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Phytochemical analysis, *in vitro* antioxidant activity and anti-inflammatory activity of *Turbinaria ornata* in human RBC model

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Turbinaria ornata methanolic extract (TOME) with more bioactive compounds has been investigated for its *in vitro* total antioxidant activity, DPPH scavenging assay, reducing power assay, and antioxidant potential. Anti-hemolysis and anti-inflammatory activities were studied in human RBC model. Our preliminary phytochemical analysis showed that TOME constitutes carbohydrates, alkaloids, saponins, phenolic compounds, flavonoids, tannins, coumarines, steroids, and terpenoids. TOME at the concentration of 100 µg showed 89.11% of total antioxidant activity. The free radicals NO, H_2O_2 and SOD scavenging activities were enhanced with an increase in the concentration of TOME. Further, TOME at selected 0.5, 0.75 and 1(mg/ml) concentrations showed significant reduction in H_2O_2-induced hemolysis. The higher dose of 500 µg/ml (among selected concentrations) showed about 81 % of anti-inflammatory activity through enhanced stabilization of RBC membrane which was significant as the positive control diclofenac sodium. Thus our study clearly elucidated that *Turbinaria ornata* with potent bioactivities inhibited free radicals assault and prevented inflammation in RBC model.

[**Keywords**: *Turbinaria ornata;* Antioxidant activity; Anti-inflammatory activity; RBC model]

Introduction

Free radicals are playing adverse role in etiology of wide continuum of disease from cancers to neurodegenerative diseases. They induce alterations in forms and function of various biological molecules which persuade to diseased conditions[1]. Oxidative stress and apoptosis have prominent role in pathogenesis of several cardiac and neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) etc[2]. Reactive oxygen species (ROS) produced enormously due to endogenous and exogenous stimuli leads to induction of signals that result in mitochondrial dysfunction and cell death[3]. Inflammation is one of the evident processes of oxidative stress and the formation of inflammatory mediators like interleukins, adhesion molecules which are having significant role in diseases like diabetes, neurodegenerative diseases, arthritis, and cardiovascular diseases therefore inhibition of inflammation is important in treating such diseases[4].

Dietary phytochemicals like phenolic and polyphenolic compounds, such as flavonoids and catechins exhibit potent antioxidant activities which are capable of removing free radicals. Consequently, they inhibit oxidative modification of lipid complex in plasma membrane of a cell. A multipotent drug with bioactive compounds has the ability to reduce the formation of free radicals, have good antioxidant capacity and extend membrane stabilization which is one of the notable process in treating most of diseases[5]. Seaweeds are rich in bioactive compounds like sulfated polysaccharides, phlorotannins and diterpenes which are valuable for human wellbeing and they are considered to be the rich source of antioxidants[6]. The potential antioxidant compounds like fucoxanthin, astaxanthin, carotenoid, phenolic acid, flavonoids and tannins are widely distributed in seaweeds[7] which are known to exhibit antioxidative activities through scavenging reactive oxygen species and inhibition of lipid peroxidation[8,9,10]. *Turbinaria ornata*, the spiny leaf seaweed has been studied for its anti oxidant, antiulcer, injury healing and hepatoprotective activities[11]. In the present study preliminary phytochemical analysis, free radicals scavenging activity, anti-inflammatory activity and anti-hemolytic activity to elucidate H_2O_2 inhibition activity of *Turbinaria ornata* have been investigated in search of new drug from its bioactive compounds.

Materials and Methods

Chemicals

Hydrogen peroxide 2,2-diphenyl-1-pricrylhydrazyl (DPPH), gallic acid, and ascorbic acid were obtained from Himedia Laboratory Ltd., Mumbai, India. Dichlofenac sodium was purchased from Sigma Aldrich, Bangalore. All other chemicals were of reagent grade; while organic solvents were of spectral grade.

Collection of samples

The marine brown alga, *Turbinaria ornata* was collected by hand picking from the intertidal waters of the Mandapam coast (Longitude 78° 8'E, Latitude 9 ° 17' N) in the Gulf of Mannar during the early hours in May 2015. The algal material was identified and authenticated in Botanical Survey of India (BSI); Coimbatore, Tamilnadu, India and a voucher specimen was maintained in our research laboratory (BSI/SRC/5/23/2015/Tech./1304). The collected algal material was washed with sea water and then with fresh water to remove sand, salts and epiphytes.

Preparation of seaweed extracts

The dried seaweed samples (25 g) were milled and extracted using 250 ml of various solvents such as ethanol, methanol and aqueous for 24 hours by employing Soxhlet apparatus. Each filtrate was concentrated to dryness under reduced pressure using rotary evaporator. The samples were lyophilized by using freeze dryer (Lark, Penguin Classic Plus, India) and stored in a refrigerator at 2-8 °C for use in subsequent experiments.

Preliminary phytochemical analysis

Preliminary phytochemical screenings of three different extracts were carried out as per the standard protocols of Harborne[12] to reveal the extract with more bioactive compounds.

Total antioxidant activity

The antioxidant activity of the seaweed extract was evaluated by the phosphomolybdenum method[13]. The assay was based on the reduction of molybdenum by the extract and consequent formation of a green complex at acidic pH. 2 ml of extract was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate); methanol was used in the place of sample as blank. The tubes containing the reaction solution were capped and incubated in water bath at 95° for 90 minutes. After the samples were incubated at room temperature, the absorbance of the solution was measured at 635 nm using a spectrophotometer against blank. The antioxidant activity was expressed as an equivalent of gallic acid (mg GA/g dried extract). All the measurements were done in triplicate.

In vitro free radical scavenging assay

TOME was screened for its free radical scavenging activity and antioxidant potential by assays, like 2,2-diphenyl-1-pricrylhydrazyl (DPPH) scavenging assay[14], reducing power assay[15], nitric oxide (NO) scavenging assay[16], hydrogen peroxide scavenging assay[17], and superoxide scavenging assay[18]. All these assays were carried out in triplicate and compared with ascorbic acid as a standard.

Prevention of H_2O_2 assault-Antihemolytic study on human RBC

To determine the effect of TOME on H_2O_2-induced hemolysis in the light of the previous study[19] with slight modifications, the following sets of spectrophotometric tubes (each four tubes) were prepared:

Group I: Control tubes: These tubes contained 1.0 ml of RBC suspension. Final volume was made to 4.0 ml with normal saline

Group II: TOME tubes: 1 ml (100 µg/ml) TOME was mixed with 1.0 ml RBC suspension . Final volume was made to 4.0 ml with normal saline (Dosage of TOME was fixed by separate study, data not shown).

Group III : H_2O_2 treated tubes: 8 mM H_2O_2 solution (0.5 ml) was mixed with 1.0 ml RBC suspension and the final volume was made to 4.0 ml with normal saline.

Group IV: H_2O_2 and TOME treated tubes: 1 ml of RBC suspension treated with 0.5 ml of 8 mM H_2O_2 solution and 1 ml of TOME (100 µg/ml). All the tubes were incubated at 37 °C for 4 hr with intermittent shaking. Absorbance of the supernatants was obtained after centrifuging the incubated tubes at 1000 rpm for 10 min and read spectrophotometrically at 540 nm.

Percententage of hemolysis was calculated by the formula,

$$\text{Percentage of hemolysis} = \frac{\text{Absorbance of individual tubes}}{\text{Absorbance with 100\%hemolysis}} \text{X100}$$

In vitro anti-inflammatory activity on human RBC membrane stabilization method

The human red blood cells (HRBC) membrane stabilization has been used as a method to study the anti-inflammatory activity[20]. Blood was collected

from healthy volunteers. The collected blood was mixed with equal volume of sterilized Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.05% citric acid, and 0.42 % sodium chloride in water). The blood was centrifuged at 3000 rpm; and the packed cells were washed with isosaline (0.85%, pH 7.2) and a 10 % v/v suspension was made with isosaline. Effect of extracts on HRBC stability was experimented with 5 mL of reaction mixture consisting of 2 ml hypotonic saline, 1 ml of sodium phosphate buffer (0.15 M, pH 7.4) and 1 ml of TOME of different concentrations (62.5, 125,250,500 µg/ml) dissolved in normal physiological saline and a positive control which replaces TOME with 1 ml of dichlofenac sodium of different concentrations (62.5, 125,250,500 µg/ml) an important NSAIDS in treating inflammatory diseases. Then 1 ml of 10% HRBC was also added. A control of 3.0 ml of isotonic saline (instead of TOME or dichlofenac or hyposaline) and 1 ml of sodium phosphate buffer (0.15 M, pH 7.4) with 1 mL of 10% HRBC. A negative control of 3 ml of hypotonic saline and 1 ml of sodium phosphate buffer (0.15 M, pH 7.4) with 1 ml of 10% HRBC were used (data not shown). The mixture was incubated at 56 °C for 30 min. The tubes were cooled under running water for 20 min and the mixture was centrifuged at 3000 rpm. The supernatants were separated; and the absorbance of the supernatants read at 560 nm. All the assay mixtures were incubated at 37 °C for 30 min and centrifuged. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100 %. The percentage of human RBC membrane stabilization or protection was calculated by using the formula,

Anti-inflammatory activity = 100- (Test OD/Control ODx100)

Results

Table 1 showed the phytochemicals present in the aqueous, methanolic and ethanolic extracts of *Turbinaria ornata.* Methanolic extract of *Turbinaria ornata (*TOME) constitutes carbohydrates, alkaloids, saponins, phenolic compounds, flavonoids, tannins, coumarines, steroids and terpenoids. Among the different extracts *Turbinaria ornata* methanolic extract (TOME) with most phytochemicals was chosen for further study.

Figure 1 showed the total antioxidant activity of TOME by phosphomolybdenum method. TOME at the concentration of 100 µg showed 89.11% of total antioxidant activity as compared with the standard gallic acid. Figure 2 and 3 showed the DPPH radical

Table 1 — Phytochemical analysis of *Turbinaria ornata* extracts in different solvents

S. No	Phytochemicals	Aqueous	Ethanol	Methanol
1	Carbohydrates	-	-	+
2	Alkaloids	+	-	+
3	Saponins	-	-	+
4.	Phenolic compounds	-	+	+
5.	Flavonoids	-	+	+
6.	Tannins	-	+	+
7.	Coumarines	-	+	+
8.	Proteins	-	-	-
9.	Steroids	-	+	+
10.	Anthroquinones	-	-	-
11.	Terpenoids	-	+	+

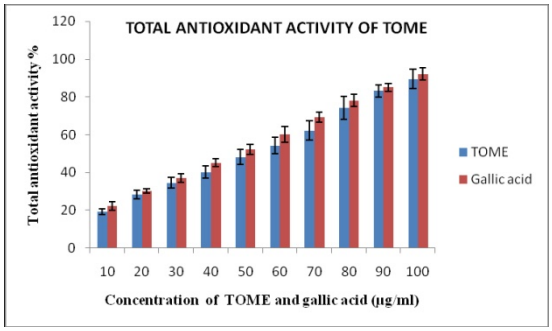

Fig. 1 — Total antioxidant activity of TOME and standard gallic acid

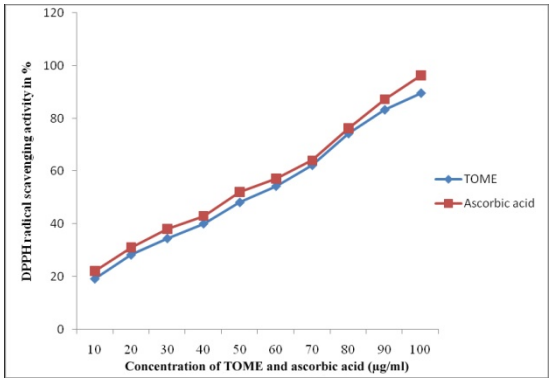

Fig. 2 — DPPH radical scavenging activity of TOME and standard ascorbic acid

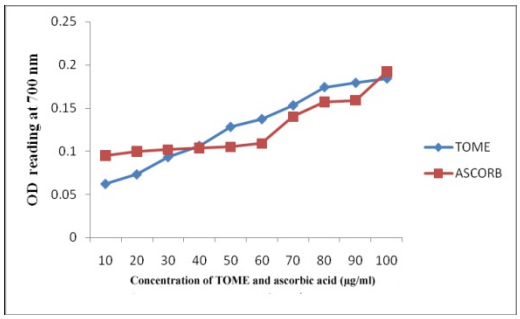

Fig. 3 — Reducing power of TOME and standard ascorbic acid

scavenging and reducing power of TOME, respectively. TOME showed the greatest DPPH antioxidant activity (~80%) at 100 µg/ml. Table 2 depicts the free radical scavenging capacity of TOME and the standard ascorbic acid.

Table 3 depicted the anti-hemolytic activity of TOME on human RBC hemolysis. Group III H $_2O_2$ treated showed high level hemolysis compared to control Group I. Group II TOME treated RBC models showed no significant hemolysis compared to Group III H $_2O_2$ treated, whereas Group IV treated with different concentrations of TOME *(*0.5 mg/ml, 0.75 mg/ml, 1 mg/ml) and H_2O_2 showed noteworthy reduction in hemolysis compared to Group III H_2O_2 treated. TOME at the concentration of 1 mg/mlinhibited the assault of H_2O_2 and about 80% of hemolysis was prevented.

Table 4 showed the anti-inflammatory activity of TOME in different concentrations of 62.5, 125, 250 and 500 µg/ml. The increase in TOME concentrations increased the human RBC membrane stabilization, the higher concentration level (500 µg/ml) showed about 81 % of anti-inflammatory activity compared to 100% lysis induced in control, which was significant as the standard diclofenac.

Discussion

Inflammation along with the reactive oxygen species have significant role in various disease conditions. Suppression of free radicals, inhibition of oxidative stress and preventing inflammation are important in treating such diseases. TOME in the present study showed promising role in treating above deleterious events and assaults. The phytochemical study showed the presence of alkaloids, flavanoids, polyphenols and tannins in methanolic extract. Our findings were well supported by the earlier study[21]. Therefore, methanolic extract of *Turbinaria ornata* was chosen for further in vitro free radical scavenging, anti-hemolysis and anti-inflammatory studies.

DPPH is a stable radical and is widely used to evaluate antioxidant activities of bioactive substances.

Table 2 — In vitro free radical scavenging assay of TOME

Concentration of TOME/Ascorbic acid µg/ml	NO scavenging capacity of TOME (%)	H_2O_2 scavenging capacity of TOME (%)	SOD scavenging capacity of TOME (%)	NO scavenging capacity of Ascorbic acid (%)	H_2O_2 scavenging capacity of Ascorbic acid (%)	SOD scavenging capacity of Ascorbic acid (%)
10	22.46±2.24	28.03±3.76	14.03±3.28	26.16±1.11	32.09±2.67	16.11±1.21
20	28.46±1.21	34.46±4.20	28.16±3.88	33.16±2.18	38.60±2.94	32.10±2.71
30	35.22±2.18	39.22±4.50	32.12±4.18	39.11±3.22	43.11±3.21	39.13±2.15
40	41.65±2.71	41.65±4.93	49.25±4.77	44.35±1.21	51.62±3.47	52.35±2.27
50	47.27±5.09	53.57±5.25	53.33±5.09	52.10±2.19	59.13±2.12	61.31±2.14
60	53.14±5.43	58.80±5.74	62.45±5.43	57.24±1.23	64.64±2.01	67.18±2.32
70	58.11±3.24	65.18±6.17	69.11±2.69	61.22±1.20	70.92±2.12	71.18±3.19
80	62.32±4.11	71.60±6.61	78.46±6.21	67.12±1.21	77.43±2.17	82.21±5.21
90	68.25±2.37	83.65±6.93	81.58±4.77	74.13±1.25	89.93±3.11	84.65±3.16
100	77.37±5.27	89.57±7.33	86.23±3.21	79.37±3.11	97.20±2.18	89.57±7.17

Table 3 — Anti-hemolytic activity of TOME on human RBC

S. No	RBC Suspension (5 X10^8 RBC/ml)	H_2O_2 Concentration	TOME 1mg/1ml	Hemolysis %
Group 1	1 ml	0.0	0.0	0.11±0.01
Group 2	1 ml	0.0	1 ml	0.12±0.02
Group 3	1 ml	8mM	0.0	93.75±7.50
Group 4	1 ml	8mM	0.5 ml	62.49±5.0
	1 ml	8mM	0.75 ml	32.47±2.60
	1 ml	8mM	1.00 ml	20.83±1.67

Table 4 — Anti-inflammatory effect of TOME *by* human RBC membrane stabilization

S. NO	RBC suspension 5 X10^8 RBC/ml	Concentration of TOME (µg/ml)	Percentage of human RBC membrane stabilization by TOME	Diclofenac sodium (µg/ml)	Percentage of human RBC membrane stabilization by Diclofenac sodium
1	1	62.5	51.00±4.08	62.5	64.80±5.18
2	1	125	68.00±5.44	125	77.0±6.16
3	1	250	72.23±5.78	250	86.00±6.88
4	1	500	81.13±2.18	500	93.17±2.23

The antioxidant activity of samples was carried out by measuring the absorbance of DPPH radical in the sample at 517 nm as against the control. The DPPH scavenging ability of TOME depends upon its concentration; increase in TOME concentration enhanced the scavenging ability. The reducing power was evaluated in the samples on the basis of their abilities to reduce Fe (III) complex to Fe (II). Greater the absorbance value, higher is the reducing power of the TOME[22]. TOME had appreciable DPPH radical scavenging activity and reducing power as comparable to the standard ascorbic acid.

The antioxidant properties of phenolics are as a result of their ability to act as reducing agents, hydrogen donors, and free radical quenchers. Phenolics can also act as metal chelators which prevent the catalytic function of metal in the process of initiating radicals. Also, it was reported that *Turbinaria ornata* have high phenolic content which was responsible for their respective antioxidant activity. Concentrations of TOME were chosen as per the earlier report[23], with slight modifications. The free radicals NO, H_2O_2 and SOD scavenging activity are increased with an increase in the concentration of TOME; and the results were well supported by the previous study[22].

The rupturing of RBC cells is known as hemolysis. Hemolysis is an indicator of cytotoxicity. In our study hydrogen peroxide affected the integrity of the erythrocyte membrane and cause hemolysis. TOME maintained the stability of human red blood cell membrane and also inhibited the H_2O_2 assault on RBC membrane and hemolysis was prevented. Our results were well supported by earlier study[24].

Inflammation is the primary defense mechanism but uncontrolled inflammation in chronic diseases leads to adverse effects. Anti-inflammatory action is one of the important strategies for treating diseases. Since, the human red blood cells (HRBC) membrane is similar to lysosomal membrane components. The prevention of hypo tonicity induced human RBC membrane lysis was considered as a measure of anti-inflammatory activity of TOME. Human RBC membrane stabilization by TOME revealed the anti-inflammatory activity of TOME and the results were well agreed with the earlier report[25].

Conclusion

In the present study, preliminary phytochemicals, antioxidant analysis of methanolic extract of *Turbinaria ornata* leaves have been done. The phytochemical analysis elucidated the presence of significant bioactive compounds in the methanolic extract of *Turbinaria ornata.* Further its antioxidant activity, anti-hemolysis and anti-inflammatory properties in RBC model from the blood collected from healthy volunteers. *Turbinaria ornata* with potent bioactive compounds showed appreciable antioxidant activity, dramatically reduced H_2O_2-induced hemolysis in human RBC model. The anti-inflammatory activity of *Turbinaria ornata* was revealed by its ability of HRBC membrane stabilization as much as appereciable to standard dichlofenac. Further *in vivo* studies, NMR assay, drug leads and molecular level investigations are in need to bring out the medicinal value of the *Turbinaria ornata* in treating different diseases due to the assault of free radicals and inflammation.

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