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Investigation on neurotoxin of sea snail meat

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ABSTRACT

Objective: To explore the neurotoxic agent tetramine and characterized with cytotoxicity studies from the chief constituents of sea food *Trochus radiatus* (*T. radiatus*) and *Thais rudolphi* (*T. rudolphi*) for coastal people of India.

Methods: Extraction was performed by following the method of Hashizume *et al.* (1987) with apposite modification. The extracted aqueous layer was chromatographed on a column of diethylaminoethyl-Sephadex and Sephadex LH-20. To analysis the toxic compound of *T. radiatus* and *T. rudolphi* has been done by high performance thin layer chromatography, gas chromatography-mass spectrometer, and the spectral data was examined by Fourier transform infrared spectrum spectroscopy. The cytotoxicity studies of the purified samples were assessed by hemolytic assay, brine shrimp assay and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – cell proliferation assay.

Results: The tetramine content was estimated as 0.4 µg/g and 1.2µg/g respectively (w/w). The maximum haemolytic activity in *T. rudolphi* was found to be 256 haemolytic unit and 16 haemolytic unit in *T. radiatus* against human erythrocytes when compared to chicken erythrocytes. The samples exhibited lethality against brine shrimps at 60 and 7 µg/100 mL, respectively. The tetramine from *T. rudolphi* and *T. radiatus* showed 54.2% and 70.8% of cytotoxicity against human lymphocyte at 2 mg/ml concentration. Further in the cell morphology studies, cell showed condensed chromatin, cytoplasmic blubbing and detachment from the surface. Furthermore, the presence of tetramine was confirmed based on the *R_f* values and it was chemically identified as Tetramethylammonium chloride (Pub Chem CID: 6379) through the gas chromatography-mass spectrometer analysis.

Conclusions: From the human health point of view, though the residue levels of N,N,N'-trimethyl-1,2-ethanediamine, Tetramethylammonium chloride and N,N-dimethylglycine detected in this study are well below the maximum residue limits of consuming level, the continuous intake may probably have side effects at the later stage. The field of marine gastropods toxin-ology is still in its infancy but the potential yields should attract more interest in the coming years and this report has been a significant development in the stoppage; control and even suppression of human hazards.

1. Introduction

Molluscs are parsimoniously and commercially important as food and non-food resources on a global scale[1-3]. The edible gastropods limpets, trochids (*Trochus* sp.), whelks (*Thais* sp.), the sacred chank (*Turbinella pyrum*), olives (*Oliva* sp.), the green snail (*Turbo*) *etc.*, are represented in the intertidal and shallow waters of Indian waters. They are consumed by fishermen and coastal people for their food

persistence due to their high nutrient content[4]. Despite the fact, not all molluscs are edible sometimes serious perils may also occur upon ingestion of some non-edible molluscs. Midst 85 species are known as poison to man either their toxin or by the way of venom secretion. Eating this kind of shellfish can cause serious illness and number of human intoxications[5].

Tetra methyl ammonium chloride, commonly called tetramine, is a neurotoxic compound of simple quaternary amine that was first isolated and identified from a sea anemone by Ackermann *et al.*[6]. Tetramine poisoning may result in number of symptoms, which include headache, dizziness, fatigue, weakness, lethargy, nausea, vomiting, perioral paresthesias (numbness around the mouth) and anorexia while high levels of exposures are characterized by seizures and even the coma[7]. The high concentrations of tetramine have been detected in variety of shellfishes. Its included *Buccinum leucostoma*[8], *Neptunea*

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antiqua (*N. antiqua*)[9,10], *Neptunea arthritica*[11-13] and *Neptunea lyrata*[14]. Several methods have been developed for the quantitative determination of tetramine in shellfish. Liquid chromatography with refractive index detection[15], proton nuclear magnetic resonance spectroscopy[16] and capillary zone electrophoresis-tandem mass spectrometry[17]. In this paper, we describe the accumulation of shellfish toxin in the form of tetramine in two edible marine molluscs such as the top shell [*Trochus radiatus* (*T. radiatus*)] and the rock-whelks [*Thais rudolphi* (*T. rudolphi*)]. The presence of tetramine was characterized through the gas chromatography-mass spectrometer GC-MS analysis. Further, cytotoxicity levels were also described to alarm the shellfish poisoning towards human consumption.

2. Materials and methods

2.1. Collection of animals

The species *T. radiatus* and *T. rudolphi* were collected from the four different landings lies between Ramanathapuram to Kanyakumari along the South East coast of India. The collected animals were transferred into laboratory in an ice box. The shells were broken and the wet tissue was stored at 4 °C.

2.2. Tissue extracts preparation

The tissue samples were used for the extraction of tetramine. Extraction was performed by following the method of Hashizume *et al.*[18] with suitable modification. Each tissue sample was extracted twice with 10 volumes of methanol and the extract was refluxed in methanol for 30 min and filtered after cooling. The filtrate containing methanol was concentrated under reduced pressure and further defatted with ether. The resulting aqueous layer was stored until use.

2.3. Purification of tetramine like compounds

The extracted aqueous layer was chromatographed on a column of diethylaminoethyl-Sephadex eluted with 0.1 N HCl at the flow rate of 1 mL/1 min. The residue was collected and dissolved with 5 mL of methanol. The elution of the follow-on syrup was attained by column chromatography on Sephadex LH-20 (16.0 × 1.5 cm) eluted with the methanol at same flow rate. The resulting elute was reduced under pressure using a rotary flash evaporator. Finally, the extracts were lyophilized and then stored in refrigerator for further analysis.

2.4. In vitro assay for cytotoxicity activity

2.4.1. Haemolytic activity

The haemolytic activity of the purified samples on chicken and human erythrocyte was evaluated by micro haemolytic method[19]. The micro haemolytic test was performed in 96 well micro-titre plates. Different rows were selected for chicken and human erythrocyte suspensions. Serial dilutions of the purified samples (100 µL) were made in 100 µL of normal saline. A total of 100 µL of 1% washed erythrocyte (RBC) was added to all the wells. Then 100 µL of 1% RBC was added with 100 µL of distilled water kept as positive control and 100 µL phosphate-buffered saline (PBS) added with 100 µL of 1% RBC kept as negative control. After, the plate was gently shaken and allowed

to incubate at room temperature for 2–3 h. Red colour suspension in the wells considered as positive hemolysis and a button formation of bottom wells was considered as lack of hemolysis. The reciprocal of the highest dilution of the purified sample shows the haemolytic pattern was taken as one haemolytic unit (HU).

2.4.2. Brine shrimp assay

The brine shrimp lethality bioassay was performed following standard adopted procedure[20]. It was evaluated using the efficiency of *Artemia nauplii* against different concentration of both purified samples. Phototrophic larvae (nauplii) were collected by a pipette and transferred (10 nos.) to vials filled with sea water (100 µL). The different concentration of sample was added to the vials. As control, group of vials were filled with sea water. The vials were maintained under illumination at room temperature 25 °C to 28 °C. After 24 h, the percentage of hatchability was calculated using Abbott's formula[21]. This assay was carried out three times with triplicates.

$$\% \text{ Death} = [(\text{Test} - \text{Control}) / \text{Survivors of control}] \times 100$$

2.4.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

To calculate the LC₅₀ (mean lethal concentration) value the results were plotted as % mortality Vs concentration of purified extract of *T. radiatus* and *T. rudolphi*. The moderate LC₅₀ value indicated higher toxicity and higher LC₅₀ values indicated lower toxicity.

The sample extracted from the target species have been tested for their cytotoxic effect on human blood cells. Blood samples were aseptically collected in heparinized sterile tubes from median cubital vein of non-smoking healthy individuals (22–25 years). Lymphocytes were isolated using Ficoll–Histopaque (Sigma, USA) by the method described by Radhakrishnan[22]. Blood was diluted with PBS (1:1) and layered against histopaque with the ratio of 4:3. The blood was centrifuged at 1340 r/min for 35 min at room temperature. The lymphocyte layer was removed and washed twice in PBS at 1200 r/min for 10 min each, and then washed with RPMI-1640 media. The number of lymphocytes was counted using a haemocytometer and the viability of the cells was assayed by the trypan blue exclusion test. Approximately, 1 × 10⁶ cells were present in 1.0 mL lymphocyte suspension. Cell viability assays were carried out as described earlier[23]. Briefly, cells were seeded at a density of 3 × 10⁴ cells/well into 24-well plate. After 24 h, samples were added to the RPMI-1640 medium at different concentrations of (1 and 2 mg/mL) and the tray was gently shaken and incubated for 24 h at 37 °C. The viable cells against dead cells were recorded using haemocytometer under a microscope. Each experiment was performed at least 6 times to ensure consistency. The percentage of viable cells was calculated by the standard formula.

2.5. Determination of tetramine like compound

2.5.1. High performance thin layer chromatography (HPTLC) analysis

Thin layer chromatography was performed on pre-coated Silica gel 60 F254 (Merck KGaA) with N-propanol: water (7:3, v/v) to separate the various constituents of the extracts. The developed plates were air dried and spots were visualized with the ninhydrin reagent (3%, 2-dihydroxy-1, 3-indanedione in acetone) and scanned digitize at 600 nm by using CAMAG software.

2.5.2. GC-MS analysis

A GC clarus 500 Perkin Elmer system interfaced to a mass spectrometer was used for GC-MS. Each sample solution (diluted with methanol) was filtered and 1 μL of the solution was injected into Elite-5 MS fused silica capillary column ($30 \times 0.25 \text{ mm ID} \times 0.25 \mu\text{m}$ film thickness). Helium (99.999%) was used as carrier gas at a constant flow of 1 mL/min, injector temperature 270 $^{\circ}\text{C}$; ion-source temperature 180 $^{\circ}\text{C}$. The oven temperature was monitored from 50 $^{\circ}\text{C}$, with an increase of 8 $^{\circ}\text{C}/\text{min}$, to 250 $^{\circ}\text{C}$ hold for 5 min. Mass spectra were taken at 70 eV and fragments were scanned from 40 to 600 Da an interval of 0.2 s.

3. Result

3.1. Estimation of tetramine compounds

The amount of the crude tetramine in the sample of *T. radiatus* and *T. rudolphi* was recorded as 920 $\mu\text{g}/\text{g}$ and 1440 $\mu\text{g}/\text{g}$. After purification using column chromatography, the yield was found to be 0.4 $\mu\text{g}/\text{g}$ and 1.2 $\mu\text{g}/\text{g}$, respectively.

3.2. In vitro assay for cytotoxicity activity

3.2.1. Haemolytic activity

The features of hemolysis were present in the purified extracts of *T. radiatus* and *T. rudolphi*. But activities differed slightly depending on the type of blood such as chicken and human. The tetramine from *T. rudolphi* showed a maximum of 256 HU in human blood and a minimum of 64 HU in chicken erythrocytes. Likewise, *T. radiatus* exhibited maximum haemolysis of 16 HU in human blood and a minimum of 8 HU in chicken erythrocytes (Table 1).

Table 1

Hemolytic activity of the purified extracts.

Study animal	Type of blood	Sample ($\mu\text{g}/\text{mL}$)	Total hemolysis up to dilution	Hemolytic titre	Specific hemolytic activity (HT/ μg)
<i>T. radiatus</i>	Chicken blood	500	3	8	0.016
	Human blood	500	4	16	0.032
<i>T. rudolphi</i>	Chicken blood	500	6	64	0.128
	Human blood	500	8	256	0.512

3.2.2. Brine shrimp assay

The brine shrimp lethality assay is considered a useful tool for the assessment of toxicity[20,24]. And it is shown in Figure 1. *T. radiatus* has LC_{50} value at 60 $\mu\text{g}/\text{mL}$ and *T. rudolphi* exhibited at 7 $\mu\text{g}/\text{mL}$. *T. radiatus* and *T. rudolphi* respectively. The mortality rate of brine shrimp nauplii was found to increase in the dose dependent manner.

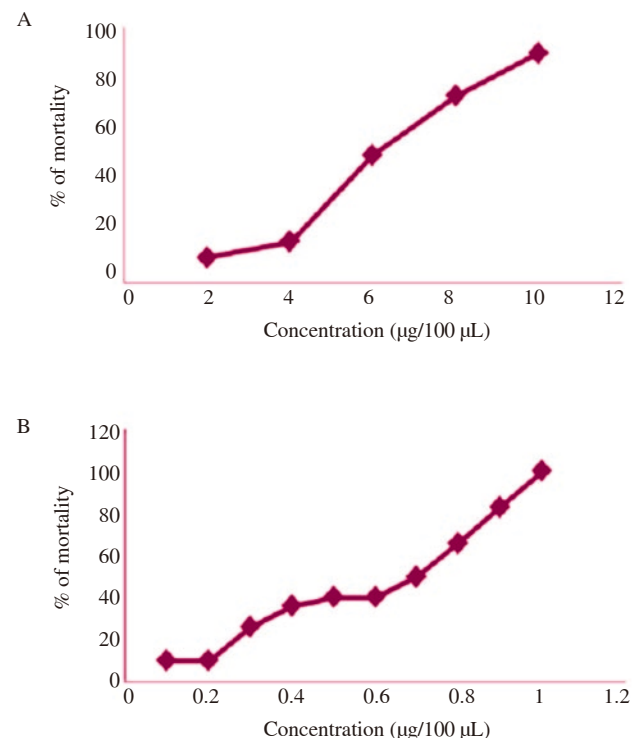


Figure 1. Brine shrimp toxicity of compound extracts of (A) *T. radiatus* and (B) *T. rudolphi*.

3.2.3. MTT assay

The viability of the cell was found to decrease with the increasing concentration of samples (Figure 2). The purified extracts of *T. radiatus* and *T. rudolphi* showed lethality (LC_{50}) against the normal

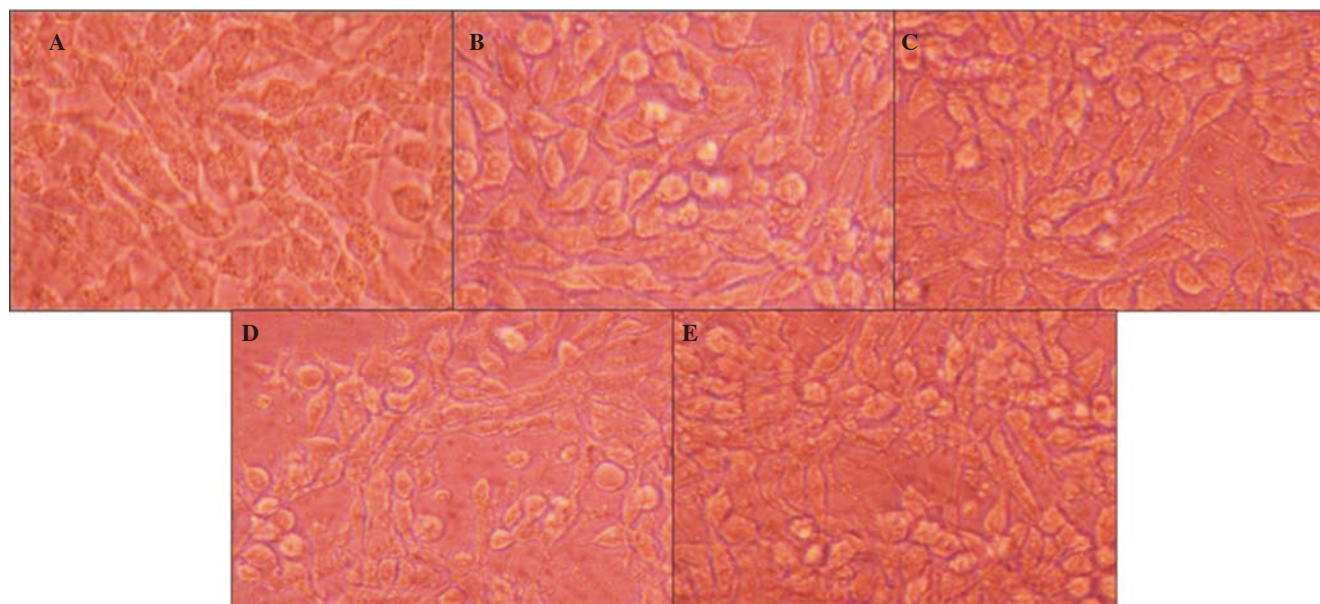


Figure 2. Morphological characteristics of lymphocytes visualized with a phase contrast microscope (a) control cells, (b) & (c) were treated with of *T. radiatus* extracts of 1 and 2 mg/mL concentration, respectively, and (d) & (e) were treated with *T. rudolphi* extracts of 1 and 2 mg/mL concentration, respectively.

cell lines at 1.87 and 1.54 mg/mL respectively. The percentage of viable cell was estimated 77.1 and 45.8 in *T. radiatus* 74.3 and 29.2 in *T. rudolphi*, respectively at the concentration of 1 and 2 mg/mL.

3.3. Determination of tetramine like compound

3.3.1. HPTLC analysis

The tetramine compounds present in both the sample extracts were separated by high performance thin layer chromatography. The HPTLC profile of the purified extract of sample *T. radiatus* exhibited single band whereas *T. rudolphi* exhibited two bands. The migrated samples were scanned at 254 nm through the HPTLC scanner and the bands with the R_f value of 0.53 and 0.55 (Figure 3.) were detected.

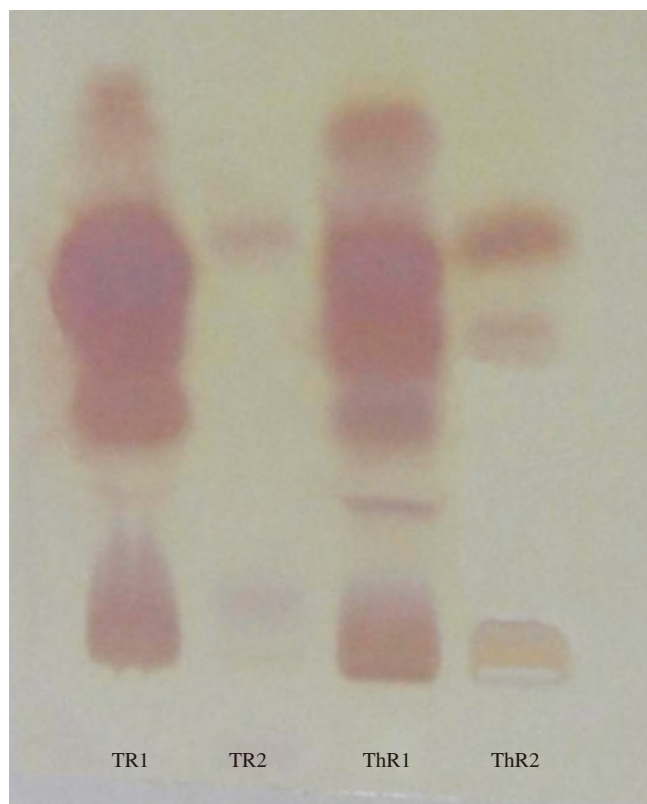


Figure 3. HPTLC profile of samples.

TR1: Crude extract of *T. radiatus*; TR2: Purified extract of *T. radiatus*;
ThR1: Crude extract of *T. rudolphi*; ThR2: Purified extract of *T. rudolphi*.

3.2.2. GC-MS analysis

The components of *T. radiatus* and *T. rudolphi* were identified through mass spectrometry with gas chromatography. The spectrum results were compared with the known spectrum components which are stored in the database of National Institute of Standard and Technology possessing more than 62000 patterns (NIST, 2005) (Figure 4). The results pertaining to GC-MS analysis leads to identification of tetramine from the purified extract of the samples which was detected at the retention time of 2.31.

4. Discussion

Seafood which includes fin fish and shellfish play a vital role in human nutrition and are particularly popular in certain parts of the world. The strong move to healthier eating habits and the substitution of meat with seafood in the diet has resulted in even greater demands for fish and shellfish[25]. Globally, 16% of all animal protein was consumed from the 66 million tons of marine species annually. They have an essential part of a balanced diet, good nutritional value and health benefits. Even though, the regular consumption of shellfish meat may perilous due to their food borne diseases, such as headache, dizziness, nausea, flaccid paralysis of skeletal muscles *etc.*,[13,26]. The numbers of cases involving intoxication due to consumption of these poisonous sea foods have increased bringing in unknown toxins or new analogue of known toxins, thus increasing the scope to investigate the intoxication of marine organisms. So far, many toxins have been isolated and studied for structure-function aspects and their applications for the humans[27]. Thus, the present study is aimed to focus the cognizance on sea food poisoning.

In the present investigation, the amount of purified tetramine content was estimated as 0.4 $\mu\text{g/g}$, 1.2 $\mu\text{g/g}$ in *T. radiatus* and *T. rudolphi*, respectively. Comparatively, the quantity of tetramine detected in the salivary glands of marine snail *N. antiqua*, was higher (5.7 mg/g)[28]. Likewise, Power *et al.*[29] avowed that the yield of tetramine ($\sim 6530 \mu\text{g/g}$) in the *N. antiqua*, varied with the season and concentrations are indirectly related to the feeding activity. Further it was reported earlier that the yield of tetramine obtained in the current study is beyond the normal level of occurrence[15,17]. Hence, it is quite evident that, the seasonal

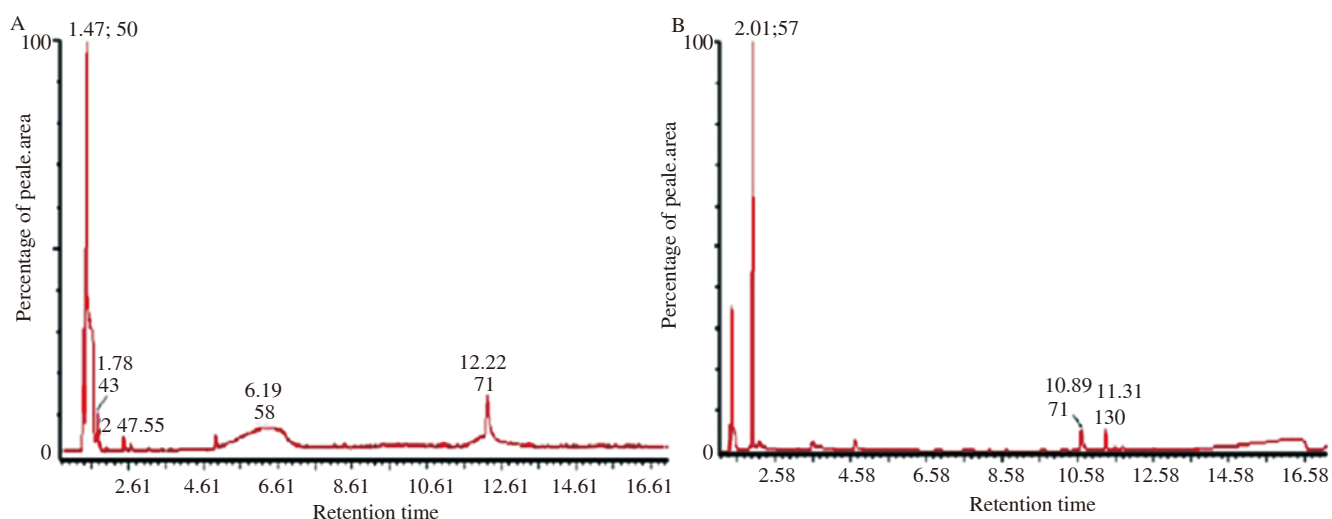


Figure 4. GC-MS chromatography spectrum *T. radiatus* (a) and *T. rudolphi* (b).

dependent concentrations of tetramine are highly dangerous for human health when it is ingested regularly.

Various investigations have proved that *T. radiatus* and *Thais* sp. contain a variety of bioactive compounds including a few toxins, which are known to possess potent haemolytic properties[30]. The purified extracts showed maximum haemolytic activity on human erythrocytes followed by poultry. Previously, Arumugam *et al.*[31] observed haemolysis effect (64 HU) in venom of mollusc *Turricula javana* and *Lophiotoma indica* against chicken blood erythrocytes. Likewise, Sarumathi *et al.*[32] reported very minimum haemolysis effect (9 HU) in a buccinidae mollusc against chicken erythrocytes tested. Further, Soletti *et al.*[33] described that the cytotoxicity effect observed in selected sea anemone was through their pore-forming cytolysins, toxin Bc2, and equinatoxin (EqTx II). Comparatively, in the present investigation, tetramine showed highest haemolysis effect against both human and chicken blood erythrocytes evidencing the pore forming cytotoxic character of the compound which are in agreement with the previous reports.

The brine shrimp lethality test was used as a surrogate tool to evaluate the toxicities and also to identify their potential for other biological activities[34]. In the case of brine shrimp lethality assay the purified sample extracts showed 50% mortality against brine shrimps at 60 and 7 µg/mL correspondingly. It was reported that, tetramine was toxic to human at the concentration of 0.1 to 0.3 mg/kg[35]. Likewise, the toxicity was observed in rabbits at concentration of 5 mg/kg, respectively[36]. This indicates that the observed toxicity against crustacean was significantly noticeable and therefore the current observation could be interpreted to mean that *T. radiatus* and *T. rudolphi* may be harmful to humans while their consumption.

In the evaluation of cytotoxic effect of tetramine on lymphocyte cell line, it indicated that the extract is effective in inhibiting the proliferation of human lymphocytes. *T. radiatus* and *T. rudolphi* showed medium toxicity against the normal cell lines at 1.87 and 1.54 mg/mL, respectively, whereas the control didn't show any considerable cell death. The morphological changes observed in the cells are concurrent with the previous reports on the cell morphology implementation of OA type toxins and azaspiracid in shellfish[37]. Likewise, Arokiyaraj *et al.*[38] isolated the triterpenes from the leaves of *Justicia gendarussa* and established the maximum inhibition (85%) against human lymphocyte proliferation. In conclusion, it is suggested to elucidate the specific virulence factor(s) of tetramine and susceptibility that cause cytotoxicity on human cells.

The HPTLC profile indicated the strong band observed at the R_f value of ~0.55, confirmed the presence of tetramine compound in both the samples. Our results are concurrent with the earlier reports of Asano and Ito[8] wherein, tetramine like compound were detected in the TLC at similar R_f . On investigation with various solvent systems for the separation of tetramine like compound, the mobile phase with N-propanol-water (7:3, v/v) showed a clear band at the R_f value of 0.55 corresponding to the presence of tetramine. Thus, the presence of tetramine like compound was evidenced in both samples through HPTLC.

The GC retention time (2.31) and the mass spectral fragmentation pattern were identical with tetramine of purified

extract of *T. radiatus* and *T. rudolphi*. Similarly, the presence of tetramine in the salivary gland of buccinid gastropods was detected at m/z 74 and this involved in numerous poisoning incidents after ingestion. Since they are distributed in trace amount in the consumed meat, sensitive and selective determination methods were needed for evaluation. The combination of liquid chromatography and electrospray ionization-single quadrupole mass spectrometry was reported as effective on their quantitative analysis[39]. The forgoing account, suggested that the shellfish meat of *T. radiatus* and *T. rudolphi* are the store-house of tetramine endowed with cytotoxic activities and may result in serious complications in regular consumption.

The Allergy Society of South Africa has classified the major edible sea foods that cause allergy to human into three groups, one among it is molluscan community. The gastropods normally feed on encrusting algae where some of these algal species have the ability to produce toxins which by then can accumulate in the shellfish. It should be noted that a small amount of tetramine may naturally occur even in various tissues of gastropod. Which may diffuse to other tissues and the broth during cooking (boiling) Shindo *et al.*[12] even though, the trace of tetramethylammonium chloride may retain on the tissues. This implies that tetramine is not denatured completely on boiling, thereby indicates its potential toxicity. At the outset, the present study implies that the impact of tetramethylammonium chloride in sea food and this may help to create the awareness among the consumers to avoid the regular consumption of these shellfish.

Conflict of interest statement

The authors declare no conflict of interest.

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