayed using a modification of the method described by Rocha-Campos *et al*. (1996). Erythrocyte suspensions [1% (v/v); in 6 mM calcium chloride] were incubated (45 min, 37 °C) with various concentrations of TTE (5 µg/ml to 120 µg/ml) and 30 µg/ml of lecithin. The reaction was stopped following

addition of 1 ml ice-cold PBS. However, in case of the control, 100% lysis was achieved with the addition of distilled water. Finally the samples were centrifuged at around 2000 rpm (5 min) and the absorbance of the supernatant was measured at 540 nm.

Haemorrhagic and necrotizing activities

The haemorrhagic activity of TTE was evaluated following the modified method of Gutierrez *et al*. (1985) TTE (5 μg), was injected in mice (intradermally) and after 2 h, the animals were sacrificed. The skin, of the mice were carefully removed and observed for the presence of haemorrhage. Different doses of TTE (10, 20, 30, 40 and 50 μg/rat) were injected into the dorsal shaved skin of six rats and after 72 h the animals were sacrificed by ether inhalation; their skins were removed and observed for the presence of necrosis (Theakson and Reid, 1983).

Pathophysiological effect of TTE (*in vitro* tissue damage)

Fresh chicken liver, heart and lungs were washed with normal saline and then the tissue samples (300 ± 10 mg) were incubated in 1 ml of 0.2 (M) K-phosphate buffer at pH - 7.4 for 45 min at 37 °C. The tissues were then washed and again incubated with TTE (50 μg/ml) in 3 ml of 0.2 (M) K-phosphate buffer (at 37 °C). After incubation, reaction mixtures were centrifuged for 5 min at 3000 r.p.m. and the absorbance of the supernatant was read at 540 nm. The percentage of haemoglobin released was calculated with respect to the tissue sample incubated with 0.1% Triton ×100 solution (Datta and Bhattacharya, 1999).

Effect of the TTE on peritoneal dye leakage

Male albino mice (18 - 22 g) were divided into different groups, which received intraperitoneal injection of normal saline, TTE or aspirin. After 3 h, the animals received 4 ml of 0.05 N acetic acid in 0.9% saline (i.p.), followed by the administration of 0.1 ml of 4% Pontamine Sky Blue (i.v.). After 1 h, the animals were sacrificed by cervical dislocation; the abdomen was opened and peritoneal exudates collected. The exudates were centrifuged for 10 min at 2000 r.p.m. and the concentration of the dye was determined at 625 nm (Sen *et al*., 2007).

Exudative inflammation

Male albino mice, randomly divided into different groups, were treated with control vehicle (normal saline), TTE or aspirin (i.p.). After a period of 1 h, 4 ml of 0.05 (N) acetic acid (in 0.9% saline) was administered (i.p.) to the different groups. The animals were sacrificed after 3 h, and the peritoneal exudates were collected (Sen *et al*., 2007). The amount of protein present in the exudates was measured by the method of Bradford (1976).

Release of Autacoids

The effect of TTE on histamine release was examined on mast cells prepared after Bloom and Hagemark, 1965. The prepared mast cells were incubated (37 °C; 30 min) with either the buffer (control), TTE (50, 100, 250 μg/ml) or compound 48/80 (100 μg/ml) as positive control. After incubation, the suspensions were centrifuged and the supernatant was used for further experimentation. For 100% release of histamine, one set of cell suspension was boiled with 0.1(N) HCl and neutralized with NaOH.

Assay of histamine was performed on atroponized (10^{-7} g/ml) guinea pig ileum (in Tyrode solution, at 32 - 35 °C, with oxygenation). The concentration dependent responses (contact time of 30 s and 5 min time cycle) were then recorded. The presence of histaminic activity was confirmed by mepyramine (10^{-8} g/ml), used as specific antagonist (Hamann, 1943). 5HT release was also studied with suspended rabbit platelets (Toh, 1956) pre-incubated with reserpine (serotonin releaser; 3×10^{-7} g/ml), TTE or normal saline, at 37 °C for 4 h. After incubation, the samples were acidified with 0.1 N HCl and were subsequently assayed on isolated rat uterus (Toh, 1956).

Rat Blood Pressure

Male Sprague-Dawley rats (250 - 300 g) were anesthetized with pentobarbitone sodium (50 mg/kg, i.p.). The blood pressure was recorded via carotid artery, using Grass Polygraph (Model 79), while the jugular vein was used as drug port (Puri and Saha, 2003).

Oedemogenic activity

Different doses of TTE (10, 20 and 50 µg; in 50µl of sterile saline) were administered into the subplantar region of the left hind paw. The contra lateral paw received the same volume of sterile saline. Edema was measured with a plethysmometer, at hourly intervals up to the 6^{th} h. Results were then calculated as the difference in paw volumes (Gutierrez *et al.,* 1986).

Evaluation of Phospholipase A_2 (PLA_2) activity.

PLA_2 activity was evaluated using a modification of the method of Lobo *et al*. (1987). TTE in predetermined doses were added to 1.5 ml of 100 mM NaCl at pH 7.6 (containing 10 mM $CaCl_2$, 7 mM Triton × 100, 0.265% Lecithin, 98.8 µM Phenol Red). The change in absorbance of the reaction mixture was measured at 558 nm.

Effect of TTE on Fluorescein diacetate (FDA) hydrolysis (Esterase activity)

Measurement of hydrolysis of the Fluorescein diacetate (FDA) was performed following the modified method of Battin (1997). Reaction mixture containing TTE (5 µg/ml to 100 µg/ml), Maclivian buffer (pH 7.20) and 5 mM FDA were incubated for 30 min. at 40 ºC. The reaction was terminated by the addition of 1 ml ice-cold Maclivian buffer. The samples were centrifuged (10000 r.p.m; 5 min) and the absorbance of the supernatants were measured at 490 nm.

The enzymatic activities of the samples were quantified as µg of fluorescein produced per µg of protein equivalent per min (µg of fluorescein/µg of test protein/min). The standard curve was prepared with different concentrations of Fluorescein di-sodium.

Determination of Acetylcholine Esterase (AChE) activity

The AChE activity was determined by colorimetric assay, according to the method of Ellman *et al*. (1961). The reaction mixture (1 ml) containing 100 mM sodium phosphate buffer (pH 7.0), 1 mM acetylthiocholine iodide (ACTI; Sigma) and TTE were incubated at 25 ºC for 10 min, followed by the addition of 1mM 5,5′-dithio bis-2-nitro benzoate (DTNB; Boehringer Mannheim). After 30 min, the absorbance was measured at 412 nm.

One unit of enzyme is defined as the amount of enzyme required to hydrolyze 1 micromole (µm) of the substrate in 1 min.

Alkaline Phosphatase activity

Reaction mixture containing TTE, 50 mM diethanolamine (pH 10.4), 50 mM NaCl, 2.5 mM $MgCl_2$, 5.0 mM p-nitrophenyl phosphate (p-NPP) was incubated at 37 ºC for 10 min. Thereafter reaction was stopped by the addition of 2 ml of 0.5 M NaOH and the amount of p-nitrophenol released was determined spectrophotometrically at 410 nm.

One unit of enzyme was defined as the amount that liberated 1 µmol/min of p-nitro phenol (Binngham *et al*. 1992).

Protease activity

The proteolytic effect of TTE was evaluated following the method of Doley and Mukerjee (2003). Casein 1% (w/v) in 20 mM phosphate buffer containing 150 mM NaCl, (pH 8.0) was incubated with different concentrations of TTE for 1 h at 37 ºC. Thereafter, 0.5 ml of 10% TCA was added to the reaction mixture and the samples were centrifuged. The amount of digested protein (µg/ml) in the supernatant was determined using Folin-Ciocalteau's reagent.

One unit (U**)** of proteolytic enzyme activity (protease) was defined as that amount which liberates 1 µg amino acids in 1 min.

Statistical analysis

Results were expressed as mean ± S.E.M (n = 10). Statistical analyses were performed with one way analysis of variance (ANOVA) followed by Student's *t* test or Post Dunnet's test, wherever applicable. $P < 0.05$ was considered to be statistically significant.

RESULTS

Acute Toxicity Study

After 24 h of study, the LD_{50} of the extract (TTE) was found to be 800 mg/kg (i.p.).

Haemolytic activity of the extract

TTE produced concentration-dependent lysis of washed rat erythrocytes (both direct and indirect). The HD_{50} dose (amount of extract which causes 50% lysis of erythrocytes) of the extract was found to be 132 µg/ml (for direct haemolysis model) and 57 µg/ml (for indirect lysis) respectively (Fig. 1). Moreover, TTE was found to be more potent in the indirect haemolytic model of the study (Fig. 1).

Haemorrhagic and necrotizing activities

Intradermal administration of the TTE (30 µg) produced prominent haemorrhagic lesions, measuring 10.75 ± 0.73 (n = 6) mm in diameter (Fig. 2). However, no significant necrotic lesions could be observed even after 72 h of observation.

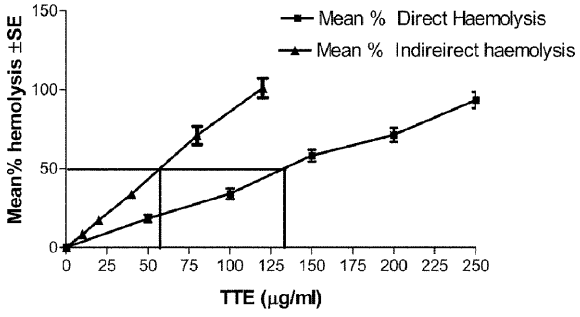

Fig. 1. Evaluation of direct and indirect haemolytic activity of the *Telescopium telescopium* extract (TTE) on washed rat erythrocytes. Values are represented as mean percentage haemolysis ± S.E. (n = 6).

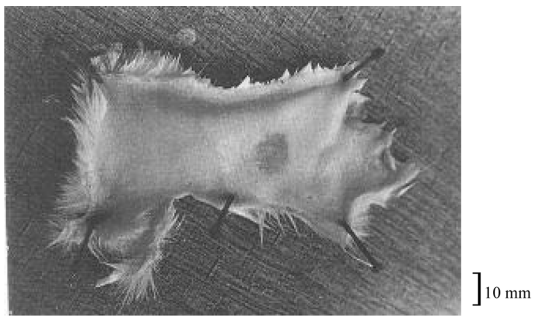

Fig. 2. Haemorrhagic lesions following intradermal administration of the TTE (30 µg) observed after 2 h.

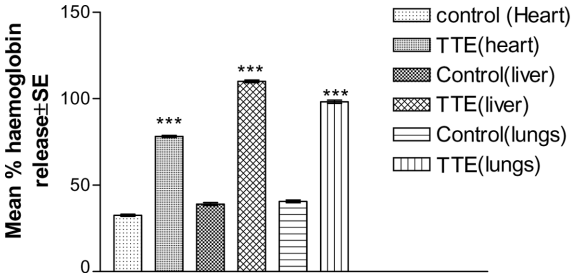

Fig. 3. Effect of the extract on *in vitro* tissue damage. Percentage of heamoglobin release by the TTE from chicken heart, lung and liver was measured at 540 nm. Values are mean percentage ± S.E. (n = 6), (*P* versus control, by "*t*" test, *** < 0.001).

Pathophysiological effect of TTE (*in vitro* tissue damage)

TTE demonstrated significant haemoglobin releasing activity (*in vitro* tissue damage) on incubation with tissue samples from chicken lung, heart and liver. From the results, it was further evident that TTE produced maximum haemoglobin release from the chicken lungs (Fig. 3).

Effect of toxin on vascular permeability (Dye permeability and Protein exudation)

TTE increased acetic acid induced peritoneal exudation and also peritoneal dye leakage. Moreover, TTE (i.p.) also produced significant increase of vascular permeability even in the absence of acetic acid (Table 1).

Release of Autacoids.

TTE caused significant and dose-dependent, release

Table 1. Effect on vascular permeability following TTE induced peritoneal inflammation

Test substances	Effect on Vascular Permeability	
	Peritoneal Dye leakage (Absorbance at 625 nm)	Peritoneal Protein Exudation (mg/ml)
N. S. (Control)	0.036 ± 0.002	02.367 ± 0.357
TTE	0.165 ± 0.011*	07.883 ± 0.323*
N.S+Acetic acid	0.305 ± 0.192*	12.080 ± 0.538*
TTE+Acetic acid	0.494 ± 0.184*	14.520 ± 0.633*
Aspirin+Acetic acid	0.091 ± 0.008	06.100 ± 0.547

NB: NS (Normal Saline); TTE (*Telescopium telescopium*).
Values are mean ± S.E. (n = 6). (*P* versus control, by Post Dunnet's test, * < 0.005).

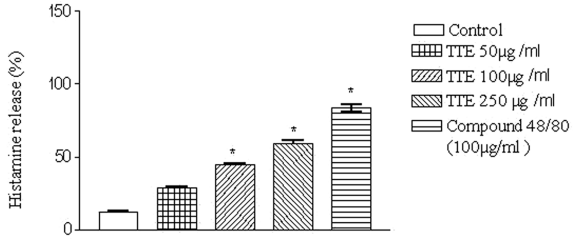

Fig. 4. Effect of TTE on histamine release from peritoneal mast cell. Values are percentage mean of histamine release ± S.E. (n = 10), (*P* versus control, by Post Dunnet's test * < 0.005).

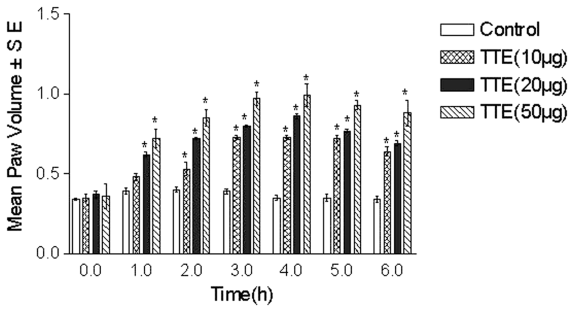

Fig. 6. Oedemogenic activity of TTE on rat paw. Test samples were injected into subplantar region of the left hind paw. Edema was calculated as the difference in paw volumes. Values were mean ± S.E.(n = 6), (*P* versus control, by Post Dunnet's test *<0.005).

of histamine from peritoneal mast cells. However, incubation of TTE (up to 1000 µg/ml) with platelets did not produce any significant change in serotonin release (Fig. 4).

Rat Blood Pressure

Intravenous administration of TTE (0.1 and 0.2 mg/kg) produced a decrease in blood pressure in anaesthetized rat. The normal blood pressure was restored after a period of 10 min (Fig. 5).

Oedemogenic activity

Intraplantar administration of TTE produced significant and dose-dependent increase of paw volume (oedema formation) up to a period of 4 h (Fig. 6).

Enzymatic activity of TTE

Incubation of TTE with different substrates revealed the presence of diverse enzymatic activities. The extract (TTE) demonstrated potent protease, AChE, phospholipase, alkaline phosphatase and esterase activities in the following order: esterase > alkaline phosphatase > phospholipase > acetylcholine esterase

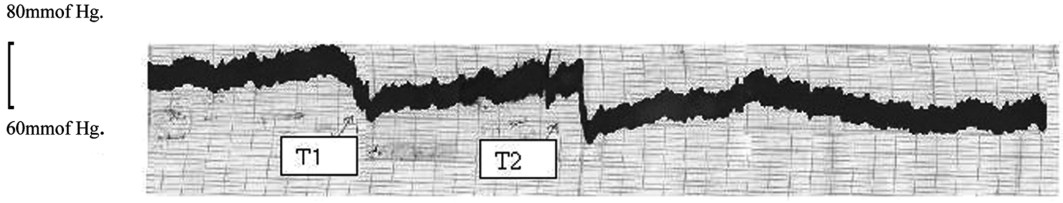

Fig. 5. Effect of TTE on rat blood pressure. Test samples (T1 = 50 mg/kg and T2 = 100 mg/kg) were administered through cannula attached with the jugular vein and blood pressure was measured by Grass Polygraph (Model 79).

Table 2. Enzymatic activity of TTE as observed on incubation of TTE with different substrates (acetyl-thiocholine iodide, lecithin, fluorescein diacetate, casein and p- nitrophenyl phosphate)

Enzymatic activity	Specific activity (U/mg of protein)
Acetylcholine Esterase activity	0.18
Phospholipase A_2 (PLA_2) activity	0.24
Esterase activity	0.32
Protease activity	0.16
Alkaline Phosphatase activity	0.26

> protease (activity represented in milligrams of protein; Table 2).

DISCUSSION

The bioactive components of marine mollusks have been reported to contain different types of bioactive components, namely neurotoxin, haemolysin, cardiotoxin, cholinesterase and also various biogenic amines (Narahasi, 1974; Roseghini *et al*., 1996). *Telescopium telescopium*, an edible gastropod, is widely found in the Eastern coastline of India. In the present study, the *Telescopium telescopium* tissue extract was tested for different pharmacological and biochemical activities.

From the results, it was observed that TTE demonstrated both direct and indirect haemolytic effects. The indirect (HD_{50} 57 μg/ml) activity was found to be more potent when compared to the direct haemolytic (HD_{50} 132 μg/ml) effect. Moreover, both indirect haemolysis as well as the oedemogenic property of TTE were effectively inhibited by different phospholipase blockers (data not shown). It is also known that phospholipases present in several animal venoms impart their action on cell membranes via indirect haemolytic action (Gary *et al*., 2000). However, a majority of PLA_2 are also known to be devoid of direct haemolytic activity (Robin Dolley *et al*., 2003). Therefore, the present findings with TTE indicate the probable involvement of a phospholipase A_2 like component.

Furthermore, from our studies it could be observed that TTE exhibited weak proteolytic activity. Since proteases are known to contribute greatly towards excessive protein degradation in intact cell membranes (Santamaria *et al*., 2002), it can be assumed that the observed proteolytic activity may be a contributing factor during the erythrocyte lysis.

TTE was found to release haemoglobin on incubation with chicken lung tissue in a manner similar to a phospholipases. NK-PLA2 isolated from Indian monocled cobra (*Naja kauthia*) venom, which has also been found to produce similar effects in chicken heart, liver and lung tissue strips (Robin Doley *et al*., 2003). Therefore, the tissue damaging activity of TTE could be attributed to the rupturing of blood capillaries, which is necessary for the release of haemoglobin. This rupturing of capillaries may be contributed by PLA_2 and the accompanying proteases present in the extract. However, the haemoglobin release was found to be tissue dependent (highest in lungs) The variable activity could be attributed to the tissue specific phospholipids/cholesterol ratio, presence of specific phospholipids and the vitamin E content of the tissue (Kini, 1997; Mukerjee *et al*., 1998).

Local oedema, redness and pain are some of the major pathophysiological manifestations that have been reported to occur with many different marine species (e.g. jellyfishes, sea anemones), including *Telescopium telescopium.* In our present study, both TTE (60% at a dose of 250 μg/ml) and compound 48/80 (82% at a dose of 100 μg/ml) were found to liberate histamine from the mast cells. Furthermore, intraperitoneal administration of TTE produced extensive peritoneal exudation as evident from increased protein content (of the peritoneal exudates), along with increased dye leakage in the peritoneal cavity, indicating the role of TTE on capillary permeability. These findings were further reinforced by the oedemogenic property, observed with TTE. Furthermore, TTE also produced hypotensive effect in anaesthetized rat, and such activity in turn could be correlated to the increased capillary

permeability and histamine liberation (Furchgott, 1984).

It was observed that TTE produced severe edema in rat hind paw within 1 h following subplantar injection and the swelling was found to persist for several hours. The oedemogenic (pro-inflammatory) activity of the extract was significantly inhibited by various phospholipase inhibitors, indicating the probable involvement of phospholipase (s) enzyme like component (s) in the extract.

The extract also produced strong esterase activity, as was evident from the breakdown of FDA and subsequent release of fluorescin (*in vitro*). The extract also demonstarted alkaline phosphatase, phospholipase, cholinesterase (a promising biomarker for environmental monitoring and a therapeutic target) and protease activities. The alkaline phosphatases (AP) from marine sources appear to be very interesting for the understanding of life forms at high salinity and are also correlated with inorganic phosphate starvation.

Therefore, on the basis of the present investigations, *Telescopium telescopium* was found to contain a variety of interesting biological activities and therefore warrants further evaluation.

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