Research Paper

Cytotoxicity Studies of the Chemical Constituents from Freshwater Algae *Chara baltica*

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Tatipamula *et al.*: Anticancer activity of *Chara baltica*

For the first time, 89 chemical constituents were identified by gas chromatography-mass spectrometry analysis of ethanol extract of *Clathria baltica*. The chemical examination of ethanol extract of *Clathria baltica* yielded three known metabolites, 1, 2 and 3, for first time from the marine algae *Clathria baltica*. All three metabolites and the ethanol extract of *Clathria baltica* were screened against cancer cell lines, MCF-7, DLD-1, HeLa, FADU, A549, and SKOV3, and one normal human cell line using sulforhodamine B assay and doxorubicin as the standard. Among all isolates, compound 3 showed significant growth inhibition of MCF-7, DLD-1 and FADU with IC_{50} values of 26.5, 15.5 and 16.5 µg/ml, respectively. Whereas, all the metabolites and the extract exerted lower growth inhibitory effect on the normal human cell line tested. This is the first *in vitro* gas chromatography-mass spectrometry analysis, as well as, cytotoxicity report on marine algae *Clathria baltica*.

Key words: GC-MS analysis, isolation, characterization, anticancer activity, sulforhodamine B assay

Globally, cancer still remains an aggressive killer that severely effecting the human population[1]. According to the report of the International Agency for Research on Cancer (IARC), it was documented that within 5 y of diagnosis - 9.6 million cancer deaths from 33.7 million people living with cancer has taken place and 18.1 million new cancer cases were reported in 2018 across the world[2]. In addition, the IARC is also estimated that about 26 million new cancer cases and 17 million cancer deaths per year may be recorded by 2030[3]. In addition, the present therapies for cancer include chemotherapy and radiotherapy, which cause lot of strain to patients and ultimately damage their health[4]. Hence, researchers are focussing on developing new, safer and effective drugs to treat cancer.

From the dawn of ancient medicine, natural products have been used to treat many deadly diseases like cancer. In addition, from the past 30 y, natural products have received great attention in attaining potential as well as novel cancer therapeutic agents[5,6]. Moreover, the mechanism of action of inhibiting various stages of tumorigenesis by most of the natural products were also well-documented[6].

In folklore, marine algae (seaweeds) are used as food, feed, fuel and livelihood in Asian countries. Based on the pigmentation, marine algae are classified as red, brown and green algae. From the past few decades, marine algae have been highly acknowledged to possess noticeable pharmacological activities, which include antineoplastic, antimicrobial and antiviral activities due to their specific functional compounds (which are not available in other plants)[7-10]. Particularly, marine algae of *Chara* genus (family: Characeae- charophyte green algae) reported to possess and antioxidant enzyme, allelophatic activities and lipid peroxidation capabilities along with high levels of photosynthetic pigment content[11]. Earlier reports from our laboratory showed phytochemical analysis along with antioxidant

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activities of marine algae *Chara baltica*[12]. Till date, complete chemical and pharmacological evaluation has not been reported on *C. baltica*. Based on the aforementioned properties of *Chara* genus, as well as, *C. baltica*, *C. baltica* specimens (in July 2018) were subjected to gas chromatography-mass spectrometry (GC-MS) analysis, chemical and cytotoxicity evaluation, which are reported in this research communication.

MATERIALS AND METHODS

The specimens of seaweed *Chara baltica* was collected near Korangi coast, Kakinada, Andhra Pradesh, India at a depth of 8-10 ft (16°80'N and 82°08'E with 3 m elevation) on 3 July, 2018. The seaweed was authenticated in the Botany Department, Andhra University, Visakhapatnam, India. A voucher specimen was deposited in the Marine Organisms Museum in the University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India (No. AU-SW-2018-947). All chemicals used in the present study were of analytical grade. Streptozotocin was from Himedia Laboratories Pvt. Ltd. (Mumbai, India); glibenclamide from the Aventis Pharma Ltd. (Mumbai, India); and rat feed from the Hindustan Lever Ltd. (Mumbai, India).

Extraction and isolation:

The seaweed was cleansed from extraneous substance and stored in ethanol-water (9:1) at the site of collection. The crude plant material (100 g) was extracted at least thrice with ethanol-water and concentrated at a reduced pressure and the combined extracts were lyophilized to obtain ethanol extract of *C. baltica*; as a reddish brown gummy residue (3.25 g). About 2 g of ethanol extract was subjected to column chromatography, afforded three fractions. Fraction I (510 mg) and fraction II (800 mg) were subjected to repeated column chromatography using hexane and ethyl acetate as a solvent system yielded 1 (240 mg) as a colourless liquid and 2 (330 mg) as a pale greenish liquid, respectively. Fraction III (400 mg) were purified by recrystallization technique using hexane and acetone as solvents yielded 3 (200 mg) as a greenish crystals.

Compound 1 (dihydrofuran-2,5-dione), colorless crystals, molecular formula: $C_4H_4O_3$; yield: 240 mg; R_f: 0.7 (hexane:ethyl acetate, 1:9); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.00 (4H, s, 2,3-CH_2); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 22.16 (C-2/C-3), 170.24 (C-1/C-4); LC-MS *m/z*: 100.

Compound 2 (3-hydroxy-4,4-dimethyldihydrofuran-2(3H)-one), pale greenish liquid, $C_6H_{10}O_3$; yield: 330 mg; R_f: 0.6 (hexane:ethyl acetate, 1:4); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.00 (6H, s, 5,6-CH_3), 2.31 (1H, s, 2-CH), 3.86-3.88 (1H, d, *J*= 8 Hz, 4a-CH_2), 4.04-4.06 (1H, d, *J*= 8 Hz, 4a-CH_2); 4.11 (1H, s, 2-OH); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 23.15 (C-5/C-6), 43.40 (C-3), 80.44 (C-2), 87.23 (C-4), 182.11 (C-1); LC-MS *m/z*: 130.

Compound 3 (2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one), pale greenish crystals, molecular formula: $C_6H_8O_4$; yield: 200 mg; R_f: 0.4 (hexane:ethyl acetate, 1:4); melting point: 163-164°; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.87 (3H, s, 6-CH_3), 2.05 (3H, s, 5-CH_3), 3.62 (1H, s, 3-OH), 3.92 (1H, s, 1-OH); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 12.14 (C-6), 22.41 (C-5), 99.46 (C-1), 139.32 (C-3), 159.75 (C-4), 192.74 (C-2); CHNS analysis: found C-50.00, H-5.60 (%), calcd. C, 50.46, H, 5.54 (%); LC-MS *m/z*: 144.

Gas chromatography-mass spectrometry (GC-MS) analysis:

The phytochemical investigation of ethanol extract of *C. baltica* was performed on a GC-MS system (GCMS-QP2010 Plus, Shimadzu, Europe). Experimental parameters set for the GC-MS were, column oven temperature- 50°; injection temperature- 250°; injection mode- split; flow control mode- linear velocity; pressure- 29.7 kPa; total flow- 7.3 ml/min; column flow- 0.72 ml/min; linear velocity- 30.7 cm/s; purge flow- 3.0 ml/min; split ratio- 5.0; oven temperature program- 50° with rate of increase of 10°/min, 50-300° with rate of increase of 7.5°/min, hold time of 2.0 min; equilibrium time- 1.0 min; ion source temperature- 210°; interface temperature- 250°; solvent cut time- 3.0 min; detector gain- 0.99+0.20 kV; threshold- 1000; start time- 4.0 min; end time- 37.30 min; ACQ mode- scan; event time- 0.50 s; scan speep- 2500; start *m/z*- 34.0 and end *m/z*- 1090.00.

Cytotoxicity assay:

MCF-7 (breast), DLD-1 (colon), HeLa (cervical), FADU (head and neck), A549 (lung), SKOV3 (ovary) and normal human mammary epithelial (NHME) were kindly provided by National Centre for Cell Science, Pune. The cancer cells were maintained in minimum essential medium (MEM), containing 10 % fetal calf serum (FBS), 5 % mixture of 100 units penicillin and 100 µg/ml streptomycin in presence of 5 % carbon

dioxide in an incubator with 90 % humidity at 37° for 72 h.

Cancer cell lines were maintained in MEM (adjusted to 10 % FBS, 1.5 g/ml $NaHCO_3$, 0.1 mM MEM non-essential amino acids and 1 mM sodium pyruvate). Three days prior to performing assay, the cells were washed with sterilized phosphate-buffered saline and grown using MEM media (supplemented with 0.25 % trypsin in versene-EDTA and 10 % FBS) and mixed to obtain homogeneous suspension of cells. The suspension was taken in a sterilized polypropylene tube and the cell count in each well was determined in a hemacytometer chamber under a microscope using 0.4 % trypan blue solution. The minimal seed density must be 1.9×10^4 cells per well. All crude extracts and the standard were dissolved in dimethyl sulfoxide (DMSO) to 100 mg/ml and 10 µg/ml, respectively. Doxorubicin and DMSO were used as a standard and control, respectively.

The sulforhodamine B (SRB) assay[13] is based on the estimation of cellular protein content. The prepared samples were taken in 96-well tissue-culture plate and added 190 µl screened ideal cell suspension and mixed occasionally and incubate at 37° with 5 % CO_2 and 90 % relative humidity for 3 h. Then 100 µl cold TCA was added to each well and incubated at 4° for 1 h. After that the plates were gently washed using water, dried using blow dryer and air-dried at room temperature. To each completely dried well, 100 µl of 0.057 % SRB solution was added, kept aside for 30 min and quickly rinse with 1 % acetic acid. To the dried plate, 200 µl of 10 mM Tris base (pH 10.5) solution was added, shake for 5 min and measure the OD at 510 nm. The blank contains only medium while the control has only cancer cells with no test samples. Percent growth inhibition was calculated using the formula, growth inhibition (%) = 100–[(S–B)/(C–B)]×100, where S is mean OD value of sample, B is mean OD value of blank, C is mean OD value of control.

RESULTS AND DISCUSSION

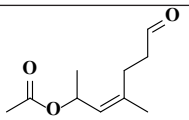
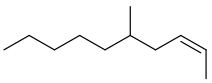
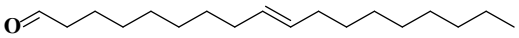
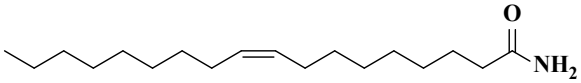
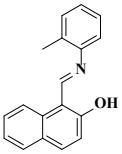
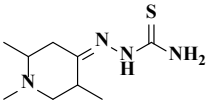
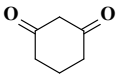
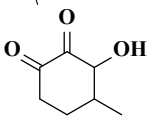
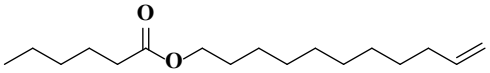
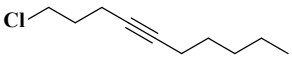
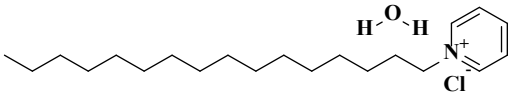
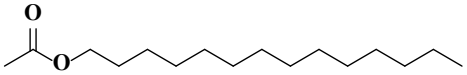
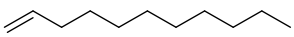
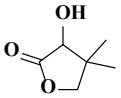
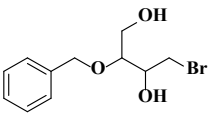
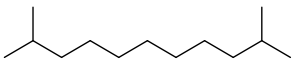
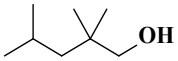
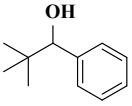
After the successful extraction of the whole marine algae material with ethanol-water, the dried ethanol extract was subjected to GC-MS analysis. The GC-MS chromatogram revealed the presence of various chemical components with different retention times, whereas the MS analyzes the compounds eluted at different times to detect its nature and structure of the compounds. The results pertaining to GC-MS analysis of the ethanol extract lead to the identification of a number of compounds. A total of 86 compounds were identified by GC-MS analysis of ethanol extract, which were tabulated in Table 1.

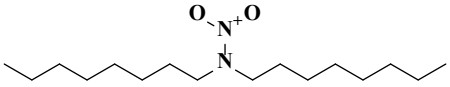
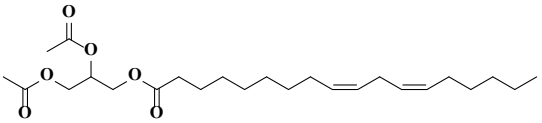
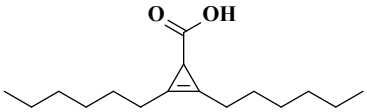
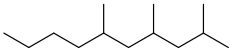
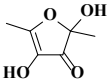
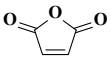
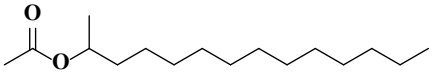
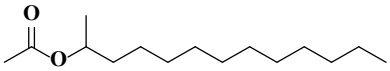
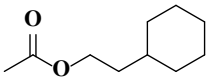
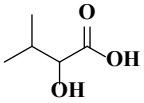
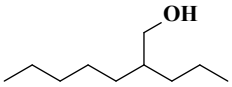
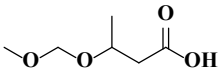
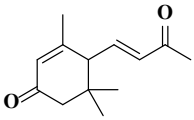
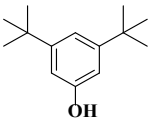
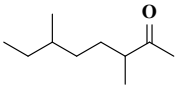
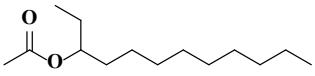
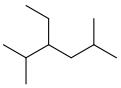
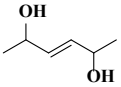
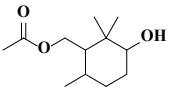
The collected seaweed was extracted with ethanol-water at room temperature. The obtained ethanol extract *C. baltica* was subjected to chromatographic purification led to isolation of three metabolites (1-3, fig. 1). Compound 1 was obtained as a colorless crystals with R_f value 0.7 (hexane:ethyl acetate, 1:9) and the LC-MS ion peak at *m/z* 100 confirmed the molecular formula as $C_4H_4O_3$. The ^1H and ^{13}C NMR data showed the presence of two methane groups at δ_H 4.00 (*s*, 4H) with corresponding carbon signal at δ_C 22.16 (C-2 and C-3). In addition, the ^{13}C NMR also revealed the presence of dione groups at δ_C 170.24 (C-1 and C-4). Hence, compound 1 was confirmed after corroboration with the existing literature[14] as a dihydrofuran-2,5-dione (fig. 1).

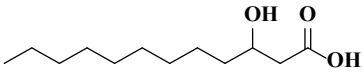
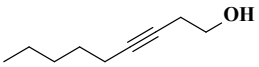
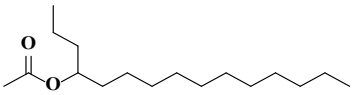
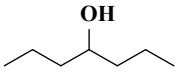
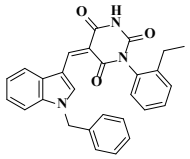
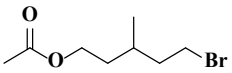
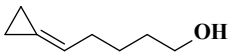
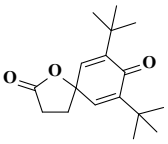
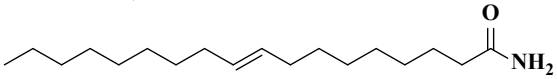
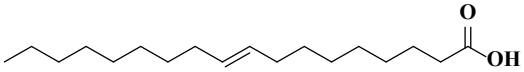
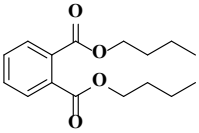
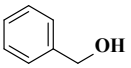
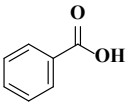
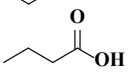
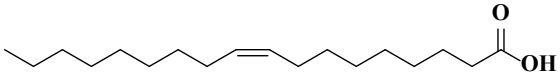
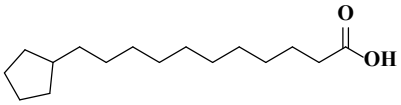
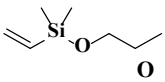
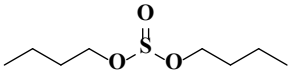
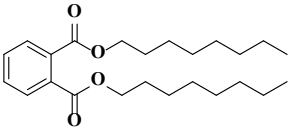
Compound 2 was obtained as a pale greenish liquid. Based on the LC-MS ion peak at *m/z* 130 confirmed the molecular formula as $C_6H_{10}O_3$. The ^1H NMR showed the presence of two singlets for two methyl groups at δ_H 1.00 (*s*, 6H) with corresponding carbon signal at δ_C 23.15 (C-5 and C-6), including one methine group at δ_H 2.31 (*s*, 1H) with corresponding carbon signal at δ_C 80.44 (C-2), and one hydroxyl group at δ_H 4.11 (*s*, 1H), additionally, two doublets at δ_H 3.86-3.88 (*d*, *J*=8 Hz, 1H) and 4.04-4.06 (*d*, *J*=8 Hz, 1H), which confirmed the presence of methane group at δ_C 87.23. In addition, the ^{13}C NMR also revealed the presence of dione group at δ_C 182.11 (C-1). By corroboration with the existing literature compound 2 was found to be 3-hydroxy-4,4-dimethyldihydrofuran-2(3H)-one (fig. 1)[15].

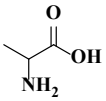
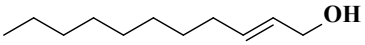
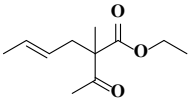
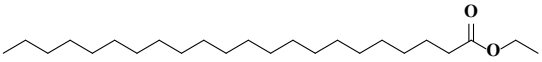
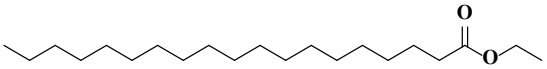
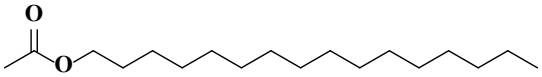
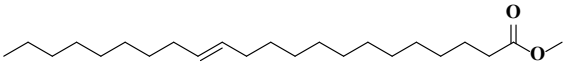
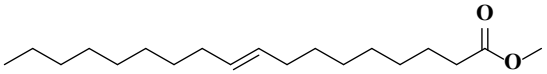
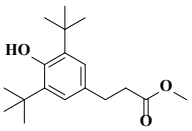
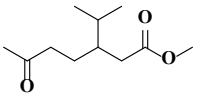
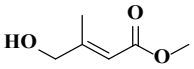
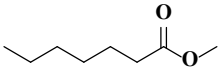
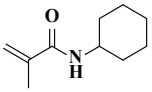
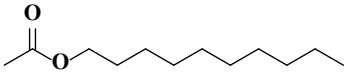
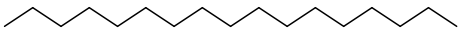
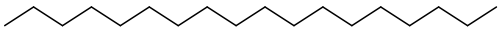
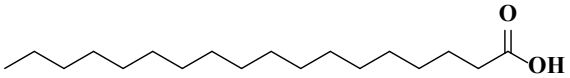
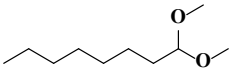
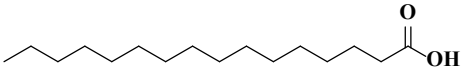
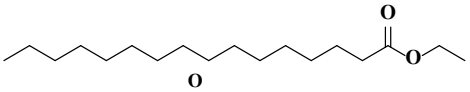
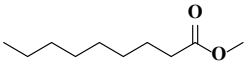
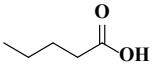
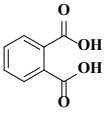
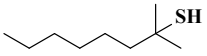
Compound 3 was obtained as a pale greenish crystals, based on the LC-MS ion peak at *m/z* 144 confirmed the molecular formula as $C_6H_8O_4$. The ^1H NMR showed the presence of four singlets for two methyl groups at δ_H 1.87 (*s*, 3H) and 2.05 (*s*, 3H), and for two hydroxyl groups at δ_H 3.62 (*s*, 1H) and 3.92 (*s*, 1H). The ^{13}C NMR revealed the presence of two methyl group at δ_C 12.14 (C-6) and 22.41 (C-5), two hydroxyl groups at δ_C 99.46 (C-1) and 139.32 (C-3), dione group at δ_C 192.74 (C-2), and one methene carbon signal at δ_C 159.75 (C-4). Based on the aforementioned data, compound 3 was compared with the existing literature on lichen metabolites and it was confirmed as a 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one (fig. 1). Compound 3 was earlier reported from *Sysepalum dulcificum*[16].

TABLE 1: CHEMICAL CONSTITUTENTS IDENTIFIED BY GC-MS ANALYSIS OF ETHANOL EXTRACTS OF *CHARA BALTICA*

S. No	Compound name	Chemical structure	Molecular formula
1	(2Z)-1,3-dimethyl-6-oxo-2-hexenyl acetate		$C_{10}H_{16}O_3$
2	(2Z)-5-methyl-2-decene		$C_{11}H_{12}$
3	(9*E*)-9-octadecenal		$C_{18}H_{34}O$
4	(9Z)-9-octadecenamide		$C_{18}H_{35}NO$
5	1-((*E*)-[(2-methylphenyl)imino] methyl)-2-naphthol		$C_{18}H_{15}NO$
6	1,2,5-trimethyl-4-piperidinone thiosemicarbazone		$C_9H_{18}N_4S$
7	1,3-cyclohexanedione		$C_6H_8O_2$
8	1,4-dimethyl-5-oxabicyclo[2.1.0] pentane		$C_6H_{10}O$
9	1,5-anhydro-6-deoxyhexo-2,3-diulose		$C_7H_{10}O_3$
10	10-undecen-1-ylester		$C_{17}H_{32}O_2$
11	1-chloro-4-decyne		$C_{10}H_{17}Cl$
12	1-hexadecylpyridinium chloride monohydrate		$C_{21}H_{40}ClNO$
13	1-Tetradecanol, acetate		$C_{16}H_{32}O_2$
14	1-undecene		$C_{11}H_{22}$
15	3-hydroxy-4,4-dimethyldihydrofuran-2(3H)-one		$C_6H_{10}O_3$
16	2-(benzyloxy)-4-bromo-1,3-butanediol		$C_{11}H_{15}BrO_3$
17	2,10-dimethylundecane		$C_{13}H_{28}$
18	2,2,4-trimethyl-1-pentanol		$C_8H_{18}O$
19	2,2-dimethyl-1-phenyl-1-propanol		$C_{11}H_{16}O$

20	2,2-dioctyl-1-oxohydrazine-1-oxide		$C_{16}H_{34}H_2O_2$
21	9,12-Octadecadienoic acid (Z,Z)-, 2,3-bis(acetyloxy)propyl ester		$C_{25}H_{42}O_6$
22	2,3-dihexyl-2-cyclopropene-1-carboxylic acid		$C_{16}H_{28}O_2$
23	2,4,6-trimethyldecane		$C_{13}H_{28}$
24	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furanone		$C_6H_8O_4$
25	2,5-furandione		$C_4H_2O_3$
26	2-acetoxytetradecane		$C_{16}H_{32}O_2$
27	2-acetoxytridecane		$C_{15}H_{30}O_2$
28	2-cyclohexen-1-one		C_6H_8O
29	2-cyclohexylethyl acetate		$C_{10}H_{18}O_2$
30	2-hydroxy-3-methylbutanoic acid		$C_5H_{10}O_3$
31	2-propylheptan-1-ol		$C_{10}H_{22}O$
32	3-(methoxymethoxy)-butanoic acid		$C_6H_{12}O_4$
33	3,5,5-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one		$C_{13}H_{18}O_2$
34	3,5-bis(1,1-dimethylethyl)-phenol		$C_{14}H_{22}O$
35	3,6-dimethyl-2-octanone		$C_{10}H_{20}O$
36	3-acetoxydodecane		$C_{14}H_{28}O_2$
37	3-ethyl-2,5-dimethylhexane		$C_{10}H_{22}$
38	3-hexene-2,5-diol		$C_6H_{12}O_2$
39	3-hydroxy-2,2,6-trimethylcyclohexyl-methyl acetate		$C_{12}H_{22}O_3$

40	3-hydroxydodecanoic acid		$C_{12}H_{24}O_3$
41	3-nonyn-1-ol		$C_9H_{16}O$
42	4-acetoxypentadecane		$C_{17}H_{34}O_2$
43	4-heptanol		$C_7H_{16}O$
44	5-(1-benzyl-1h-indol-3-ylmethylene)-1-(2-ethyl-phenyl)-pyrimidine-2,4,6-trione		$C_{28}H_{23}N_3O_3$
45	5-bromo-3-methylpentyl acetate		$C_8H_{15}BrO_2$
46	5-cyclopropylidene-1-pentanol		$C_8H_{14}O$
47	7,9-ditert-butyl-1-oxaspiro[4.5] deca-6,9-diene-2,8-dione		$C_{17}H_{24}O_3$
48	9-octadecenamide		$C_{18}H_{35}NO$
49	9-octadecenoic acid		$C_{18}H_{34}O_2$
50	Acetic acid		$C_2H_4O_2$
52	Araldite		$C_{16}H_{22}O_4$
53	Benzenemethanol		C_7H_8O
54	Benzoic acid		$C_7H_6O_2$
55	Butanoic acid		$C_4H_8O_2$
56	Cis-oleic acid		$C_{18}H_{34}O_2$
57	Cyclopentaneundecanoic acid		$C_{16}H_{30}O_2$
58	Dimethyl(ethenyl)propoxysilane		$C_7H_{16}OSi$
59	Di-*n*-butyl sulphite		$C_8H_{18}O_3S$
60	Di-n-octyl phthalate		$C_{24}H_{38}O_4$

61	*DL*-alanine		$C_3H_7NO_2$
62	*E*-2-undecen-1-ol		$C_{11}H_{22}O$
63	Ethyl-(4E)-2-acetyl-2-methyl-4-hexenoate		$C_{11}H_{18}O_3$
64	Ethyl docosanoate		$C_{24}H_{48}O_2$
65	Ethyl nonadecanoate		$C_{21}H_{42}O_2$
66	Hexadecyl acetate		$C_{18}H_{36}O_2$
67	Methyl-(*E*)-docos-13-enoate		$C_{23}H_{44}O_2$
68	Methyl-(9*E*)-9-octadecenoate		$C_{19}H_{36}O_2$
69	Methyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate		$C_{18}H_{28}O_3$
70	Methyl-3-isopropyl-6-oxoheptanoate		$C_{11}H_{20}O_3$
71	Methyl-4-hydroxy-3-methyl-2-butenoate		$C_6H_{10}O_3$
72	Methyl heptanoate		$C_8H_{16}O_2$
73	*n*-cyclohexyl methacrylamide		$C_{10}H_{17}NO$
74	*n*-decyl acetate		$C_{12}H_{24}O_2$
75	*n*-heptadecane		$C_{17}H_{36}$
76	Octadecane		$C_{18}H_{38}$
77	Octadecanoic acid		$C_{18}H_{38}O_2$
78	Octanal dimethyl acetal		$C_{10}H_{22}O_2$
79	Palmitic acid		$C_{16}H_{32}O_2$
80	Palmitic acid ethyl ester		$C_{18}H_{36}O_2$
81	Pelargonic acid methyl ester		$C_{10}H_{20}O_2$
82	Pentanoic acid		$C_5H_{10}O_2$
83	Phthalic acid		$C_8H_6O_4$
84	Tert-nonanethiol		$C_9H_{20}S$

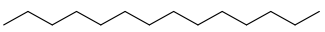
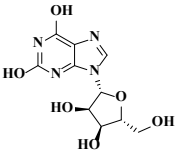
85	Tetradecane		$C_{14}H_{30}$
86	Xanthosine		$C_{10}H_{12}N_4O_6$

TABLE 2: CYTOTOXICITY STUDIES OF COMPOUNDS 1-3 AND ETHANOL EXTRACT OF *CHARA BALTICA*

Sample	Percent growth inhibition at 30 µg/ml						
	MCF-7	DLD-1	HeLa	FADU	A549	SKOV3	NHME[c]
1	1.46±0.18	5.64±0.65	3.97±0.70	13.80±1.06	21.37±1.94	5.69±0.87	0.52±0.01
2	7.17±0.24	11.91±0.50	5.12±0.18	16.41±1.26	14.57±0.39	8.14±1.05	1.04±0.16
3	56.86±4.78	76.37±5.34	22.27±6.23	64.08±3.92	34.07±4.84	46.11±4.74	9.40±0.33
Ethanol extract*	71.53±4.71	87.11±3.58	40.90±2.57	70.46±4.96	57.62±5.58	48.24±4.44	2.91±0.31
Doxorubicin[#]	84.40±0.80	66.71±0.71	90.71±0.13	88.34±0.27	65.40±0.60	77.05±0.22	10.08±0.95

Mean±SEM values (n=3); [#]10 µg/ml; *100 µg/ml; [c]normal human mammary epithelialium

TABLE 3: GROWTH INHIBITION AND IC_{50} VALUES OF COMPOUND 3 AND ETHANOL EXTRACTS OF *CHARA BALTICA*

Cell line sample	Concentration (µg/ml)				IC_{50} values (µg/ml)
	5	10	20	30	
MCF-7					
Compound 3	18.09±2.87	28.17±2.87	38.59±1.74	56.86±4.78	26.5
Ethanol extracts**	25.06±1.78	36.58±2.68	50.05±3.57	71.53±4.71	75
Doxorubicin*	30.87±0.61	45.45±0.18	67.08±0.11	81.25±0.27	5.5
DLD-1					
Compound 3	22.55±4.84	39.77±2.83	57.92±7.78	76.37±5.34	15.5
Ethanol extract **	29.75±1.77	42.57±2.49	59.22±1.77	87.11±3.58	61
Doxorubicin*	19.75±4.61	31.58±6.85	44.75±2.84	52.57±7.97	5.4
FADU					
Compound 3	29.68±2.68	40.88±4.06	54.87±4.54	64.08±3.92	16.5
Ethanol extract **	26.47±2.97	37.18±2.52	50.17±3.58	70.46±4.96	74.5
Doxorubicin*	41.01±3.85	52.14±2.84	68.88±1.77	85.55±6.24	4.5
A549					
Ethanol extract **	26.47±2.97	37.18±2.52	50.17±3.58	57.62±5.58	74.5
Doxorubicin*	41.01±3.85	52.14±2.84	68.88±1.77	85.55±6.24	4.5

Each value is a mean+SD of % growth inhibition of the cell line tested with n=3; *2.5, 5.0, 7.5 and 10 µg/ml concentrations; **25, 50, 75 and 100 µg/ml concentrations

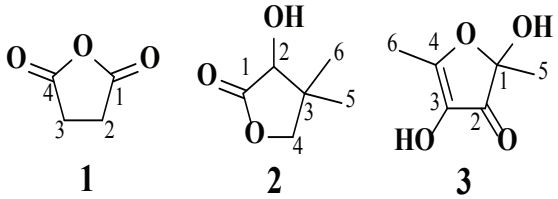


Fig. 1: Known secondary metabolites from ethanol extracts of *Chara baltica*

Earlier reports of *C. baltica* indicated free radical scavenging activity[13]. Based on the report, the isolated metabolites (1-3) were screened along with ethanol extract against six different cancer cell lines using SRB assay with doxorubicin as standard. Initially, compounds (1-3) at 30 µg/ml concentration, extract at 100 µg/ml concentration and doxorubicin at 10 µg/ml concentration were screened against the cell lines tested and percent inhibition of cell growth was tabulated in Table 2.

Further, the samples that caused 50 % or more of cell death were further screened at 5, 10, 20 and 30 µg/ml concentrations for pure compounds; 25, 50, 75 and 100 µg/ml concentrations for extracts; 2.5, 5.0, 7.5 and 10 µg/ml concentrations for doxorubicin. The results were plotted to obtain IC_{50} values. The lower IC_{50} value indicated better inhibitory profile against cancer cell lines.

During the initial screening, the isolate 3 and ethanol extract depicted prominent degree of specificity against MCF-7, DLD-1, FADU and A549. Besides, all the samples exhibited very low toxicity towards NHME cell

lines (Table 2). From the outcomes of the SRB assay, it is interesting to note that compound 3 (30 µg/ml) and ethanol extract (100 µg/ml) showed prominent degree of specificity against DLD-1 with 76.37±5.34 and 87.11±3.58 % growth inhibition which was found to be better than activity of 66.71±0.71 % shown by the standard at 10 µg/ml (Table 2).

From the Table 3, the IC_{50} values of 3 and ethanol extract against MCF-7 were 26.5 and 75.0 µg/ml, respectively, whereas standard value was 5.5 µg/ml. In addition, the concentration of 3 and ethanol extract needed for 50 % inhibition of DLD-1 were found to be 15.5 and 61.0 µg/ml, respectively, while for doxorubicin it was 5.4 µg/ml (Table 3).

From the SRB assay of FADU (Table 3) it can be calculated that the IC_{50} values of 3 and ethanol extract against FADU were 16.5 and 74.5 µg/ml, respectively, whereas standard value was 4.5 µg/ml. Additionally, the concentration of ethanol extract needed for 50 % inhibition of A549 was found to be 74.5 µg/ml, while standard (doxorubicin) was 4.5 µg/ml (Table 3).

Hence, from the overall SRB assay it can be concluded that the key agent responsible for anticancer activity is compound 3. In addition, the growth inhibitory properties of compound 3 is mainly due to the higher levels of oxygenated substituents present in its chemical structure, this higher levels of oxygenated content help in irradiating free radicals that are usually formed during massive cell division of cancer cells, which ultimately lead to the cell death[13,17].

In conclusion, this is the first report of GC-MS analysis, chemical and cytotoxicity profile of marine algae *C. baltica*. The GC-MS analysis of ethanol extract *C. baltica* revealed presence of 89 chemical constituents. In addition, the chemical examination of ethanol extract showed the presence of three metabolites 1-3. Additionally, the cytotoxicity studies of ethanol extract indicated specificity towards MCF-7, DLD-1, FADU and A549 without affecting NHME. The SRB assay screening proved that compound 3 has greater cytotoxicity. The results of the current study could be useful for further research on cancer to identify potential bioactive molecules from different aquatic fauna, such as marine algae like *C. baltica.*

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Conflict of interest:

No conflict of interest between any of the authors.

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