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Potential future applications of spermatheca extract from the marine snail *Telescopium telescopium*

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Abstract: Cytosol fraction of spermatheca and/or the ovotestis gland from the marine snail *Telescopium telescopium* was found to have antimicrobial, antiprotozoal, antifertility, and immunomodulation properties. The present experiment was conducted to explore the biochemical nature, composition, and pharmacological properties of the cytosol fraction along with its toxicity on the hematopoietic system in vivo. This glandular extract was analyzed and found to contain enzymes, hormones, minerals, vitamins, and other biologically active components like higher vertebrates. The present study showed that the extract was able to restrict the induced tumor (sarcoma-180) growth in mice concomitantly with an increased life span of the tumor bearing mice, but without any hematopoietic toxicity. This antitumor property was thought to be mediated immunologically as also found in other aspects of pharmacological actions of this extract; hence, it could be developed as a potent immunomodulator to be used for various pathological conditions in general, but in cancer in particular.

Key words: Anticancer, immunomodulation, snail, spermatheca extract

Introduction

The marine ecosystem has been recognized as an important source of bioactive compounds having medicinal potential. The research on marine natural products in the last 3 decades has led to the discoveries of many chemically and biologically interesting molecules (1). The major sources of biomedical compounds obtained from invertebrates include sponges (37%), coelenterates (21%), microorganisms (18%), algae (9%), echinoderms (6%), tunicates (6%), molluscs (2%), and bryozoans (1%) (2). Mollusc, one of the most abundant marine species, yielded some important anticancer compounds like dolastatin (3), acharan sulphate (4), kahalalide F (5), and kulolide (6).

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Telescopium telescopium, a marine mollusc (snail), holds an interesting role for many reasons. Fascinating previous works with the cytosol fraction of spermatheca and/or ovotestis, a complex organ of the marine mollusc *T. telescopium*, exhibited antimicrobial (7), antiprotozoal (8), reversible antifertility, (9) mammalian sperm agglutinating, immobilizing, and spermicidal properties (10). The strong immunomodulatory effect on the female reproductive system of rats was also observed (11). The antiserum of the spermatheca extract was found to cross-react with the human sperm plasma membrane, as well as with the rat testicular tissues, respectively (12).

The crude extract of the spermatheca gland, therefore, appeared to be inherent with pharmacobiological and immunomodulatory properties, which hypothetically could further be employed for many pathological conditions in general, including cancer in particular. Hence, it seems important to analyze the biochemical nature of this glandular extract, and study its toxicity on the hematopoietic system and explore any other additional pharmacobiological properties, especially for cancer biology as marine invertebrates, in particular molluscs, are emerging as one of the most important sources to develop novel chemotherapeutic agents against tumor cells.

Materials and methods

Preparation of the glandular extract:

The adult snail *T. telescopium* was collected from the estuaries of the Bay of Bengal near Sagar Island (22°19′N; 80°03′E), West Bengal, India. The outer shell of the snail was broken carefully. The whole body was removed from the shell and washed thoroughly with 0.15 M phosphate buffer saline (PBS) solution, pH 7.4. The spermatheca/ovotestis gland was dissected out from the body and collected in a sterile beaker containing ice cold PBS. The dissected glands from 50 snails were homogenized collectively, and sonicated at 4 °C for 30 s, each time with a pause of 60 s at 6 μ amplitude for 1 h. The sonicated material was then centrifuged (15,000 rpm) at 4 °C, and the collected supernatant was filtrated through a membrane filter. The collected filtrate (*Telescopium* bioactive compound (TBC)) was aliquoted, lyophilized, and preserved at -20 °C.

Biochemical analysis of the TBC

Biochemical analysis of the TBC was performed with commercial kits.

Identification of eluted protein by SDS-PAGE

Polyacrylamide gel electrophoresis (10%) was performed (13) under reducing conditions and approximately 40 μg of protein was loaded in each lane. Molecular weight markers (Bio-Rad) were also run in parallel.

Analysis of neutral sugar

For gas liquid chromatography, a Hewlett-Packed 5890 series II gas chromatograph with a flame-ionization detector was used. Resolutions were performed with a glass column (1.8 mm × 2 mm) containing 3% of SP-2340 on Supelcoport (100-200 mesh) at 200 °C for the alditol acetate of neutral sugars. A recording integrator HP 3396 A (Hewlett-Packed) was used to determine the peak areas. The TBC 100 μg (TBC stock solution, 1 mg/mL in distilled water) was hydrolyzed with 2 M trifluoro acetic acid (1) in a sealed tube at 120 °C for 1.5 h, then myo-inositol was added as an internal standard. The acid was removed by co-distillation with water by evaporation in vacuo. The hydrolysate was then reduced with sodium borohydride and acetylated with acetic anhydride/pyridine (1:1; v/v) and converted into alditol acetates (14).

Animals

In total, 72 healthy, adult Swiss mice were selected from a breeding colony and kept under isomanagemental conditions. The animals were divided into 12 groups, containing 6 mice in each group.

Transplantation of Sarcoma 180 (S-180)

Of the healthy, adult Swiss mice, 60 were selected and developed with S-180. The present form of the S-180 ascites tumor was developed by Charlotte Friend by i.p. injection of minced S-180 tissues into the Swiss albino mice. The ascites fluid contains about 7-10 × 10^7 tumor cells/mL on the seventh day after tumor transplantation. Ascites fluid was withdrawn from the peritoneal cavity of donor tumor bearing mice, 10 days post-transplantation of tumor cells and diluted

with sterile saline so that 1 mL of fluid contained 3.5 $\times 10^7$ cells. In addition, 0.5 mL of this fluid containing 1.75 $\times 10^7$ cells/mL was injected subcutaneously in the lateral side of the thigh region. After 7-8 days, a tumor was palpable in that region and considered for experimental purpose.

Evaluation of antitumor property

The animals were divided into 10 groups, containing 6 mice in each group. Of these 10 groups, 4 (III, IV, V, and VI) received the TBC at the dose rate of 1, 2, 3, and 5 mg/kg body weight, respectively, as a single dose, while the same amount was inoculated as a multiple dose on every alternative day for 3 injections to the other 4 groups (VII, VIII, IX and X). The first 2 groups (I and II) received normal saline as single and multiple doses, respectively, as a placebo like the corresponding doses for each design and volume of the tumor ($\pi/6$ LW^2 where L and W are length and width of tumor) in all the groups were measured and calculated (15).

Histopathological Examination of Tumor Tissue

Sections (5 μ) of the tumor tissues from 6th and 21st day of post-transplantation and treatment groups were prepared. All the tissue sections were processed and stained with hematoxylin and eosin stain and mounted with DPX. The stained sections were observed under a light microscope (400×).

Evaluation of toxicity in hematopoietic system:

The study group (XI) received the said amount of the TBC (3 mg/kg) as a single intraperitoneal dose, whereas the control group (XII) was administered 1 mL of double distilled water for injection by the same route. For the counting of total bone marrow cells, 2 mice from each group were sacrificed, the femur was collected, and marrow cells were flushed with normal saline and collected in a centrifuge tube after removing the femoral condyles and trochlear. Total leukocyte count (TLC), differential leukocyte count (DLC) and bone marrow count were performed by standard Giemsa staining procedure after preparing the smear on the microscopic glass slides. All the stained slides were observed under a microscope (400×; 1000×) and cell counts were recorded and analyzed.

Results

Biochemical analysis of the TBC

The TBC contains a wide range of bioactive compounds, which are presented in Table 1.

Table 1. Biochemical analysis of the TBC.

SL. No.	Composition	Concentration	SL. No.	Composition	Concentration
1	Inorganic phosphorus	0.96 mg/dL	18	Glucose	25 mg/dL
2	Iron	212 μg/dL	19	Insulin	6.2 μIU/mL
3	Magnesium	10.6 mEq/L	20	GGT	16.0 U/L
4	Copper	55 ppm	21	CPK	2.0 IU/L
5	Lithium	0.4 mEq/L	22	TSH	0.05 μIU/mL
6	Non-Protein Nitrogen	1.7 mg/dL	23	SGPT	148 U/L
7	Urea	12 mg/dL	24	SGOT	352 U/L
8	Creatinine	0.2 mg/dL	25	LDH	17 IU/L
9	Cholesterol	38 mg/dL	26	AP	10 U/L
10	Triglycerides	34 mg/dL	27	ALP	179 U/L
11	HDL	8 mg/dL	28	Testosterone	1.75 ng/mL
12	Total protein	286 mg/dL	29	Progesterone	5.0 ng/mL
13	Albumin	180 mg/dL	30	Estradiol	201 pg/mL
14	Globulin	106 mg/dL	31	Amylase	2.0 IU/L
15	Sodium	82 mEq/L	32	Lipase	29.0 IU/L
16	Potassium	4.5 mEq/L	33	Vitamin B12	1504.0 pg/mL
17	Bicarbonate	12 mEq/L	21	CPK	2.0IU/L

Identification of eluted protein by SDS-PAGE:

SDS-PAGE of the TBC revealed that nearly 20 Coomassie blue-positive components had an estimated molecular weight between 90 kD and 16 kD, of which 68, 64, 55, 53, 51, 46, 37, 34, and 23 kD proteins showed major polypeptide bands; however, 90, 87, 84, 78, 72, 47, 45, 30.5, 28, 26, 24, 18, and 16 kD proteins had minor polypeptide bands, respectively (Figure 1).

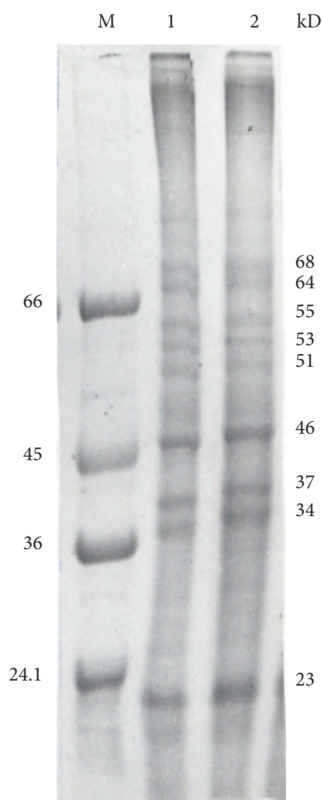


Figure 1. Electrophoretic analysis (10% SDS-PAGE) of the TBC (Lanes 1 and 2) showing the Coomassie blue positive polypeptide bonds (M = Biomarker).

Analysis of neutral sugar

The neutral sugar was detected as alditol acetates by gas liquid chromatography, and was found to contain approximately 96% glucose as neutral sugar (Figure 2).

Tumor growth inhibition study

In the treatment modality, different amounts of the TBC as single intraperitoneal dose were evaluated. A significant reduction ($P < 0.5$) in the tumor growth was observed with a single dose (3 mg/kg); however, the growth inhibition was not dose dependent (Table 2). The other doses in single and multiple designs (1 mg/kg, 2 mg/kg, and 5 mg/kg) at the initial phase showed significant ($P < 0.05$) reductions in tumor growth; however, in the later phases these growth inhibition patterns were not as significant as the 3 mg/kg body weight dose (Tables 2 and 3). A single intraperitoneal dose of 3 mg/kg of body weight exhibited a constant inhibition pattern of tumor growth concomitantly with an enhanced life span of the tumor-bearing host (Table 4).

Evaluation of toxicity in the hematopoietic system

Total leukocytes count (TLC)

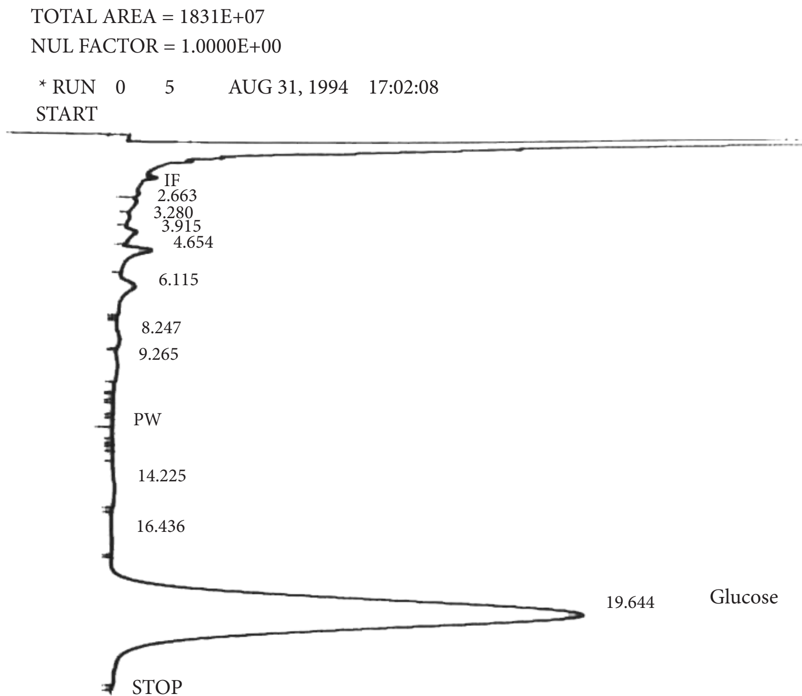
The TBC, when administered intraperitoneally at the dose rate of 3 mg/kg body weight in normal, healthy, Swiss mice, initially the total leukocyte count increased significantly ($P < 0.05$), followed by gradual diminution; however, it maintained a significant ($P < 0.05$) incremental gap from the initial one (Table 5).

Differential leukocytes count (DLC)

The differential leukocyte count did not alter significantly in Swiss mice treated intraperitoneally with the TBC at the dose rate of 3 mg/kg body weight (Table 6).

Table 2. Tumor volume in single dose design on different observation days (different superscript denotes significant difference exists at 5%).

Dose of the TBC (mg/kg)	Tumor Volume (mm^3) on different days					
	9	11	13	16	18	21
Control (Untreated group)	427^a	455^a	632^a	975^a	1719^a	2209^a
1	214^c	285^b	403^d	568^d	1149^c	2096^e
2	103^b	158^c	147^c	153^c	286^b	622^d
3	234^c	261^b	233^b	205^b	228^b	252^c
5	93^b	214^b	214^b	271^b	282^b	492^b



Closing signal file M: SIGNAL . BNC
Storing. processed peaks to M: Q8F04A10. PRO

SIGNAL FILE: M: SIGNAL.BNC
PEAK FILE: M: Q8F04A10.PRO
AREA%

RT	AREA	TYPE	WIDTH	AREA%
3.915	22207	PV	.323	.59284
4.654	53325	VP	.328	1.42358
6.115	44772	PP	.499	1.19525
8.247	11580	VV	.564	.30914
9.265	12837	VP	.626	.34270
14.225	13922	VP	.917	.37167
19.644	3587197	PV	1.424	95.76480

TOTAL AREA = 3745838
MUT FACTOR = 1.0000E+00

Figure 2. Neutral sugar glucose present in "TBC" detected by GLC.

Table 3. Tumor volume in multiple dose design of the TBC on different groups and observation days (different superscript denotes significant difference exists at 5%).

Dose of the TBC (mg/kg)	**Tumor Volume (mm^3) on different days**				
	0	**11**	**15**	**19**	**25**
Control (Untreated group)	219.73^{a}	1031.17^{a}	1768.73^{a}	2674.18^{a}	3664.95^{c}
1	300.67^{d}	1038.41^{a}	1630.9^{b}	2560.75^{a}	3300.37^{a}
2	168.31^{c}	1136.92^{c}	1680^{b}	2487.63^{b}	3196.55^{a}
3	108.57^{b}	690.24^{b}	1266.81^{c}	1690.97^{c}	2551.65^{b}
5	200.83^{a}	1026.99^{a}	1600.25^{b}	2328.09^{b}	2825.67^{b}

Table 4. Life span of the different groups treated with different doses of the TBC (different superscript denotes significant difference exists at 5%).

Dose of the compounds(mg/kg)	**Life span after tumor transplantation (days)**	
	Multiple dose	**Single dose**
Control (Untreated group)	34[a]	37[a]
1	41[b]	47[b]
2	43[b]	68[c]
3	54[c]	72[c]
5	46[b]	63[c]

Table 5. Total leukocyte count on different days in intervals after inoculation with the TBC compared with the control group (different superscript denotes significant difference exists at 5%).

Category	**Total Leukocyte Count ($\times 10^7$) in days of observations**			
	3	**6**	**21**	**29**
Control Group (I)	3.2[a]	3.2	3.0	3.2
Experimental Group(II)	1[b]	4	3.0	3.2

Table 6. Differential leukocyte count on different days in intervals after inoculation with the TBC compared with the control group.

Category	**3rd day**		**6th day**		**21st day**	
	Study group	**Control**	**Study group**	**Control**	**Study group**	**Control**
Neutrophil	77	77	78	73	77	77
Lymphocyte	20	21	20	24	20	19
Monocyte	1	1	1	1	1	2
Eosinophil	2	1	1	2	1	2
Basophil	0	0	0	0	1	0

Bone marrow count

Intraperitoneal inoculation with the TBC at the same dose rate increases the bone marrow count significantly ($P < 0.05$), unlike most other commonly used chemotherapeutic drugs (Table 7).

Histopathological findings

Histopathological observations revealed that the blood vessels and nerves were free from malignant cell invasion after treatment with the TBC (Figures 3 and 4).

Discussion

The present biochemical analysis revealed that the TBC is enriched with many biologically active compounds. The reversible antifertility effect in male rats that was initiated by IgG fraction of antiserum raised against spermatheca extract could be explained by the fact that glycoprotein epitopes of rat testicular tissue resemble those of spermatheca/ovotestis of *T. telescopium* (9). However, this cross-reactivity might suggest the presence of common sperm surface antigenicity between the species, as well as that

Table 7. Bone marrow count on different days in intervals after inoculation with the TBC compared with the control group (different superscript denotes significant difference exists at 5% level between groups).

Days of observation	Count in different groups (× 10^6/mL)	
	Control	Treated
0 day (before treatment)	13.5	13.7
6th day after treatment	14^a	37.75^b

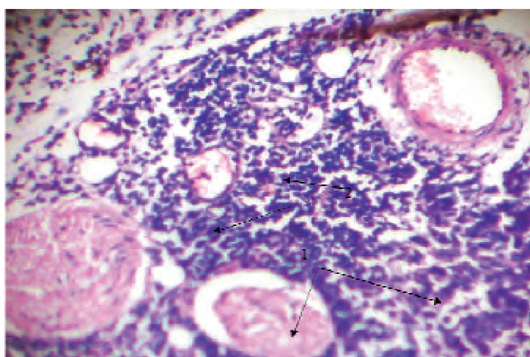


Figure 3. Photomicrograph showing the section of the 21st day post-transplantation tumor tissue in control group (HE ×100) 1. Myonecrosis. 2. intermascular space.

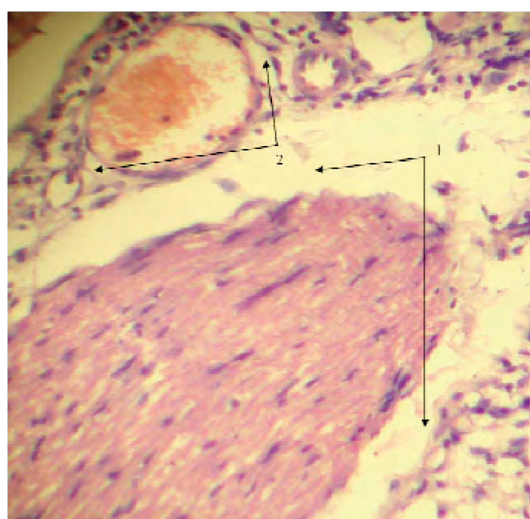


Figure 4. Photomicrograph showing the 21st day post-transplanted section of tumor tissue in the treatment group (HE ×400) 1. Nerve bundle appearing completely free from invasion. 2. Blood vessels without malignant invasion.

spermatheca extract possesses species-specific carbohydrate sequences, which were responsible for the initial recognition steps between these diversified species, and that the antigen(s) was/were related immunogenically.

Among the current interest in immune responses and malignancy is the hope that the immune system might be used to help cancer patients (16,17), because both cellular and humoral immunity are important in controlling the growth of malignant cell populations, and that it is the interactions between them that are most important (18).

Kinetic studies from the tumor growth, following the administration of the compound, showed that 3 mg/kg body weight single dose schedule of the compound significantly restricts the tumor growth in a manner that indicates the tumor static property of the compound. This may be due to the antiangiogenic property of the compound rather than its direct tumor cell killing ability; though from the available results the exact underlying mechanism is not clear. Additionally, it may have an immunomodulator property without any hematopoietic toxicity as the TBC increases the total leukocyte count, but did not alter the differential leukocyte count. This strongly suggests that the compound possesses a strong immunostimulating property, which may play some vital role in immunological control of neoplastic cell division. This immunostimulation property is again reconfirmed by an increased bone marrow count in the treated group. Moreover, the TBC is able to enhance the life span of tumor bearing hosts, which may be the result of the inhibition of suffering, pain, less nutritional compromise, reduced cancer associated immunosuppression, and opportunistic infection. Furthermore, chance of metastasis seemed

to be reduced as the blood vessels and nerves were found to be free of malignant cell invasion after the TBC treatment, and possibly be the another reason for the increased life span of the treated animals.

Therefore, this unique, novel, and crude extract isolated from *T. telescopium,* which was found to have different dimensions of pharmacological actions many of which from immunological pathways could be developed in the future as potent and effective immunomodulators against various disease conditions in general, and as an antitumor agent(s) in particular.

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