Modulatory effects of *Terminalia arjuna* against domoic acid induced toxicity in Caco-2 cell line

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Abstract Domoic acid is a potent marine algal toxin produced by diatomic genus of *Pseudo-nitzschia* causing amnesic shell fish poisoning. Domoic acid toxicosis mainly involves excitotoxic effects coupled with oxidative stress. The present study was aimed to evaluate the protective effects of hydro-alcoholic extract of *Terminalia arjuna* (TA) against domoic acid induced toxic effects in Caco-2 cell line. It was observed that the toxicity induced by domoic acid in Caco-2 cells was mediated by oxidative insult leading to morphological changes, DNA damage and apoptosis. In our study pre-treatment of the cells with TA (10, 20 and 30 µg/ml) showed significant protection against domoic acid induced morphological, oxidative and apoptotic damages in a dose dependent manner. The effect of phytochemicals present in TA viz., kaempferol and arjunenin showed significant protection against domoic acid induced toxicity in Caco-2 cell line. Hence, it could be inferred that the protective effect of TA extract against domoic acid induced toxicity could be due to the individual or synergistic effects of kaempferol and argungenin. However, further clinical studies are warranted to consider TA

as a natural remedy to prevent amnesic shell fish poisoning.

Keywords Domoic acid · Oxidative stress · *Terminalia arjuna* · Apoptosis · Morphological changes · Phytochemicals

Abbreviations

TA	<i>Terminalia arjuna</i> hydro-alcoholic extract
MMP	Mitochondrial membrane potential
DAPI	2-(4-amidinophenyl)-1H-indole-6-carboxamide
ROS	Reactive oxygen species
NO	Nitric Oxide
CAT	Catalase
GR	Glutathione reductase
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

Introduction

Domoic acid belonging to kainoid group of bio-toxins is produced by the diatomic species of *Pseudo-nitzschia* causes amnesic shell fish poisoning. The structure of domoic acid closely resembles glutamic acid and kainic acid. Due to the rigid structure of domoic acid, it binds with higher affinity to glutamate receptors, thereby causing neuro-excitatory behavioural effects in humans, marine mammals and birds

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(Pulido 2008). Domoic acid is reported to interact with various receptors such as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid, N-methyl-D-aspartate (NMDA) and activate different subtypes of voltage-dependant Ca^{2+} channels to promote influx of Ca^{2+} , which causes neuronal degeneration (Berman and Murray 1997; Hampson and Manalo 1998). In humans, an oral exposure to domoic acid leads to a variety of gastro-intestinal and neurological disorders including nausea, vomiting, diarrhoea, abdominal cramps, excitotoxicity, seizures and memory impairment (Lefebvre and Robertson 2010).

Memory impairment and associated neuropathology are the prominent effects of domoic acid toxicity (Sutherland et al. 1990). The toxicosis of domoic acid is associated with generation of large amount of reactive oxygen species which in turn causes, tissue damage, impairment of mitochondrial potential, apoptosis and leads to cell death (Giordano et al. 2008). Oxidative stress is the imbalance between the oxidants and antioxidants in the biological system that leads to accumulation of reactive oxygen species (ROS) in the body (Buttke and Sandstrom 1992). Accumulated ROS adversely affects cell homeostasis by oxidising vital components of the cell and leads to many metabolic disorders. Thus toxic insult disturbs the antioxidant defense system of the body resulting in damage to all macromolecules such are DNA, protein, lipid etc. These adverse effects in the body could be neutralized by providing supplementary antioxidants from natural sources (Uttara et al. 2009).

Herbal drugs make headway in modern research because of growing public acceptance due to their lesser side effects and increased effectiveness. A recent survey reveals that about 63.9% of the studied population endorse the use of alternative medicine (including herbal) and this trend is similar throughout the world Oluwatoyin et al. (2007). *Terminalia arjuna* (Roxb.) Wight and Arn. belongs to the family Combretaceae and is known as *Arjuna* in *Ayurveda*, the traditional Indian medicine. Bark of the plant is well known for its medicinal importance as cardio protective agent (Jain et al. 2009). *Terminalia arjuna* bark is frequently used to treat hypertension, ischemic heart diseases, hypercholesterolemia and it is known to possess anti-microbial, anti-mutagenic, anti-helminthic, anti-fertility and anti-HIV properties (Dwivedi 2007). *Terminalia arjuna* has shown in vitro

antioxidant potential and DNA damage protective activity in PC12 cells (Kumar et al. 2013).

The aim of the present study was to evaluate modulatory effects of hydro-alcoholic extract of *Terminalia arjuna* bark (TA) against domoic acid induced toxicity in Caco-2 cells, the human epithelial colorectal adenocarcinoma cells. Cell morphology, cell viability, mitochondrial membrane potential and other oxidative stress markers were targeted to study the effect of TA pre treatment on domoic acid induced cytotoxicity.

Materials and methods

Chemicals and reagents

Domoic acid, ursolic acid, kaempferol, arjungenin, arjunolic acid, ellagic acid, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 2',7'-Dichloro dihydrofluorescein diacetate (H2 DCFDA), rhodamine 123, DAPI, Dulbecco's modified Eagle's medium (DMEM), L-glutamine, trypsin, protease and phosphatase inhibitor cocktail, luminol and p-coumaric acid, primary antibodies for catalase, glutathione reductase, glyceraldehyde 3-phosphate dehydrogenase, anti-mouse polyvalent secondary antibody were obtained from Sigma (St. Louis, MO, USA). Foetal bovine serum was obtained from HyClone™ (Logan, UT, USA).

Plant material

Terminalia arjuna bark was collected from Mysore, India and authenticated by the Department of Botany, University of Mysore, Mysore, India (Voucher number: AND 1357). The shade dried bark material was defatted with petroleum ether and then extracted with 70% ethyl alcohol for 72 h by using soxhlet apparatus. The *T. arjuna* bark extract (TA) was concentrated under reduced pressure (Heidolph, Schwabach, Germany) and lyophilised for further studies.

Phytochemical studies

LC-ESI-MS/MS analysis of TA

The LC-ESI-MS/MS analysis of TA was performed using 6520 Accurate Q-TOF (Agilent Santa Clara, CA, USA) mass spectrometer coupled to HPLC

equipped with a UV–VIS detector. Zorbax SBC18 Rapid resolution column of 4.6×150 mm, 3.5μ particle size was used under the following conditions: (A) formic acid (0.1%v/v) and 10 mM ammonium fluoride and (B) acetonitrile +0.1% Formic acid; gradient (in solvent B): (i) 30%, from 0 to 15 min, (ii) 55%, from 15 min, (iii) 95%, from 25 to 45 min, and (iv) 35%, at 45–48 min; flow rate: 0.2 ml/min; injection volume was 5 μ L. Both negative and positive ion modes of ESI parameters was used; mass range 100–1200 m/z; 4 kV spray voltage; gas temperature 325 °C; gas flow 10 L/min; and nebulizer 40 psi.

Estimation of total polyphenols and flavonoids

Total polyphenolic content present in TA was estimated using Folin-Ciocalteu reagent as mentioned by Slinkard and Singleton (1977) and expressed as mg/g gallic acid equivalents (GAE). The quantification of flavonoids was carried out as described by Ordon et al. (2006) and was expressed as mg/g rutin equivalents.

Free radical scavenging activity

DPPH radical scavenging activity

The free radical scavenging activity of TA was measured using DPPH (1, 1-diphenyl 2-picrylhydrazyl) assay. Briefly, 100 μ L of different concentrations of extract was added to 2.9 ml of DPPH (0.1 mM). The tubes were incubated for 30 min and OD was taken at 515 nm. The results were then expressed as percentage inhibition of sample.

Hydrogen peroxide (H₂O₂) radical scavenging activity

The H₂O₂ radical scavenging activity assay was performed as mentioned by Ruch et al. (1989). A solution of hydrogen peroxide (2 mmol/l) was prepared in phosphate buffer (pH 7.4). TA was added to hydrogen peroxide solution (0.6 ml) in different concentrations and the absorbance was read at 230 nm.

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The working reagent was prepared by mixing of 7 mM ABTS and 2.4 mM potassium persulfate (K₂S₂O₈) in

equal quantities and then incubated for 12 h at room temperature in dark. 1 ml of the working reagent was added to different concentrations of TA extract and absorbance was measured at 734 nm.

Ferrous reducing antioxidant power assay

Briefly, to TA (100 μ L, various concentrations) 2.9 ml of FRAP reagent (a mixture of 25 ml acetate buffer (300 mM; pH 3.6), 2.5 ml TPTZ (2, 4, 6-tripyridyl-s-triazine; 10 mM) in HCl (40 mM), 2.5 ml FeCl₃ (20 mM)) was added. The mixture was then incubated for 15 min in dark and then absorbance was measured at 593 nm for the coloured product of ferrous tripyridyltriazine complex; FeSO₄ was used as the standard.

Cell culture and maintenance

Human colorectal adenocarcinoma cell line (Caco-2) was obtained from National Centre for Cell Sciences (NCCS), Pune, India. The cells were grown in culture plates (24 well and 96 well plates) and culture flasks (25 and 75 cm²) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and L-glutamine, and anti-mycotic and antibiotic solution (Sigma) at 37 °C in a humidified atmosphere of 5% CO₂. The medium was replaced every alternate day and 80% confluent cells were used for all assays.

Cytotoxicity by MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was done to determine cell viability, since it is widely accepted and reliable cytotoxicity assay (Fotakis and Timbrell 2006). The Caco-2 cells were seeded in 96 wells plate. To observe the effects of domoic acid, TA and phytocompounds viz., ursolic acid, ellagic acid, kaempferol, arjunolic acid and arjungenin on cell viability, individual treatment was done at varying concentrations. After 24 h of individual treatments (10–100 ng ml⁻¹ of domoic acid and TA), 100 μ L of MTT (0.7 mg/ml) was added and incubated for 4 h at 37 °C. The formazan crystals formed after incubation were dissolved in DMSO and the absorbance was read at 570 nm using VERSA max Hidex plate chameleonTMV (Turku, Finland). Based on the above results, the toxin (IC₅₀)

was added to the plates, which were 1 h prior incubated with different concentrations of TA (10, 20 and 30 µg/ml) or the phytocompounds. The protective effect of plant extract and the phytocompounds against domoic acid induced toxicity on Caco-2 was determined. The cells without any treatments served as control. Results were expressed as percentage viability, where absorbance of untreated well (control) was taken as 100%.

Morphological studies

Morphological studies under bright-field microscope

Approximately, 1×10^6 cells/ml were seeded in 25 cm² cell culture. The cells were exposed with domoic acid (DA) (75 ng/ml) for 24 h with or without prior treatment for 1 h with different concentrations of TA (10, 20 and 30 µg/ml) or the phytocompounds such as ursolic acid, ellagic acid, kaempferol, arjunolic acid and arjungenin. The cells without any treatments were considered as control. After incubation period, cells were photographed (Olympus, Tokyo, Japan) to observe the morphological changes.

Morphological studies under scanning electron microscope

Approximately, 1×10^6 cells/ml were seeded on cover slip placed in 12-well cell culture plate and were exposed to domoic acid (75 ng/ml) for 24 h with or without pre-treatment of TA. After incubation period, medium was discarded and cells were fixed with 2.5% of glutaraldehyde and washed with gradient of ethanol (70–100%). The cover slip was pasted on dual side glue carbon tape and sputter-coated with gold. Cells without domoic acid exposure or TA pre-treatment were considered as control. The morphological features of cells were observed under SEM (FEI, Hillsboro, OR, USA) at 20 kV and, images were captured under 500× and 3000× magnification.

Estimation of apoptotic damage

Mitochondrial membrane potential (MMP)

MMP was determined using fluorescent probe rhodamine 123. Cells seeded in 24 wells plate were exposed to domoic acid (75 ng/ml) for 24 h with or

without prior treatment of various concentrations of TA for 1 h. The medium was discarded and incubated with rhodamine 123 (10 µg/ml) for 1 h. The cells were then washed twice with PBS and fluorescence was read at excitation wavelength, 485 nm and an emission wavelength, 535 nm using Hidex plate chameleonTMV.

DNA damage using DAPI staining

Caco-2 seeded on cover slips were left to adhere and were exposed to domoic acid (75 ng/ml) for 24 h with or without prior treatment of various concentrations of TA for 1 h. The medium was removed and cells were washed with phosphate buffer and fixed using 100% ethanol. To this, 100 µl of DAPI stain (1 µl/ml ethanol) was added and incubated for 5 min. The stain was removed and mounting solution was added to it before visualization. The fluorescence microscope was used to take the images of the treated cells to analyze the nuclear features.

Estimation of oxidative stress status

Reactive oxygen species (ROS) production

Caco-2 cells (80% confluent) grown in 24 well plates were exposed to domoic acid (75 ng/ml) for 24 h with or without prior treatment for 1 h with various concentrations of TA (10, 20 and 30 µg/ml) or phytocompounds viz., ursolic acid, ellagic acid, kaempferol, arjunolic acid and arjungenin. After incubation, the medium was removed and dichlorodihydrofluorescein diacetate (H2 DCFDA), at 5 mg/ml was added and incubated for 30 min. The cells were washed thrice with phosphate buffer and increase in fluorescence was measured at excitation wavelength of 485 nm and an emission wavelength of 535 nm using Hidex plate chameleonTMV.

Nitric oxide (NO) production

Caco2 cells (80% confluent) grown in 24 well plates were exposed to domoic acid (75 ng/ml) for 24 h with or without prior treatment for 1 h with various concentrations of TA (10, 20 and 30 µg/ml) or phytocompounds viz., ursolic acid, ellagic acid, kaempferol, arjunolic acid and arjungenin. After incubation, an equal amount of Griess reagent was added to the supernatant and the pink colour developed was read at 540 nm.

Western blot analysis

Cells grown in 75 cm² flasks were treated with domoic acid for 24 h with or without prior treatment with various concentrations of TA (10, 20 and 30 µg/ml) for 1 h, washed with phosphate buffer and collected in centrifuge tube. The proteins from the cells were extracted using HEPES buffer (pH 7.4) containing protease and phosphatase inhibitor cocktail (10 µl/ml) and estimated using the method published by Lowry et al. (1951). Equal amount of protein was loaded to each well in SDS-PAGE gel and separated. The protein bands were then transferred on to a nitrocellulose membrane (Pall Scientific Pvt. Ltd., Mumbai, India) and blocked with 5% non-fat milk at 4 °C for 24 h. Primary antibodies to CAT and GR (1:1000) were individually added to the blot and kept during shaking at room temperature for 3 h. The membranes were washed with Tris-buffered saline with Tween 20 for 15 min each and HRP conjugated anti-mouse secondary antibody (1:10,000) was added. Following incubation for 2 h the membranes were washed and the blot was developed by chemiluminescence method using luminol and p-coumaric acid. The bands developed were transferred on to an x-ray film to analyse the band intensity (Jayaraj et al. 2006).

Statistical analysis

The data are expressed as mean ± SEM for at least three independent experiments (triplicate). Statistical differences between treatment groups were determined by ANOVA test. Differences were considered significant at $p < 0.05$.

Results

In vitro free radical scavenging potential of TA

TA is found to be rich in polyphenols ($47 \pm 0.16\%$) and flavonoids ($5.8 \pm 0.05\%$). Scavenging activity of TA against various free radicals i.e. DPPH, H₂O₂, ABTS, NO and ferric reducing antioxidant power are shown in Table 1.

LC-ESI-MS/MS analysis of TA

LC-ESI-MS/MS analysis of TA was carried out and compounds were confirmed by mass fragmentation

Table 1 In vitro free radical scavenging activity of TA

Parameters	TA
DPPH radical scavenging activity ^a	85 ± 5
H ₂ O ₂ radical scavenging activity ^a	180 ± 9
ABTS radical scavenging activity ^a	13.3 ± 2
Nitric oxide radical scavenging activity ^a	109 ± 5
Ferric reducing antioxidant power ^b	683 ± 12
Total polyphenols ^c	470 ± 16
Total flavonoids ^d	58 ± 5

^a Expressed in IC₅₀ values in µg/ml

^b Expressed in units of mmol Fe (II)/g

^c Expressed in units of mg/g of gallic acid equivalents

^d Expressed in units of mg/g of rutin equivalents

analysis. Some of the major phytochemicals identified are arjunolic acid, arjungenin, kaempferol 3-glucuronide, ursolic acid, ellagic acid and its derivatives etc. (Fig. 1).

Effect of TA and on domoic acid induced cytotoxicity

Caco-2 cells treated with TA (10–50 µg/ml) did not show any significant change in cell viability, when compared to the control cells (Fig. 2a). This result confirmed that TA alone did not cause any effect on cell viability. Caco-2 cells were exposed to different concentrations of domoic acid to observe the mortality of cell line (Fig. 2b). Domoic acid treatment markedly decreased cell viability in a dose dependent manner and IC₅₀ was found to be 75 ± 3 ng/ml. Domoic acid exposed cells were significantly protected by pre-treatment with TA, and improved cell viability in increasing order from 10 µg/ml, to 20 µg/ml, and 30 µg/ml, respectively (Fig. 2c). Cells pre-treated with phytochemicals, kaempferol and arjungenin showed significant increase ($p < 0.05$) in cell viability with respect to cells exposed to domoic acid without any pre-treatment (Fig. 2d).

Effect of TA on domoic acid induced cell morphology and SEM characterisation

Caco-2 cells were observed for general morphological differences in various groups i.e. control group, domoic acid exposed group and domoic acid with TA or the phytochemicals pre-treatment groups

Fig. 1 LC-ESI-MS/MS chromatograms of some identified bioactive compounds from TA

Fig. 1 continued

(Fig. 3a, c). Domoic acid exposed cells showed loss of adherence to the flask. Caco-2 cells pre-treated with TA, kaempferol and arjungenin showed improvement in their adherence property. Domoic acid exposure showed considerable morphological apoptotic characteristics, however the same was decreased with pre-treatment of TA, kaempferol and arjungenin. Cells pretreated with phytochemicals viz., ellagic acid, ursolic acid and arjunolic acid did not show any significant difference in cell morphology with respect to the domoic acid treated group. SEM analysis showed that domoic acid exposure led to ultra structural changes like detached cells, shrinkage and reduction in cell size when compared to control group cells (Fig. 3b). Moreover, pre-treatment with TA (30 $\mu\text{g/ml}$) protected cells from ultra structural morphological changes induced by domoic acid (Fig. 3b).

Effect of TA on domoic acid induced apoptosis

Effect of TA on domoic acid induced loss of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential is one of the major indicators of apoptosis detection in cell line studies. In the present study, domoic acid exposure induced apoptosis at a greater extent assessed by means of fluorescence intensity ($41.2 \pm 3.5\%$), when compared with untreated cell line (Fig. 4a). Different doses of TA pre-treatment attenuated domoic acid induced loss of mitochondrial membrane potential and improved the fluorescence intensity by $49.5 \pm 4.3\%$ (TA-10 $\mu\text{g/ml}$), $64.8 \pm 3.38\%$ (TA-20 $\mu\text{g/ml}$) and $86.2 \pm 6.1\%$ (TA-30 $\mu\text{g/ml}$).

Effect of TA on domoic acid induced nuclear damage

Caco-2 cells were examined for nuclear damage using DAPI staining. As shown in Fig. 4b, domoic acid exposed cells were observed with condensed nuclei in greater numbers; however, TA pre-treatment reduced the number of cells with nuclear damage in a concentration dependent manner. Cells pre-treated with TA-30 $\mu\text{g/ml}$ appeared similar to those of control cells.

Effect of TA on domoic acid induced oxidative stress

Potential effect of TA on domoic acid induced ROS production

Caco-2 cells treated with domoic acid showed a three-fold increase in ROS generation when compared with the control group (Fig. 5a). The value of ROS was expressed as relative fluorescence compared to group which is treated with only domoic acid, which was taken as 100%. The intensity of fluorescence in cells treated with TA, kaempferol and arjungenin showed a significant reduction ($p < 0.05$) of ROS production compared to the group which was treated with only domoic acid (Fig. 5a, b).

Potential effect of TA on domoic acid induced NO release

NO production by Caco-2 cells culture medium was determined by the measurement of total nitrite in culture medium. Domoic acid exposure triggered significant release of nitrite with respect to the control group ($p < 0.05$). Pre-treatment of cells with TA, kaempferol and arjungenin significantly inhibited domoic acid induced NO release ($p < 0.05$) (Fig. 5c, d).

Effect of TA on domoic acid induced changes in glutathione reductase and catalase

Domoic acid exposure significantly down regulated the levels of antioxidant enzymes ($p < 0.05$), such as, glutathione reductase and catalase when compared to control Caco-2 cells (Fig-6). Pre-treatment of TA showed protective effect in a dose depended manner on domoic acid induced oxidative stress with respect to levels of glutathione reductase and catalase (Fig. 6).

Discussion

Exposure to domoic acid through contaminated sea food consumption, particularly shell fish causes several toxic effects to humans sometimes proving fatal. One report on such an intoxication indicated that consumption of domoic acid contaminated mussel led to three deaths and neurological abnormalities in more than hundred individuals (Lelong et al. 2012). Few other studies observed the symptoms of illness with respect to domoic acid consumption including gastrointestinal symptoms within 24 h viz., vomiting, abdominal cramps, diarrhoea and followed by neurological symptoms up to 48 h like behavioural changes, seizures and memory loss (Lefebvre and Robertson

Fig. 2 Protective effect of TA against domoic acid induced cytotoxicity in Caco2 cells. **a** Response to pre-treatment of TA. Caco-2 cells were treated with hydro-alcoholic extract of *Terminalia arjuna* at different concentrations (10–50 µg/ml) for 24 h. **b** Cytotoxicity of domoic acid. Cytotoxicity of domoic acid was assessed in various doses of domoic acid i.e. 20–150 ng/ml and 75 ± 3 ng/ml was found to be the IC_{50} value. **c** Effect of TA on domoic acid induced toxicity. Pre-treatment with TA (10–30 µg/ml) followed by domoic acid exposure (75 ng/ml) for 24 h. **d** Effect of phytocompounds present in TA against domoic acid induced cytotoxicity. Cell

Fig. 3 Protective effect of TA pre-treatment on domoic acid induced structural alterations of Caco-2 cells. **a** Phase-contrast microscopy: (i) Control Caco-2 cells, (ii) Domoic acid (75 ng/ml) exposed cells for 24 h, (iii–v) Pre-treatment with TA (10 µg/ml, 20 µg/ml and 30 µg/ml) followed by domoic acid (75 ng/ml) exposure for 24 h. **b** Scanning electron microscopy: I and (i) Control Caco-2 cells showing normal morphology and normal cell size, II and (ii) Domoic acid exposed cells showing cell damage, III and (iii) Pre-treatment with TA (30 µg/ml) followed by domoic acid exposure showing protective effect. Images from I to III at $500\times$ magnification and images from (i–iii) are at $3000\times$ magnification. **c** Effect of pre-treatment with phytocompounds on domoic acid induced morphological changes in Caco-2 cells. (i): Control Caco-2 cells, (ii): Domoic acid (75 ng/ml) exposed cells for 24 h, (iii–vii): Pre-treatment with ursolic acid (UA) 250 ng/ml, kaempferol (K) 200 ng/ml, ellagic acid (EA) 300 ng/ml, arjunolic acid (AA) 150 ng/ml, arjungenin (AG) 200 ng/ml respectively, followed by domoic acid (DA) exposure (75 ng/ml) for 24 h

viability on pre-treatment of ursolic acid (UA) 250 ng/ml, kaempferol (K) 200 ng/ml, ellagic acid (EA) 300 ng/ml, arjunolic acid (AA) 150 ng/ml, arjungenin (AG) 200 ng/ml, followed by domoic acid (DA) exposure (75 ng/ml) for 24 h. Data are expressed as mean $\pm$ SEM of three independent experiments. In the TA treated groups Asterisk indicates statistically significance $p < 0.05$ vs control and Double asterisk indicates $p < 0.05$ vs domoic acid, whereas in phytocompounds treated groups Asterisk indicates significant difference $p < 0.05$ with respect to domoic acid; # indicates significant difference at $p < 0.05$ with respect to control

Fig. 3 continued

2010; Ramsdell and Gulland 2014). The present study was conducted to investigate the role of TA against diverse toxic responses following domoic acid exposure on Caco-2 cells. The entry of toxin in the food chain can primarily exert its effect on the digestive system. Several studies concerning the role of domoic acid on primary neuronal cells have been carried out (Berman and Murray 1997; Berman et al. 2002). The present study is focused on Caco-2 cells, since intestinal cells are likely to be more sensitive to domoic acid in vivo in the first few hours of exposure.

Domoic acid exposure decreased cell viability in a concentration dependent manner (20–150 ng/ml), indicating the sensitivity of Caco-2 cell line to the toxic effects of domoic acid. The estimated IC_{50} (0.2 μ M) of domoic acid on Caco-2 cells was in accordance with the previous findings of Carvalho et al. (2008). However, pre-treatment with TA increased cell viability up to a certain extent and prevented domoic acid induced cell death. Bright field microscopic observation and SEM analysis of the present study revealed that domoic acid (75 ng/ml) caused structural changes in Caco-2 cells which include shrunken cells, decreased cell size, irregular cell borders and formation of intra-cytoplasmic vacuoles. Pre-treatment with TA at 30 μ g/ml prevented ultra-structural alterations in the colonic cell line Caco-2 subsequent to domoic acid exposure

suggesting its protective role against domoic acid induced toxicity.

Mitochondria play a key role in integration and transmission of cell death signals by activating caspases or programmed cell death pathways. Effect of domoic acid on cell apoptosis has been observed by a decline in the mitochondrial membrane potential ($\Delta\Psi_m$). TA pre-treatment increased $\Delta\Psi_m$ in a dose dependent manner. The increase in $\Delta\Psi_m$ may be due to active regulation of pro-apoptotic/apoptotic/anti-apoptotic factors like cytochrome c (Cyt c), caspase-2, caspase-9 or Bcl-2 proteins (Samraj et al. 2007). Carvalho et al. (2008) demonstrated that exposure of Caco-2 cells to domoic acid (60–100 ng/ml), damaged membranes (lysosomes, and mitochondria) and induced apoptosis through up-regulation of Bax. Present findings of domoic acid induced cytotoxicity are in accordance with the results of previous studies of Carvalho et al. (2008) on the intestine cell line. Earlier studies by Ananth et al. (2001) strongly suggest that domoic acid induces apoptosis by the modulation of gene expressions such as, of Bcl-2, Bax, and caspase-3 in degenerating neurons of the hippocampus.

Present observation on in vitro free radical scavenging activities showed that *T.arjuna* bark extract scavenged free radical generation of DPPH $\cdot$, ABTS $\cdot$, NO $\cdot$, H $_2$ O $_2$ and has high ferric reducing antioxidant

Fig. 4 Protective effect of TA pre-treatment on domoic acid induced apoptosis. **a** Effect of TA on domoic acid induced loss of mitochondrial membrane potential. Pre-treatment with TA (10 µg/ml, 20 µg/ml and 30 µg/ml, respectively) followed by domoic acid (75 ng/ml) exposure for 24 h. The value of MMP was expressed as relative fluorescence intensity compared to control. *Statistically significant differences, $*p < 0.05$ vs

control and $**p < 0.05$ vs domoic acid. **b** Effect of TA on domoic acid induced nuclear damage using DAPI staining. (i) Control Caco-2 cells, (ii) Domoic acid (75 ng/ml) treated cells exposed for 24 h, (iii–v) Pre-treatment of TA (10, 20, and 30 µg/ml, respectively) followed by domoic acid (75 ng/ml) exposure for 24 h. Arrow marks in the image represent cells undergoing apoptosis

power. Hydro-alcoholic extract of TA showed presence of high quantity of polyphenols and flavonoids. Our previous studies on bioactivity guided fractionation of TA showed presence of triterpenoids, tannins, polyphenols and flavonoids (Kumar et al. 2013). Further, LC–MS results of the present study identified bioactive polyphenols and terpenoids i.e. ellagic acid, ursolic acid, kempherol-3-glucuronide, trocotrienol, arjungenin, arjunolic acid, etc. Upon exposure to domoic acid, Caco-2 cell lines showed chromatin condensation as revealed by DAPI staining in the present study. Moreover, administration of TA decreased the number of condensed cell in a dose

dependent manner. In our previous studies on in vitro PC12 cell line, different fractions of TA has shown a protective role on DNA damage (Kumar et al. 2013). Previous studies of Carvalho et al. (2008) also confirmed the genotoxicity of domoic acid through DNA damage, strand breaks and chromatin condensation.

The amount of free radical production is determined by the balance of many factors, and ROS are produced both endogenously and exogenously. The endogenous sources of ROS include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation. (Rahman 2007). The

Fig. 5 Effect of pre-treatment of TA and phytochemicals on domoic acid induced oxidative stress. **a, b** Reactive oxygen species (ROS). **c, d** Nitrite production (NO). Pre-treatment of TA (10, 20, and 30 μg/ml, respectively) and phytochemicals (ursolic acid (UA) 250 ng/ml, kaempferol (K) 200 ng/ml, ellagic acid (EA) 300 ng/ml, arjunolic acid (AA) 150 ng/ml, arjungenin (AG) 200 ng/ml.) followed by domoic acid (75 ng/ml) exposure for 24 h. The values of ROS were expressed as relative fluorescence intensity and NO was expressed as relative absorbance compared to domoic acid treatment. In TA treated groups *indicates statistically significance $p < 0.05$ vs control and ** indicates $p < 0.05$ vs domoic acid, whereas in phytochemicals treated groups * indicates significant difference $p < 0.05$ with respect to domoic acid; # indicates significant difference at $p < 0.05$ with respect to control

ROS and NO, respectively, in Caco-2 cells. Our present results demonstrated that ROS and NO generation were significantly triggered ($p < 0.05$) by domoic acid exposure, and the same were decreased by administration of TA in a dose dependent manner. This domoic acid induced oxidative stress was also confirmed by others in in vitro and in vivo studies (Giordano et al. 2008; Vranjac-Tramoudanas et al. 2011; Pulido 2008). A previous study on California sea lions suggested that oxidative stress may occur during excitotoxicity induced by domoic acid (Giordano et al. 2008; Silvagni et al. 2005). Lu et al. (2013) demonstrated that domoic acid induces inflammatory markers, mitochondrial damage and generates oxidative species, which results in amnesia in domoic acid treated mice through PKC- α -dependent K-ras/Raf/MEK/ERK1/2 pathway.

Catalase (CAT) is one of the antioxidant enzymes that catalyzes the conversion of H_2O_2 into H_2O and O_2 and is important during elimination of ROS. Glutathione Reductase (GR) is a flavo-protein oxidoreductase, GR catalyzes the reduction of GSH and is involved in defense against ROS. Present protein expression studies revealed that both enzymes i.e. CAT and GR were down regulated by the domoic acid exposure. However, these enzyme activities were up regulated two fold by TA-20 and TA-30 pre-treatments even after the domoic acid exposure. Previous studies on experimental animal models also suggested that the *T. arjuna* alcoholic extract may help in altering the levels of cytochrome P-450 and other antioxidant enzymes like SOD, CAT, GST and GSH in different organs. The exogenous antioxidants from *T. arjuna* may act directly or indirectly with the internal

accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can cause decreased oxidative phosphorylation and damage to mitochondrial DNA resulting in mitochondrial dysfunction. In the present study, DCFDA fluorescence and Griess reagent were used to determine the accumulation of

Fig. 6 Protective effect of TA on domoic acid induced modulation of glutathione reductase and catalase. 1. Control 2. Domoic acid (75 ng/ml) 3–5. TA-10, 20 and 30 µg/ml, respectively. GAPDH - Glyceraldehyde 3-phosphate dehydrogenase, GR-glutathione reductase, CAT-catalase. Relative band intensities are shown in separate bar diagrams for GR and CAT

antioxidant system for synergistic effects to protect against several diseases linked to oxidative stress (Das et al. 2010; Kumar et al. 2013).

Attempt was also made to identify the most promising phytochemicals in TA extract against domoic acid toxicity. Morphological studies using bright field microscopy, MTT assay, ROS and nitrite estimation were carried out using arjungenin, arjunolic acid, ellagic acid, ursolic acid and kaempferol. The study showed that kaempferol and arjungenin at the concentration of 200 ng/ml showed protective effect against domoic acid induced toxicity in terms of increase in cell viability, decrease in cell damage with respect to cell morphology, reduced ROS and nitric oxide production in Caco-2 cell line. Arjungenin and kaempferol are known to have antioxidant potential to combat against a wide range of oxidative stress related diseases (Ghosh et al. 2010; Rajendran et al. 2014). Kaempferol belongs to the flavonol group which has attracted great interest owing to its apparent beneficial effects on human health attributed to its antioxidant activities (Wattel et al. 2003). A previous study by Choi (2011) strongly evidenced that kaempferol can help in reducing or preventing osteoblasts degeneration in degenerative disorders by decreasing reactive oxygen species generation and mitochondrial membrane dissipation. Further Hong et al. (2009) demonstrated modulatory effect of kaempferol on cellular antioxidant defense capacity and cell apoptosis by regulating HO-1 expression and MAPK signal

transduction. Since oxidative stress is one of the major outcomes of amnesic shell fish poisoning, arjungenin and kaempferol can act as promising therapeutic compounds to combat the toxic insult caused by domoic acid.

To date, however, only a few studies have examined drugs/phytochemicals/herbal extracts to ameliorate domoic acid induced toxicity. In the present study TA prevented domoic acid induced toxic effects in Caco-2 cells to a greater extent. The present study inferred that the presence of kaempferol and arjungenin in the TA extract might have individually or synergistically affected the observed protective activity against domoic acid induced toxicity in Caco-2 cell line. Moreover, our study offers the possibility of potential therapeutic use of *T. arjuna* bark for the prevention domoic acid induced damages.

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Compliance with ethical standards

Conflicts of interest The authors declare that there is no conflict of interest.

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