

Phytochemical Analysis, *In-vitro* Antioxidant and Anti-Hemolysis Activity of *Turbinaria ornata* (turner) J. Agardh

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Abstract: The assault of free radicals and imbalance in oxidant and antioxidant status leads to the induction of diseases from cancers to neuro-degenerative diseases. Natural antioxidants can put a secured check over free radicals and the damages induced by them at various levels. Seaweeds are rich in bioactive compounds like sulfated polysaccharides, phlorotannins and diterpenes which are benefit for human health applications. *Turbinaria ornata*, the spiny leaf seaweed has been studied for its antioxidant, antiulcer, wound healing and hepatoprotective activities. In the present study, phytochemicals analysis, *in vitro* antioxidant and anti-hemolysis activity of *Turbinaria ornata* methanolic extract (TOME) in RBC model was done. The results reveal the presence of carbohydrates, alkaloids, saponins, phenolic compounds, flavonoids, tannins, coumarines, steroids and terpenoids. The UV-Vis, FTIR and GCMS analysis also elucidates the presence of phenolics and important bioactive compounds in TOME, which exhibits appreciable antioxidant activity and prevents H₂O₂ induced hemolysis in human RBC model by maintaining the cell membrane integrity.

Key words: *Turbinaria ornata*, *in vitro* antioxidant activity, antihemolysis, bioactive compounds.

INTRODUCTION

Free radicals are playing adverse role in etiology of wide spectrum of diseases from cancers to neuro-degenerative diseases. They induce alterations in forms and function of various biological molecules to bring about the diseased conditions [1]. Oxidative stress is having prominent role in pathogenesis of cancer and several neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease etc [2]. Reactive oxygen species (ROS) produced enormously due to endogenous and exogenous stimuli leads to induction of signal transduction that results in mitochondrial dysfunction and cell death [3]. Antioxidants can put a check over free radicals and the damages induced by them at various levels. A multipotent drug which has the ability to reduce the free radicals formation, good antioxidant capacity and membrane stabilizing potential is of great use in treating most of diseases [4]. Seaweeds are rich in bioactive compounds like sulfated polysaccharides, phlorotannins and diterpenes which are benefit for both human and animal health applications and are considered to be a rich source of antioxidants [5]. *Turbinaria ornata*, the spiny leaf seaweed has been studied for its anti-oxidant, antiulcer, wound healing and hepatoprotective activities[6]. In the present study the phytochemical screening, free radicals scavenging activity and antihemolytic activity of *Turbinaria ornata* have been investigated in search of new drug from natural bioactive compounds.

MATERIALS AND METHODS

Chemicals

Hydrogen peroxide, 1, 1-diphenyl-2-picrylhydrazyl, gallic

acid, and ascorbic acid were obtained from Himedia laboratory Ltd., Mumbai, India. All other chemicals were of reagent grade and 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 month of May 2015 in the early hours. The algal material was identified and authenticated in BSI coimbatore, Tamilnadu and a voucher specimen was maintained in our research Laboratory (BSI/SRC/5/23/2015/Tech./1304), collected algal material was washed with sea water and then with fresh water and freed from 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 h by using 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 Screening.

Preliminary phytochemical screenings of three different extracts were carried out as per the standard protocols of Harborne [7].

UV-Vis and FTIR analysis of TOME

UV-Vis analysis was made by scanning in the range of 200-800 nm using Shimadzu spectrophotometer and

characteristic peaks were detected [8]. Fourier transform infrared spectroscopy (FTIR) analysis was done with the solid samples of TOME [9]. Turbinaria ornata (10mg) were mixed with 100mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc. The disc was then read spectrophotometrically (Bio-Rad FTIR-40, USA). The frequencies of different components present in each sample were analyzed.

GC-MS analysis for phytochemical constituents

A high resolution mass spectrum equipped with a data system in combination with Gas Chromatography was used for the chemical analysis of seaweeds. The aqueous methanolic extract of Turbinaria ornata was examined for its chemical composition using GC-MS THERMO GC - TRACE ULTRA VER: 5.0, THERMO MS DSQ II. The DB 5 - MS capillary standard non - polar column, with dimension of 30 Mts, ID : 0.25 mm, FILM : 0.25 µm was used and injection volume was one micro litre. The extract was diluted in methanol and injected in the split mode. The carrier gas was helium and the flow rate was 1.0 ml/min. The temperature was programmed to an oven temperature of 70° C raised to 260°C at 6°C /min. The run time was 40.50 min. The chemical constituents were identified after comparison with the data available in the library search results attached to the GC-MS instrument.

Total antioxidant activity

The antioxidant activity of the seaweed extract was evaluated by the phosphomolybdenum method [10]. The assay is based on the reduction of molybdenum by the extract and subsequent formation of a green complex at acidic pH. 2 ml extract was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate), methanol was used in 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 cooled to 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 measured in triplicates.

In vitro Free Radical Scavenging Assay

TOME was screened for its free radical scavenging and antioxidant potential by the following assays 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay [11, 12], Nitric oxide (NO) scavenging assay [13], hydrogen peroxide scavenging assay [14], superoxide scavenging assay [15]. All these assays were carried out in triplicates and compared with ascorbic acid as a standard.

Antihemolytic study on human RBC:

To determine the effect of TOME on H₂O₂ induced hemolysis in the light of the previous study [16] with slight modifications, the following sets of spectrophotometric tubes (each four tubes) prepared:

Group I: Control tubes: These tubes were contained 1.0 ml RBC suspension made to 4 ml with normal saline.

Group II: TOME tubes: 1ml (100µg/ml) TOME was mixed with 1.0ml RBC suspension. Final volume was made to 4.0ml with normal saline

Group III: H₂O₂ treated tubes: 8mM H₂O₂ solution (0.5ml) was mixed with 1.0ml RBC suspension and the final volume was made to 4.0 ml with normal saline.

Group IV: H₂O₂ and TOME treated tubes: 1 ml of RBC suspension treated with 0.5ml of 8mM H₂O₂ solution and 1ml (100µg/ml) TOME of 0.5ml, 0.75ml and 1ml. 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 1000rpm for 10 minutes and read spectrophotometrically at 540 nm. Percentage of hemolysis was calculated by the formula given below

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

Figures and Tables

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	-	+	+

Table1. Phytochemical analysis of Turbinaria ornata extracts in different solvents

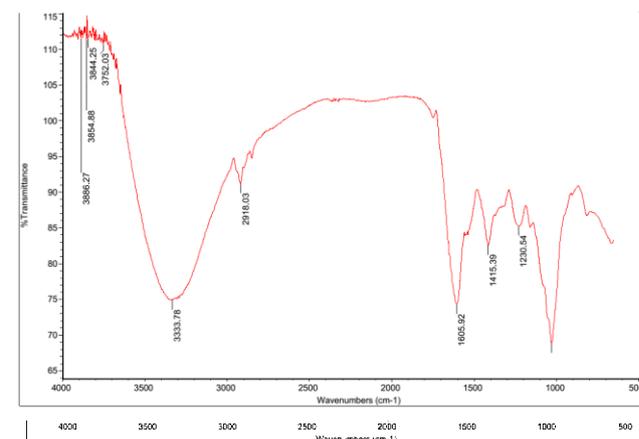
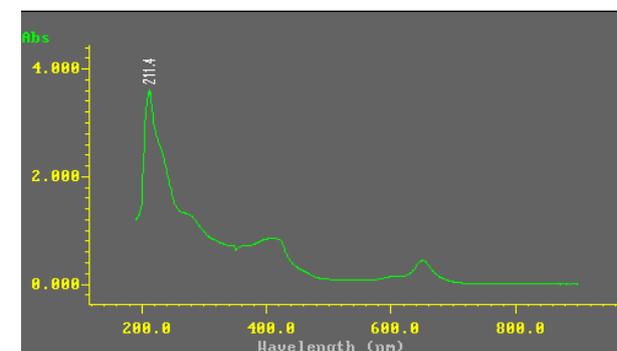


Fig. 2 FTIR spectrum of TOME

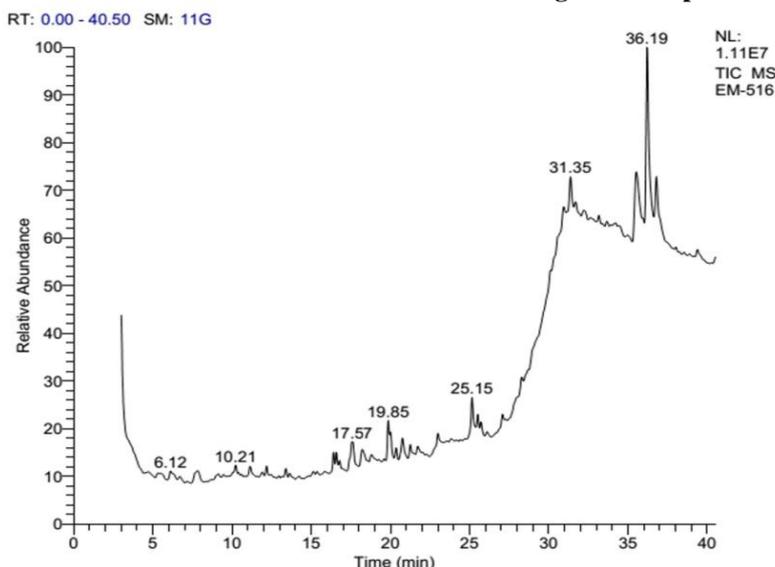


Fig.3. GC-MS Spectra of TOME

Retention Time	Compound	Formula	Molecular weight
6.21	trans-3-Chloro-1-isopropyl-2-(4-methylphenyl) azetidene	C ₁₃ H ₁₈ ClN	223
10.21	3-Acetyl-2-methyl-3a,4,5,8,9,9a-theahydro-cyclooctafuran	C ₁₄ H ₁₈ O ₂	218
17.57	1,1-Dimethyl-2-(1'-hydroxyethyl)-3,5bis(hydroxymethyl) cyclopentane	C ₁₁ H ₂₂ O ₃	202
19.85	Neophytadiene	C ₂₀ H ₃₈	278
25.15	Phytol	C ₂₀ H ₄₀ O	296
31.35	Di-(2-ethylhexyl) phthalate	C ₂₄ H ₃₈ O ₄	390
36.19	2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23 hexamethyl- (CAS)	C ₃₀ H ₅₀	410

Table2. Phytochemical composition of TOME

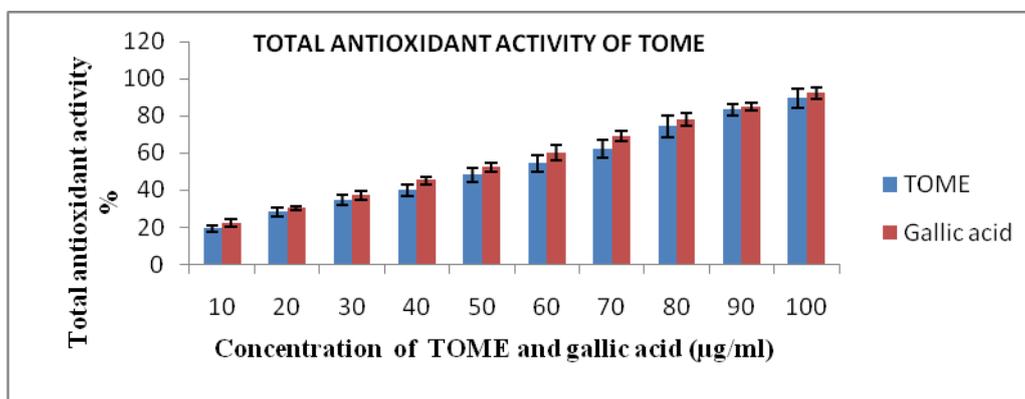


Fig. 4 Total antioxidant activity of TOME and gallic acid

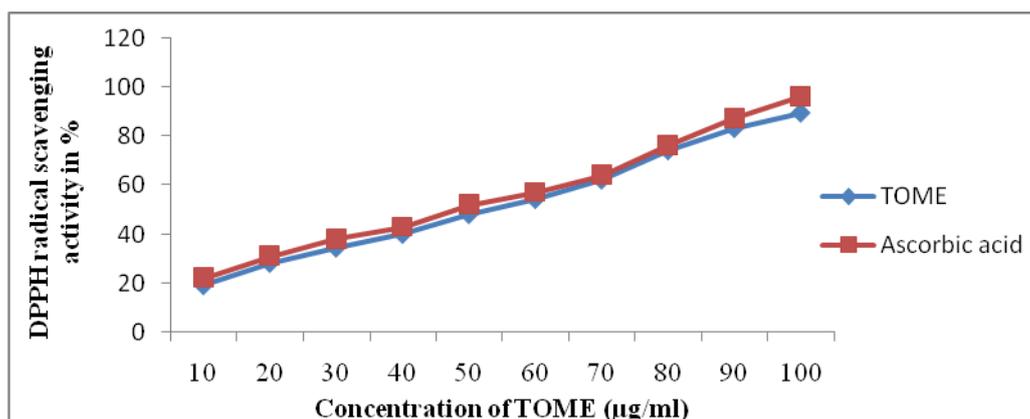


Fig. 5 DPPH free radical scavenging activity of TOME and Ascorbic acid

Concentration of TOME/Ascorbic acid µg/ml	NO scavenging capacity of TOME (%)	H ₂ O ₂ scavenging capacity of TOME (%)	SOD scavenging capacity of TOME (%)	NO scavenging capacity of Ascorbic acid (%)	H ₂ O 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

Table3. In vitro free radical assay of TOME

S.No	RBC Suspension (5X10 ⁸ RBC/ml)	H ₂ O 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

Table4. Ameliorative effect of TOME on hemolysis of RBC

RESULTS AND DISCUSSION

Phytochemical analysis of methanolic extract of Turbinaria ornata (TOME):

Table 1 shows 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 our findings are supported by the earlier study [17], therefore TOME was chosen for further in vitro free radical scavenging effects.

UV-Vis and FTIR analysis of TOME

Figure.1 depicts the UV-VIS analysis of TOME, In UV-Vis spectra TOME extract exhibited good absorption at 214nm, this absorption maximum indicates the presence of phenolics which is a promising evidence for the antioxidant activity investigated in this study our results are well supported by the previous study [18]. Figure.2 depicts the FTIR analysis of TOME. The FTIR spectrum spectrum of TOME in the region 3995.85-455.13 constitutes the following bioactive compounds Chondroitin sulfate, Streptomycin sulfate, 6-deoxy-d-glucose crystalline, Isomaltose, Limonene, 11-ketoetio cholanolone glucuronid, Kanamycin monosulfate 2-fluoroethanol, Alpha-bromo-p-xylene, benzyl alcohol, p-nitrophenyl-b-d-mannopyranoside Pinacolone, Methyl alcohol, Silicone oil, Risticetin sulfate, Ethylbenzyl alcohol, para-Toluenesulfonyl fluoride, Adenosine 5'-diphosphoglucose butyramide. Alcohol: O-H (stretch, H-bonding) 3200-3600cm⁻¹, O-H Stretch, free 3500-3700 cm⁻¹, C-O stretch 1050-1150 cm⁻¹, Alkane: C-H stretch

2850-3000 cm⁻¹, -C-H bending 1350-1480 cm⁻¹, Alkene =C-Hstretch 3010-3100 cm⁻¹ =C-Hbending 675-1000 cm⁻¹

¹, C=Cstretch 1620-1680 cm⁻¹, Alkyne C-H stretch 3300 cm⁻¹, -C=C- stretch 2100-2260 cm⁻¹, Amines: N-H stretch 3300-3500 cm⁻¹, C-H stretch 1080-1360 cm⁻¹, N-H bending 1600 cm⁻¹, Aromatic : C-H stretch 3000-3100 cm⁻¹, C=Cstretch 1400-1600 cm⁻¹, Carbonyl C=Ostretch 1670-1820 cm⁻¹, Nitro compounds: N-O stretch 1515-1560&1345-1385 cm⁻¹. IR ABSORPTION :Carbonyl C=Ostretch 1670-1820 cm⁻¹, Acid: C=Ostretch 1700-1725 cm⁻¹,O-H stretch 2500-3300 cm⁻¹, C-O stretch 1210-1320 cm⁻¹, Aldehyde: C=Ostretch 1740-1720 cm⁻¹, =C-Hstretch 2820-2850 and 2720-2750 cm⁻¹, Amide: C=Ostretch 1640-1690 cm⁻¹, N-H stretch 3100-3500 cm⁻¹, N-H bending 1550-1640 cm⁻¹. FTIR spectrum strongly elucidates the promising bioactive compounds in TOME the present study results well supported by the previous study [19].

GC- MS analysis: Figure.3 elucidates the GC- MS analysis of crude methanolic extract of Turbinaria ornata showed the presence of mixture of bioactive compounds. A total of 7 peaks were observed with different retention times as presented in Fig. The molecular formula and molecular weight for the compounds identified were fetched from the library search results of the GC-MS systems and are given in Table 2, our results are well supported by the previous study [20].

Total antioxidant activity and free radical scavenging activity of TOME:

Figure.4 shows the total antioxidant activity of TOME by phosphomolybdenum method, TOME at the concentration of 100µg shows 89.11% of total antioxidant activity as

compared with the standard gallic acid which is supported by the previous reports [21]. Figure.5 shows the DPPH radical scavenging activity of TOME. DPPH is a stable radical and is widely used to evaluate antioxidant activities of bioactive substances. The antioxidant activity of a samples was carried out by measuring the absorbance of DPPH radical in the sample at 517 nm as against to that of control. The scavenging ability of TOME was concentration dependent. The TOME shows the greatest DPPH antioxidant activity (~80%) at 100 µg/ml [22]. TOME having appreciable DPPH radical scavenging activity. Table 3 depicts the free radical scavenging capacity of TOME and the standard ascorbic acid. Concentrations of TOME were chosen as per the earlier report [23], with slight modifications. 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 is reported that *Turbinaria ornata* have high phenolic content which is responsible for their respective antioxidant activity. The free radicals NO, H₂O₂ and SOD scavenging activity are increased with increase in the concentration of TOME and the results are well supported by the previous study¹⁹.

Hemolytic activity of TOME on human RBC:

Table 4 shows the hemolytic activity of TOME on human RBC hemolysis. The rupturing of RBC cells is known as hemolysis. Hemolysis is a good indicator of cytotoxicity. Hydrogen peroxide affects the integrity of the erythrocyte membrane. Thus Group III H₂O₂ treated shows high level hemolysis compared to control Group I. Group II TOME treated RBC models shows no significant hemolysis compared to Group III H₂O₂ treated which is similar to the earlier different study [24]. Whereas Group IV treated with different concentrations of TOME (0.5 mg/ml, 0.75 mg/ml, 1mg/ml) and H₂O₂ treated shows significant reduction in hemolysis compared to Group III H₂O₂ treated. TOME maintains the stability of human red blood cell membrane also inhibits H₂O₂ assault RBC membrane.

CONCLUSION

In the present study preliminary phytochemicals, in vitro antioxidant activity analysis of methanolic extract of *Turbinaria ornata* (TOME) have been done and investigated further for its anti-hemolysis activity in RBC model of blood collected from healthy volunteers of 22-25 years age. The phytochemical analysis elucidates the presence of significant bioactive compounds in the methanolic extract of *Turbinaria ornata*, similarly the UV-Vis, FTIR and GCMS analysis elucidates the presence of bioactive compounds with potent antioxidant activity. Thus *Turbinaria ornata* with potent bioactive compounds shows appreciable antioxidant activity prevents H₂O₂ induced hemolysis in human RBC model by maintaining the cell membrane integrity. Further in vivo studies, NMR assay, drug leads and molecular level investigations will be done to bring out the medicinal value of the *Turbinaria ornata* in treating different diseases due to the assault of free radicals and oxidative stress.

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