PHARMACEUTICALS IN THE AQUATIC ENVIRONMENT

2,3,7,8-TCDD-mediated toxicity in peripheral blood mononuclear cells is alleviated by the antioxidants present in *Gelidiella acerosa*: an in vitro study

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Abstract Seaweeds have been used as a source of traditional medicine worldwide for the treatment of various ailments, mainly due to their ability to quench the free radicals. The present study aims at evaluating the protective effect of methanolic extract of *Gelidiella acerosa*, an edible red seaweed against 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced toxicity in peripheral blood mononuclear cells (PBMC). For evaluating the protective effect of *G. acerosa*, PBMC were divided into four groups: vehicle control, TCDD (10 nM), TCDD+*G. acerosa* (300 μg/ml), and *G. acerosa* alone treated. Scavenging of intracellular reactive oxygen species (ROS) induced by TCDD was assessed by the dichloro-dihydrofluorescein diacetate (DCFH-DA) method. Alterations at macromolecular level were quantified through lipid peroxidation (LPO) level, protein carbonyl content (PCC) level, and comet assay. The cellular morphology upon TCDD toxicity and *G. acerosa* treatment was obtained by light microscopy and histopathological studies. The chemical composition present in the methanolic extract of *G. acerosa* was determined by gas chromatography-mass spectrometry (GC-MS) analysis. The results reveal that 10 nM TCDD caused significant ($P<0.05$) reduction in cell viability (94.10±0.99), and treatment with 300 μg/ml extract increased the cell viability (99.24±0.69). TCDD treatment resulted in a significant increase in the production of ROS, LPO (114±0.09), and PCC (15.13± 1.53) compared to the control, whereas co-treatment with *G. acerosa* significantly ($P<0.05$) mitigated the effects. Further, *G. acerosa* significantly ($P<0.05$) prevented TCDD-induced genotoxicity and cell damage. GC-MS analysis showed the presence of *n*-hexadecanoic acid (retention time (RT) 13.15), cholesterol (RT 28.80), α-D-glucopyranose, 4-*O*-α-D-galactopyranosyl (RT 20.01), and azulene (RT 4.20). The findings suggest that *G. acerosa* has a strong protective ability against TCDD-induced cytotoxicity, oxidative stress, and DNA damage.

Keywords *G. acerosa* · 2,3,7,8-TCDD · PBMC · Comet assay · Histopathology · Oxidative stress markers

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Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is considered to be one of the most toxic environmental contaminants formed as a by-product in the process of paper and pulp bleaching, in the incineration of municipal and hospital wastes, and during the production of chlorophenoxy herbicides (Skene et al. 1989). It is resistant to degradation and hence tends to accumulate in the environment, in animals, and even in human tissues (Olson 1994). TCDD has a long biological half-life in humans (≥7 years) and is classified by the International Agency for Research on Cancer (IARC, 1997) as a human carcinogen (Baccarelli et al. 2004). TCDD and its related aromatic hydrocarbons have received increasing attention over the past years due to their ability to induce hepatotoxicity, neurotoxicity, cardiotoxicity, teratogenesis, and immunotoxicity (Ciftci and Ozdemir 2010).

The immune system which is one of the most liable targets of TCDD is affected mainly through the activation of the aryl hydrocarbon receptor (AhR) (Inouye et al. 2005). The activation of this transcription factor is potentially involved in the expression of several genes including cytochromes P450 (CYPs) along with response to oxidative stress (Aly and Khafagy 2011). Reactive oxygen species (ROS) formed upon oxidative stress induced by TCDD is involved in

macromolecular damages such as peroxidation of lipids, protein oxidation, and DNA damages (Sul et al. 2009; Turkez et al. 2012). Immunological examinations of dioxin-exposed populations have revealed that TCDD causes alterations in human peripheral blood mononuclear cell (PBMC) functions, including mitogen-induced proliferative responses and immunoglobulin secretion (Lang et al. 1996). Since PBMC, which contains a mixture of immune cells, play an important role in the human immune system and protect humans from infectious agents, maintenance of the integrity of PBMC is vital. An exogenous supply of antioxidant compounds has been shown to protect the cell membrane integrity by quenching the free radicals formed upon oxidative stress (Uttara et al. 2009). Hence, in the present study, the effect of the seaweed *Gelidiella acerosa*, which possesses antioxidant activity (Devi et al. 2008; Suganthy et al. 2010), was assessed for its protective potential against TCDD-induced toxicity in PBMC.

Seaweeds, which are a part of the human diet in Asia, have received much attention as a source of natural antioxidants (Yuan et al. 2005; Ganesan et al. 2008). It is rich in bioactive compounds such as carotenoids, dietary fiber, protein, essential fatty acids, vitamins, and minerals (Namvar et al. 2012; Ganesan et al. 2008). The edible seaweed *G. acerosa*, the perennial red algae in the family Gelidiellaceae, mainly occurs in the west and southeast coast of India. It has been reported as a source of good-quality agar (Prasad et al. 2007). Previous studies from our laboratory have showed that *G. acerosa* has a very potent antioxidant activity (Devi et al. 2008; Suganthy et al. 2010). The present study demonstrates the beneficial effects of *G. acerosa* extract against TCDD-induced toxicity via determination of oxidative, DNA, and histopathological damages in the peripheral blood mononuclear cell model.

Materials and method

Preparation of methanolic extract of *G. acerosa*

G. acerosa was collected during the month of September from the Gulf of Mannar, Tamil Nadu, and was identified according to the references Oza and Zaidi (2001) and Krishnamurthy and Joshi (1970). The voucher specimen of the sample is deposited at the Department of Biotechnology, Alagappa University [AUDBTGA20100101]. The collected seaweeds were washed with water and alcohol (70 %), cut into small pieces, shade dried for 5 to 6 days, and powdered. Ten grams of powdered seaweed was suspended in 50 ml of methanol for 72 h. The extract was filtered and evaporated to dryness under reduced pressure in a desiccator (Tarsons Products Pvt. Ltd., India). The yield of the extract was 5 %. The

dried extract powder was dissolved in sterile Milli-Q water and stored at −20 °C.

% of yield of extract

$$= \frac{\text{Wt of beaker with dried extract} - \text{Wt of beaker}}{\text{Wt of plant material packed}} \times 100$$

PBMC isolation

PBMC were isolated by the density gradient method using HiSep LSM 1077 (HiMedia, India) according to the method described earlier (Kiruthika et al. 2010). After isolation, PBMC were adjusted to 1×10^6 cells/ml in a complete medium [Roswell Park Memorial Institute (RPMI) with 10 % of FBS and 2 mM of antibiotic (L-glutamine-streptomycin)] and cells of 98–100 % viability were used for all the experiments. The protocol was approved by the Institutional Ethics Committee of Alagappa University, Karaikudi, India (No. IEC/ALU/1-2008).

Cell viability

The viability of cells was assessed using trypan blue exclusion assay. To an equal volume (10 μl) of PBMC, trypan blue suspensions (1.6 mg/ml in saline solution) was added, and the live cells were counted in a light microscope using a Neubauer counting chamber to calculate the percentage of cell viability. Cells with damaged cell membrane stained blue (dead cells), while cells with intact plasma membrane integrity remained unstained (live cells).

$$\% \text{ of cytotoxicity} = \frac{\text{Number of live cells}}{\text{Total number of cells}} \times 100$$

Determination of non- cytotoxic concentration/safe dose of *G. acerosa* extract in PBMC

PBMC were treated with various concentrations of crude methanolic extract of *G. acerosa*, ranging from 200 to 1000 μg/ml, at a regular interval of 200 μg/ml for 12 and 18 h at 37 °C in a CO_2 incubator supplied with 5 % CO_2. The cell viability was assessed by trypan blue exclusion assay.

Assessment of cytotoxicity induced by TCDD at an appropriate time interval

For fixing the optimum doses for TCDD, PBMC were treated with 10, 15, 20, and 25 nM and the cell viability was determined by trypan blue assay at 18 and 24 h. The

PBMC were maintained at 37 °C in a CO_2 incubator supplied with 5 % CO_2.

Fixing the optimum protective dose for *G. acerosa*

The PBMC were treated with 20–100 μg/ml (at intervals of 20 μg/ml) and 100–500 μg/ml (at intervals of 100 μg/ml) of crude methanolic extract of *G. acerosa*, and toxicity was assessed against 10 nM of TCDD. The cells were kept at 37 °C in a CO_2 incubator supplied with 5 % CO_2 for 18 h, and the cell viability was assessed by trypan blue exclusion method.

Experimental setup

For further experiments, PBMC (1×10^6 cells/ml) were divided into four different groups as follows: group 1, vehicle control (VC)—PBMC in RPMI medium; group 2, TCDD treated (T)—PBMC treated with TCDD (10 nM dissolved in less than 0.1 % DMSO) in RPMI medium; group 3, seaweed extract+TCDD (ST)—PBMC treated with TCDD (10 nM)+ seaweed extract (300 μg/ml dissolved in sterile Milli-Q water) in RPMI medium; and group 4, seaweed extract alone (SA)— PBMC treated with 300 μg/ml seaweed extract alone in RPMI medium. The cells which were treated with TCDD/seaweed extract were maintained in 5 % CO_2 at 37 °C for 18 h.

Assay of cytotoxicity

Cell viability was determined by trypan blue exclusion assay.

Assessment of oxidative stress markers

Detection of intracellular ROS

The reactive oxygen species (ROS) produced by PBMC was analyzed using fluorescent dye dichloro-dihydro-fluorescein diacetate (DCFH-DA) as per the method of Chen et al. (2004). In brief, after incubation of cells for 18 h, the cells were washed with Kreb's-Ringer solution for 5 min and 10 μM of DCFH-DA was added; the cells were incubated for 15 min at 37 °C in a CO_2 incubator. Then, they were washed three times with Kreb's-Ringer solution. For capturing the representative image, the stained cells were mounted onto a cover slip and the images were viewed in a confocal laser scanning microscope (model: LSM 710, Carl Zeiss, Germany).

Estimation of lipid peroxidation

Thiobarbituric acid reactive substance (TBARS), which is a breakdown product of lipid peroxidation (LPO), was estimated by the method of Nabavi et al. (2012), using malondialdehyde (MDA) as a standard. The assay mixtures contained 0.3 ml of cell lysate and 0.6 ml of stock solution (0.37 % TBA and 15 % trichloroacetic acid suspended in 2.5 M HCl). The samples were vortexed and heated to 100 °C for 15 min in a boiling water bath and cooled. Precipitate was removed by centrifugation at 3000 rpm for 15 min, and the absorbance was measured at 532 nm against the blank. The LPO was expressed as nanomolars of TBARS per milligram of protein.

Determination of protein carbonyl content

Protein carbonyl content was assessed by Kiruthika et al. (2010) with mild modifications. Initially, 0.6 ml of cell lysate was taken in two tubes, and one tube was marked as "test" and the other as "control." An equal amount of 10 % TCA was added and the mixture was centrifuged at 3000 rpm for 10 min. To the test sample was added 0.6 ml of 0.2 % 2,4-dinitrophenylhydrazine (DNPH) prepared in 2.5 M HCl, and 0.6 ml of 2.5 M HCl alone was added to the control sample. The contents were mixed thoroughly and incubated in the dark (room temperature) for 1 h with intermittent shaking every 15 min. Then, 10 % TCA was added to both tubes and centrifuged for 20 min at 3500 rpm to obtain the protein precipitates. The supernatant was carefully aspirated and discarded. The pellets were washed using 1 ml of ethanol/ethyl acetate (1:1, *v/v*) to remove unreacted DNPH. The final protein pellet was dissolved in 6 M guanidine hydrochloride and was incubated at 37 °C for 10 min. Any insoluble material was removed by centrifugation, and the carbonyl content was measured by taking the absorbance of the representative supernatant of samples at 370 nm. Each sample was read against the control (treated with 2.5 M HCl). The carbonyl content was calculated using the absorption coefficient of DNPH (22,000 M/cm) and expressed in micromolars per milligram of protein.

Measurement of genotoxicity by alkaline comet assay

The comet assay which is a rapid and quantitative method used to evaluate DNA strand breaks in individual cells visually was assessed by the method of Gandhi (2013). After incubation for 18 h, PBMC were centrifuged at 2500 rpm for 15 min to remove the medium. PBMC were then washed thrice with PBS by centrifugation at 2000 rpm for 4 min. The cell pellet suspended in 200 μl of 0.75 % low melting agarose was spread over a thin 1 % normal melting agarose gel on a dried microscopic slide, and the slides were allowed to solidify with cover slips for 5 min at 40 °C. After solidification, the cover slips were removed and dipped in lysis buffer overnight. After lysis, the slides were placed on a horizontal electrophoresis box filled with freshly made electrophoresis buffer, and the embedded cells were electrophoresed in the solution for 20 min at 25 V to allow DNA unwinding and expression of

alkali-labile damage. The slides were then neutralized by gently washing in neutralization buffer, pH 7.5. After staining with 10 μg/ml of ethidium bromide, the cells were examined in a confocal laser scanning microscope (model: LSM 710, Carl Zeiss, Germany) equipped with an excitation filter of 515–560 nm and a magnification of ×20. Imaging was performed using AutoComet™ scoring software to determine the percentage of DNA in the tail, tail moment, and olive tail moment. The significance was evaluated by Mann-Whitney *U* test.

Morphological assessment of cells by microscopic evaluation

Light microscopy

After the incubation period, PBMC were mounted in a glass slide and viewed in a light microscope to visualize the morphology. The photographs were taken with a digital camera (Nikon H550L) at a magnification of ×20.

Histopathology

For histopathological evaluation, a smear was prepared on a clean glass slide with few drops of PBMC suspension and stained with Leishman stain. The stained slides were examined under a light microscope for characteristic histological changes.

GC-MS analysis of the *G. acerosa* extract

Gas chromatography-mass spectrometry (GC-MS) analysis was performed on a GC Clarus 500 PerkinElmer apparatus, fitted with Elite-5MS (5 % diphenyl/95 % dimethylpolysiloxane), 30×0.25 mm×0.25 μm df column, coupled to mass detector TurboMass Gold (PerkinElmer), operating with electron impact mode at 70 eV. The oven temperature was held at 110 °C for 2 min, then increased up to 280 °C with 5 °C/min increments, and held at this temperature for 9 min. Three microliters of *G. acerosa* methanol extract sample was injected into the GC-MS instrument for analysis. Helium was used as carrier gas at a flow rate of 1 ml/min. The injector temperature was held at 250 °C, and interface temperature and ion-source temperature were kept at 200 °C. The mass range selected was set at 45–450 (*m/z*). The chemical components of the extract were identified by comparing their retention indices (RI) and mass fragmentation patterns with those stored in the NIST (National Institute of Standards and Technology) library, year 2005.

Determination of protein content

Protein content was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis

All the values are expressed as mean±SD. The significance of the difference between groups [T vs VC; ST vs T] was assessed by Student's *t* test. *$p < 0.25$, **$p < 0.10$, and ***$p < 0.05$ were used as the criteria for significance.

Results and discussion

TCDD is a highly toxic environmental pollutant which produces a broad range of acute and long-term toxic and biochemical effects in experimental animals (Byers et al. 2006). Many reports demonstrated that TCDD-induced toxicity is associated with increased oxidative stress and free radical-mediated tissue damage in experimental animals (Hassoun et al. 2002; Latchoumycandane et al. 2003). Toxic effects caused by TCDD are mediated via aryl hydrocarbon receptor (AhR)-mediated signaling pathways. In the presence of TCDD, AhR gets translocated into the nucleus and induces the transcription of phase I (CYP1A1 and 1B1) and phase II drug-metabolizing genes such as glutathione *S*-transferases (GSTs), uridine diphosphate-glucuronosyltransferases (UGTs), NAD(P)H quinine oxidoreductase (NQO1), and UDP-glucuronyltransferase (Hung et al. 2006; Ishimura et al. 2009). Activation of CYP450 results in the generation of ROS and alters the level of antioxidant enzymes like SOD, CAT, GPx, and GR and thereby disrupts the cells. Hence, novel antioxidant-rich pharmacological strategies that prevent the TCDD-mediated toxicity might represent a powerful therapeutic tool.

An earlier finding in our laboratory showed that *G. acerosa* extract scavenges free radicals in vitro (Devi et al. 2008; Suganthy et al. 2010). The purpose of the present study is to elucidate the role of *G. acerosa* against TCDD-induced oxidative stress. The results suggest that the crude methanolic extract of the edible red seaweed *G. acerosa* alleviates TCDD-induced oxidative stress, cell damage, and DNA damage in human PBMC.

Determination of the safety dose for *G. acerosa*

Before fixing the optimum dosage, the non-cytotoxic concentration of *G. acerosa* was determined with varying concentrations (200–1000 μg/ml) and time intervals (12–18 h). The cell viability data confirmed that *G. acerosa* extract, at the tested concentrations, was non-cytotoxic on PBMC (data not shown).

Fixation of optimum dosage for TCDD

In order to select the toxic concentration of TCDD, 18- and 24-h dose-response studies were conducted by exposing the
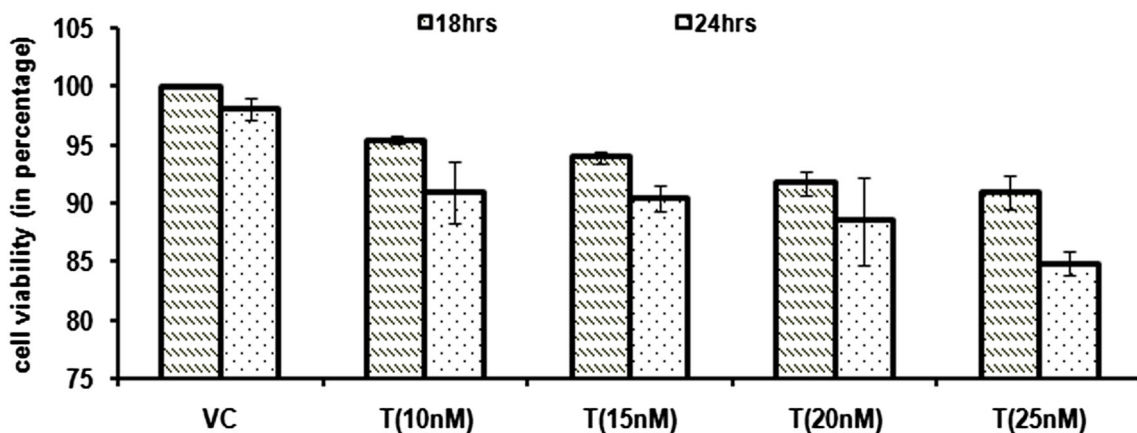
Fig. 1 Fixation of optimum dosage for TCDD. PBMC were incubated with increasing concentration of TCDD (10 to 25 nM) for 18 and 24 h. Values are presented as percentage of the control (mean±S.D., $n=3$)

cells to different concentrations of TCDD (10–25 nM). Cell viability was measured by trypan blue exclusion assay. Figure 1 reveals that increasing the concentration of TCDD induced a dose- and time-dependent increase in toxicity as shown by the decrease in cell viability. Cells treated with TCDD (10–25 nM) for 18 h showed decreased viability (95.44–90.95 %) compared to the control (100 %). After 24 h of treatment with different concentrations of TCDD, the cell viability was reduced more (90.97–84.87 %) compared to the control (98.10 %). A change in morphology was observed in PBMC incubated for 24 h in both the control and

TCDD-treated groups. Based on the results obtained and the earlier reports (Baccarelli et al. 2004; Landi et al. 2005), 10 nM concentration of TCDD was chosen for further experiments.

Fixation of optimum dosage for *G. acerosa* extract

PBMC were treated with different concentrations of crude methanol extract of *G. acerosa* (20–100 and 100–500 μg/ml) and 10 nM of TCDD for 18 h. Co-treatment with the extract suppressed TCDD-induced cell death in PBMC in a dose-

Fig. 2 Protective effects of different doses of *G. acerosa* extract [20–100 μg/ml (**a**) and 100–500 μg/ml (**b**)] against 10 nM of TCDD. Viability of PBMC was determined by trypan blue exclusion assay. Values are presented as percentage of the control (mean±S.D., $n=3$)
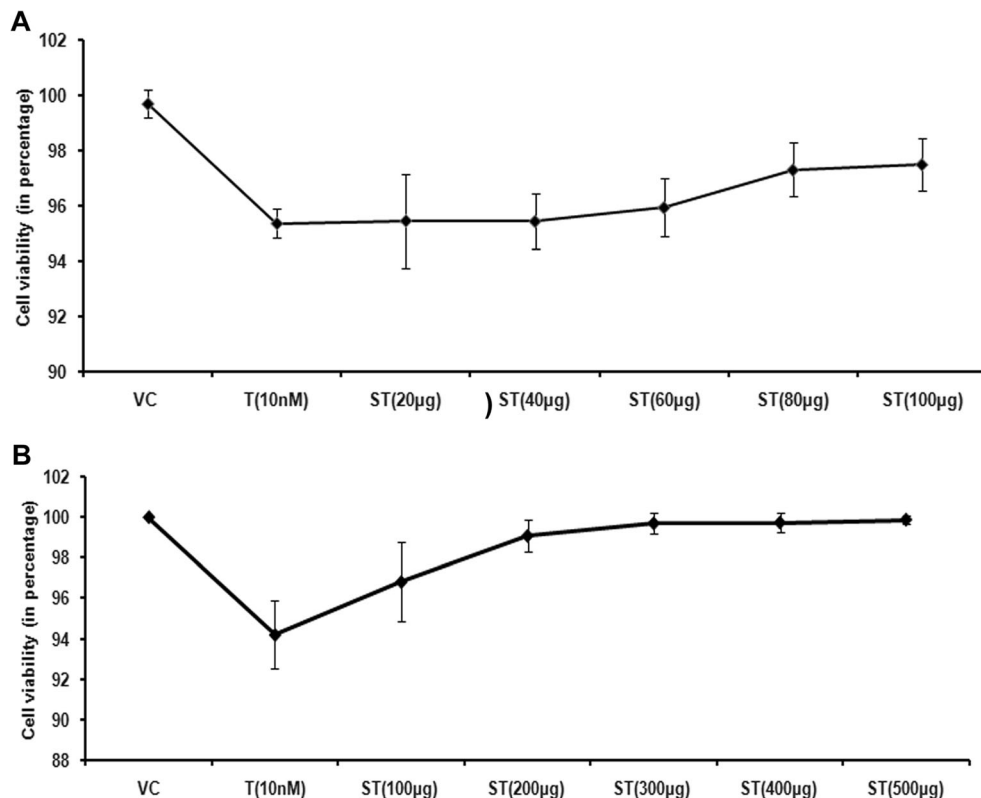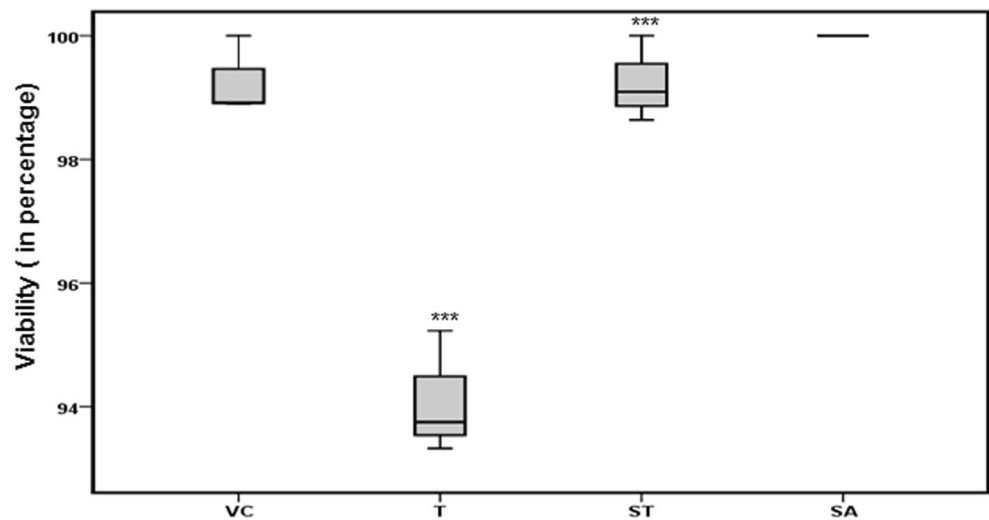
Fig. 3 Suppressive effect of
G. acerosa extract against TCDD-
induced cell death. PBMC were
incubated with TCDD (10 nM)
and *G. acerosa* (300 μg/ml) for
18 h, and cell viability was
assessed by trypan blue assay.
Values are mean±S.D. from three
independent experiments
(***$p<0.05$)

dependent manner (95–100 %) (Fig. 2a, b). Results showed
that 300 μg/ml of extract showed 99.70 % cytoprotection in
PBMC; hence, 300 μg/ml of *G. acerosa* extract against 10 nM
TCDD at 18 h was used for further studies.

G. acerosa extract protects PBMC against TCDD-induced cytotoxicity

The protective effect of *G. acerosa* was studied by trypan blue
exclusion assay. The total number of viable cells was

significantly reduced ($p<0.05$) in TCDD-treated cells relative
to untreated control cells (Fig. 3), which might be due to the
production of ROS, which plays an important role in TCDD-
mediated toxicity. ROS reacts with the cellular components
including nucleic acids, lipids, and proteins and can cause
disintegration of cell membranes. Earlier reports indicate that
TCDD reduced the viability of PC12 cells (Martin et al. 2010)
and induced a time-dependent increase in cytotoxic response
in human breast cancer (MCF-7, MDA-MB-231) and pitui-
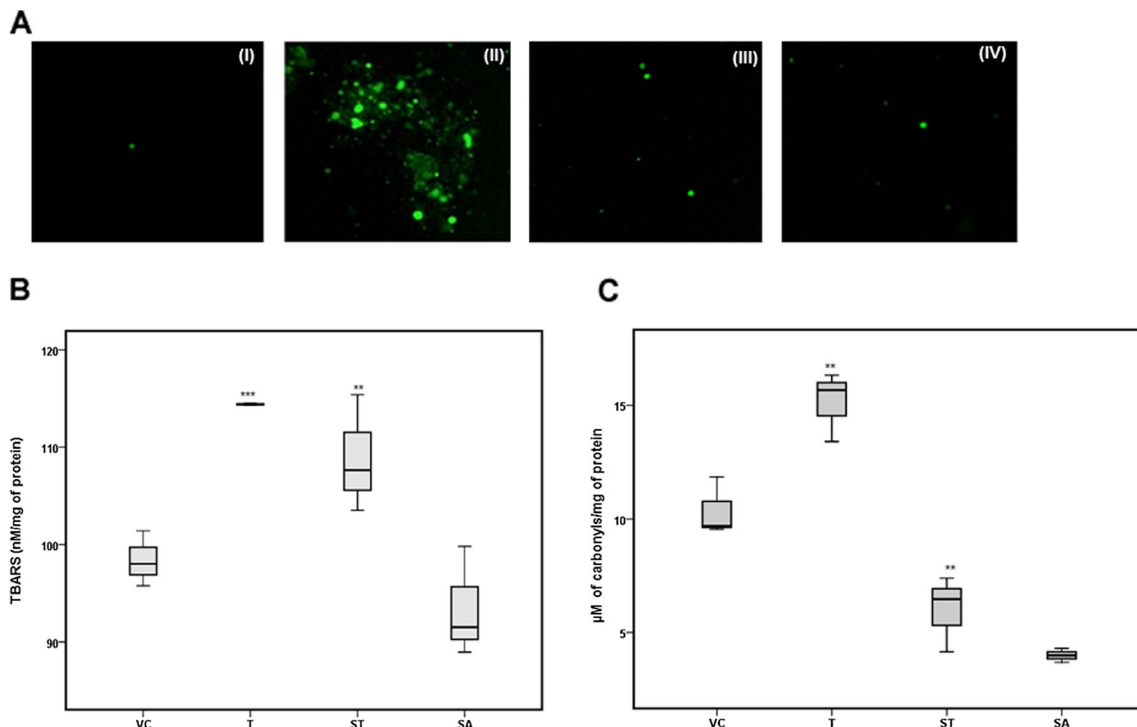tary (AtT-20) cells (Lin et al. 2007; Hung et al. 2006). TCDD-

Fig. 4 a Confocal microscopic image showing the effect of *G. acerosa*
extract against TCDD-induced intracellular ROS production in PBMC.
(I) VC, *(II)* T, *(III)* ST, *(IV)* SA. Protective effect of methanol extract of
G. acerosa on TCDD-induced increase in **b** LPO (nM/mg of protein) and
c PCC (μM of carbonyls/mg of protein) levels. Values are mean±S.D.
($n=3$)

induced cytotoxicity was efficiently decreased ($p<0.05$) by *G. acerosa* (300 μg/ml) extract co-treatment, suggesting that the observed cytoprotection might be attributed to its ROS inhibitory potential. Earlier, Suganthy et al. (2010) reported that *G. acerosa* displayed a protective effect against H_2O_2-induced oxidative stress in PBMC.

### *G. acerosa* extract reduces ROS production in PBMC induced by TCDD

The accumulation of ROS after TCDD exposure was measured by using fluorescent probe DCFH-DA. Results demonstrated that there was a significant change in ROS production following treatment with TCDD (10 nM) at 18 h (Fig. 4a). Increase in ROS production in rat Sertoli cells (Aly and

Khafagy 2011) and rabbit chondrocytes (Yang and Lee 2010) caused by TCDD is attributed to the induction of CYP4501A1. Confocal microscope analysis revealed that *G. acerosa* extract reduced the fluorescence level induced by TCDD treatment (Fig. 4a), which may be due to the inhibitory effect of the extract on hydroxyl and nitric oxide radicals and its scavenging effect on hydrogen peroxide (Devi et al. 2008; Suganthy et al. 2010).

G. acerosa extract protects PBMC against TCDD-induced lipid and protein oxidation

As shown in Fig. 4b, the LPO level was significantly increased in the TCDD-treated group ($p<0.05$) compared to the control. Free radical-induced LPO causes loss in cell

Fig. 5 a The tail length, percentage of DNA in tail, and olive tail moments of PBMC treated with TCDD and *G. acerosa* extract. The effect of *G. acerosa* and TCDD on DNA damage was studied using comet assay to measure single-strand DNA breaks at a single-cell level (***$p<0.00$). b Comet images of PBMC stained with Et-Br. The DNA damage was detected by confocal microscopy. (I) VC (cells treated with vehicle), (II) T (cells+10 nM TCDD), (III) ST (cells+10 nM TCDD+300 μg/ml seaweed extract), (IV) SA (cells+ 300 μg/ml seaweed extract)
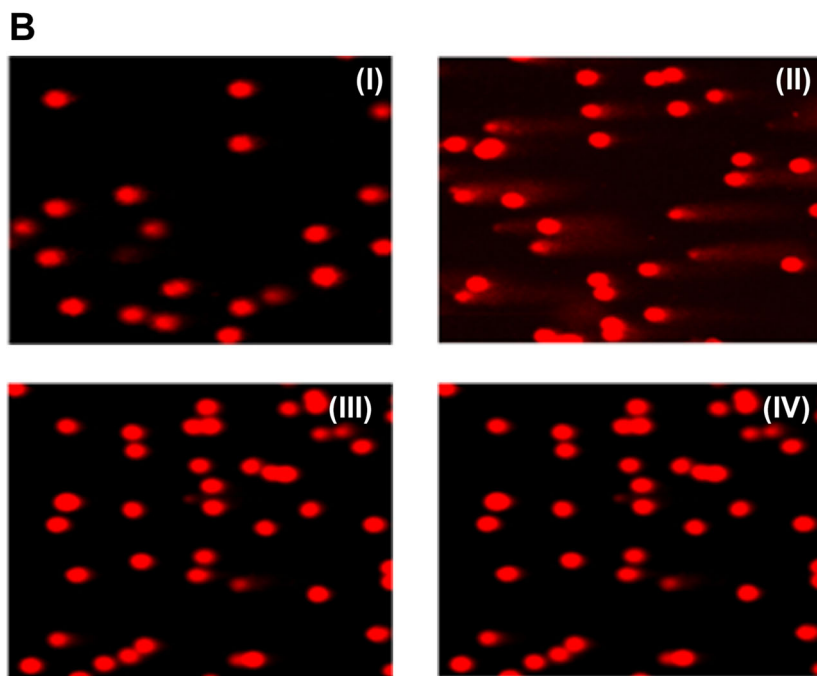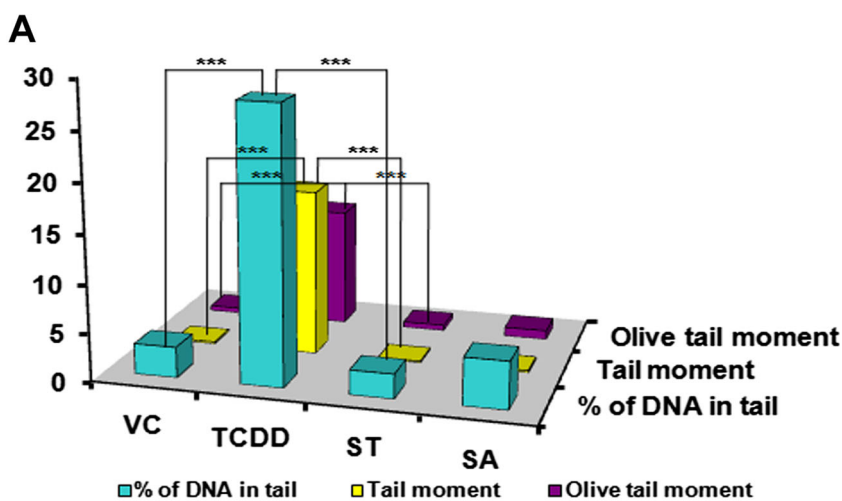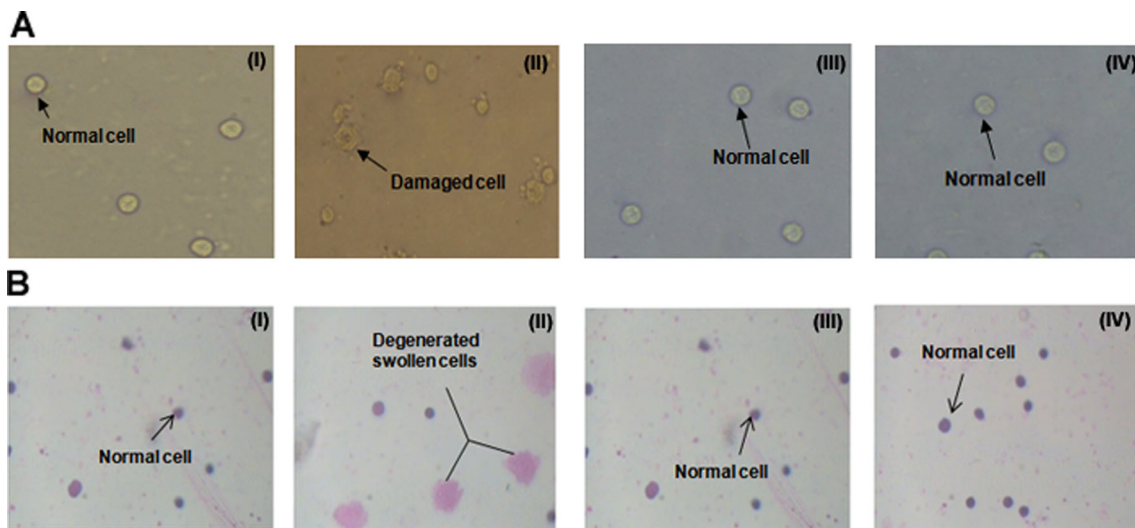
Fig. 6 Morphology was assessed by light microscopy (**a**) and histopathology (**b**) after incubation for 18 h with TCDD and *G. acerosa*. *(I)* VC—shows the normal cells, *(II)* T—shows the degenerate and swollen cells, *(III)* and *(IV)* ST and SA—show the normal cell morphology

homeostasis by modifying the structure and functions of the cell membrane. Hence, compounds which show a protective effect against lipid peroxidation are of great importance for proper cell function, since lipid peroxide products interact with protein and DNA molecules, consequences of which are involved in the development of various diseases. The cells co-treated with seaweed extract diminished the levels of LPO ($p<0.1$), which may be due to the quenching of ROS by the seaweed extract. Devi et al. (2008) reported that *G. acerosa*

inhibited H_2O_2-induced lipid peroxidation in RBC and also showed its free radical scavenging activity.

TCDD ($p<0.25$) increased the protein carbonyl content (PCC) in PBMC (Fig. 4c), which is supported by the results of Hung et al. (2006), Lin et al. (2007), and Aly and Domenech (2009) who observed an increased PCC level in the mitochondria of rat hepatocytes treated with TCDD. Protein carbonyl content is the most commonly used marker of protein oxidation. The generation of ROS damages several

Table 1 GC-MS analysis of the methanolic extract of *G. acerosa*

S. No.	RT	Name of the compound	Molecular formula	MW	Peak area (%)
1.	4.2	Azulene	$C_{10}H_8$	128	3.24
2.	6.47	2-Undecanol	$C_{11}H_{24}O$	172	0.65
3.	8.91	2-Dodecanol	$C_{12}H_{26}O$	186	0.68
4.	7.24	2-Octenal, (E)-	$C_8H_{14}O$	126	0.18
5.	10.12	Eicosane	$C_{20}H_{42}$	282	2.68
6.	10.85	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.12
7.	11.13	5-Octadecene, (E)	$C_{18}H_{36}$	252	0.83
8.	11.68	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	268	2.80
9.	13.15	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	35.38
10.	14.95	Phytol	$C_{20}H_{40}O$	296	1.79
11.	15.37	Oleic acid	$C_{18}H_{34}O_2$	282	2.87
12.	16.93	4,6-*O*-Ethylidene-α'-D-glucose	$C_8H_{14}O_6$	206	0.65
13.	20.01	α'-D-Glucopyranose, 4-*O*-α'-D-galactopyranosyl-	$C_{12}H_{22}O_{11}$	342	6.07
14.	20.62	Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330	2.90
15.	20.85	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278	1.54
16.	25.42	Cholesta-4,6-dien-3-ol, (3α')-	$C_{27}H_{44}O$	384	1.36
17.	28.80	Cholesterol	$C_{27}H_{46}O$	386	36.27

RT retention time, *MW* molecular weight

amino acid residues, and such oxidative damage of amino acid residues or peptide backbone of proteins generates PCC products. The lowered PCC in the *G. acerosa* extract co-incubated group indicates scavenging of oxygen free radicals and reduction of oxidative stress by the seaweed.

G. acerosa protects PBMC against TCDD-mediated genotoxicity

Single cell gel electrophoresis (SCGE) or comet assay is a simple, rapid, and sensitive technique for measuring DNA damage. As shown in Fig. 5, the mean of the tail length, olive tail moment, and the percentage of DNA in the tails were increased in the TCDD incubated group ($p < 0.00$). TCDD has been shown to induce an elevation in the excretion rate of 8-oxoguanine, the major repair product of 8-oxo-2-deoxyguanosine (8-oxo-dG) in mouse hepatoma cells, in human HepG2 hepatoma cells, and in rats chronically exposed to TCDD (Park et al. 1996; Knerr et al. 2006). DNA damage was not observed in the *G. acerosa* incubated group, which is supported by the earlier work of Suganthy et al. (2010) who reported that different solvent fraction of *G. acerosa* extract protects DNA against oxidative stress.

Maintenance of morphological integrity of PBMC by *G. acerosa* extract

The morphological changes induced by 10 nM of TCDD toxicity as well as the protective effect of 300 µg/ml *G. acerosa* were evaluated by light microscopic observation

Fig. 7 Summary of the protective effect of *G. acerosa* against TCDD-induced toxicity in PBMC
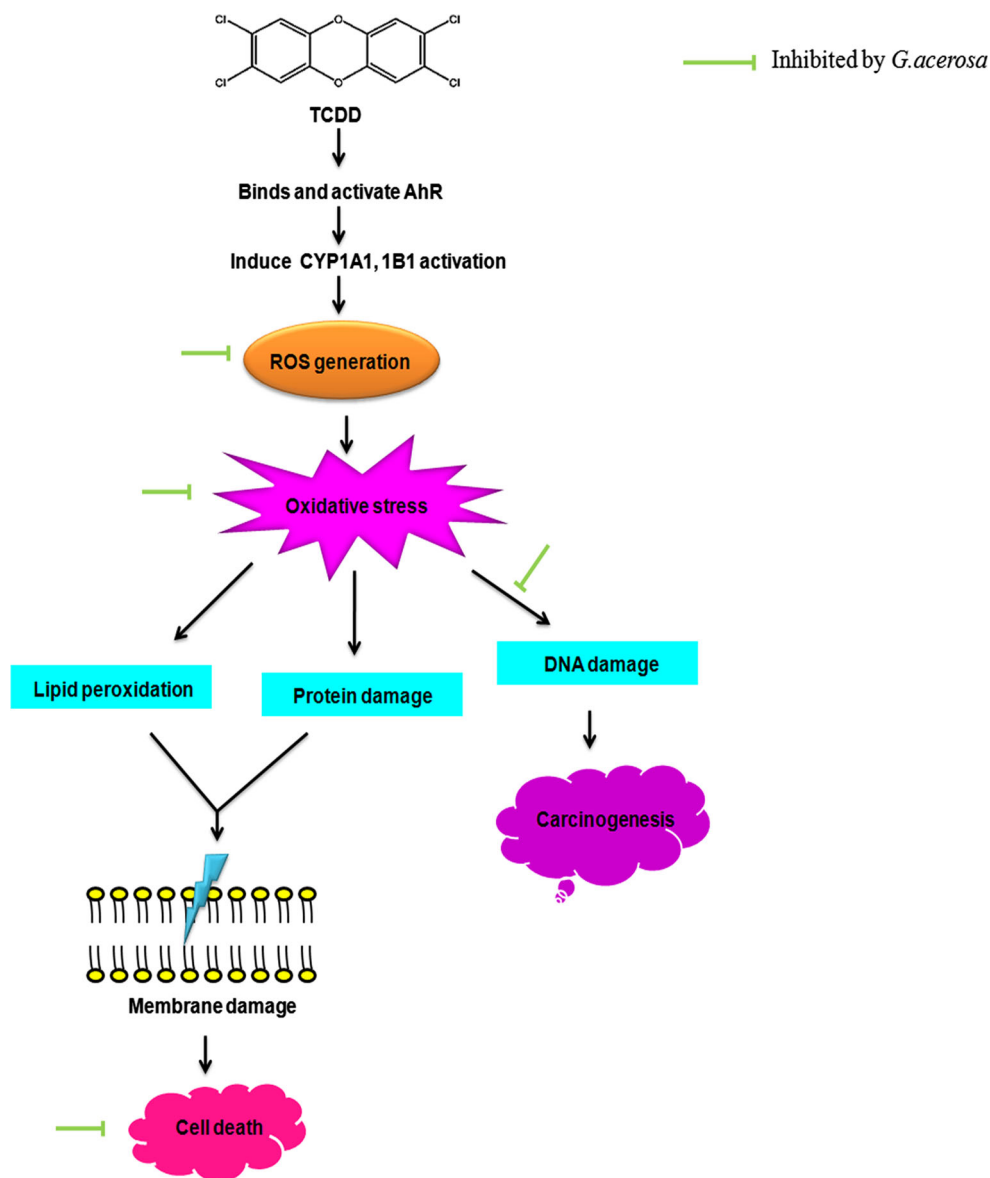
and histopathology. After 18 h of PBMC incubation with 10 nM of TCDD, the clear indicators of cell death such as cell shrinkage, plasma membrane blebbing, and degeneration were observed (Fig. 6). The observed morphological changes may be due to the oxidation of lipid and protein by TCDD, which induces alteration in the structural integrity in PBMC. It has been already proved that TCDD produces ROS, which induces morphological changes in PBMC (Ilavarasi et al. 2011). Normal morphology observed in *G. acerosa* extract-treated cells indicates the protective potential of the seaweed against TCDD-induced damages.

Assessment of chemical composition of *G. acerosa* extract by GC-MS

GC-MS analysis of the methanolic extract of *G. acerosa* revealed the presence of a total of 17 constituents. The identified compounds with their retention time and their percentage are listed in Table 1. The compounds are arranged in the order of their retention time. The analysis showed that the methanolic extract of seaweed is a complex mixture consisting mainly of saturated fatty acids, cholesterol, sugars, and terpenoids. The major compounds in the *G. acerosa* methanol extract were cholesterol (36.27 %), *n*-hexadecanoic acid (35.38 %), α-D-glucopyranose, 4-O-α-D-galactopyranosyl (6.07 %), azulene (3.24 %), hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester (2.90 %), oleic acid (2.87), 2-pentadecanone, 6,10,14-trimethyl- (2.80 %), eicosane (2.68 %), and phytol (1.79 %). Accumulated evidence demonstrates the antioxidant activity of essential fatty acid components extracted from various plants, of which *n*-hexadecanoic acid identified in *G. acerosa* possesses antioxidant and antimicrobial activities (Kala et al. 2011; Bai et al. 2011). The reduction of lipid peroxidation products by *G. acerosa* may be due to the presence of oleic acid, which is reported to prevent the oxidative modification of lipoproteins (Sola et al. 1997). In the present study, the remarkable antioxidant activity of the methanol extract of *G. acerosa* may be attributed to the presence of the abovementioned compounds.

Conclusion

The present study revealed that *G. acerosa* extract exhibited a protective effect against TCDD-induced toxicity, which may probably be related to its scavenging effect on ROS production and ability to maintain cell membrane integrity and to reduce free radical-dependent lipid, protein, and DNA damages (Fig. 7). The observed beneficial effect of *G. acerosa* extract may be attributed to the presence of large amounts of saturated fatty acid (*n*-hexadecanoic acid), oleic acid, and

terpenoids in the seaweed, which acts as a free radical quencher. However, further studies need to be carried out at the molecular level, explaining the role of *G. acerosa* extract and its active components on specific targeted sites including the expression of CYP1A1 and 1B1 and their role in improving the antioxidant status to protect the cells from TCDD-induced toxicity.

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