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ORIGINAL ARTICLE

Caulerpa taxifolia inhibits cell proliferation and induces oxidative stress in breast cancer cells

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Abstract

Caulerpa taxifolia (M. Vahl) C. Agardh or *killer alga* is known to possess several bioactive secondary metabolites with unique structural modifications. We investigated anti-oxidant and anti-proliferative activity of *C. taxifolia* extract (CTE) on breast and lung cancer cells, along with possible effects on mitochondrial membrane potential (MMP) and cell cycle progression. The results revealed up to 6-folds increase in reactive oxygen species (ROS), 2-folds increase in glutathione reductase (GR) activity, 1.7-fold increase in superoxide dismutase (SOD) activity and 1.8-fold change in catalase activity w.r.t. untreated cells i.e. 10.72 to 21.44 nmol/min/mL, 2.0 to 3.49 U/mL and 37.51 to 69.26 U/min/g FW, respectively, in MDA-MB-cells. Likewise, selective anti-proliferative activity with IC_{50} 0.19 ± 0.1, 0.27 ± 0.1, and 0.43 ± 0.1 μg/μL, was recorded in MDA-MB-231, T-47D, and H1299 cells. In addition, dose-dependent increase in MMP of up to 40% and G_1/S phase mitotic arrest was documented by CTE treatment in MDA-MB-231 cells. The results suggest an anti-proliferative and oxidative stress inducing activity of CTE. Changes in MMP and cell cycle arrest further support the anti-cancer effects of CTE. It is believed that *C. taxifolia* may be considered as a potent source of anti-cancer drugs, subject to further validations.

Keywords *Caulerpa taxifolia* · Marine alga · Killer alga · Cancer · Oxidative stress

Abbreviations

CTE	*Caulerpa taxifolia* extract
CYN	Caulerpenyne
ROS	Reaction oxygen species
MMP	Mitochondrial membrane potential
GR	Glutathione reductase
SOD	Superoxide dismutase
hPBMCs	Human peripheral blood mononuclear cells
JC-1	5,5',6,6'-tetraethylbenzimidazolylcarbocyanine iodide
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
PBS	phosphate saline buffer
MTT	3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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Background

C. taxifolia (M. Vahl) C. Agardh or 'killer alga' is a species of the coenocytic macroalga genus *Caulerpa* and is considered one of the largest known single-celled species (Bhushan et al. 2016). It is distributed in tropical waters of Caribbean, Africa, Japan, India, Hawaii, Australia and usually grows on rocky, sandy substrates or deceased seagrass rhizomes. Thallus of this alga is green/yellowish-green, non-septate, feather-like and arises from a creeping stolon which is anchored to substrate by numerous rhizoids (Guiry and Guiry 2016). The aesthetic appearance of its feathery fronds and high adaptability, instigated its use in aquaria. An accidental introduction of this species in Mediterranean waters in 1984 led to its unrestrained growth, with subsequent invasion in the surrounding coasts (Papini et al. 2013). Thereafter, this invasion created havoc, and took over most of the native flora in the invaded coasts of France,

Monaco, Spain, Croatia, Italy, Tunesia, California, Australia, and earned the name- *killer alga* (Aplikioti et al. 2016; Jousson et al. 2000). International Union for Conservation of Nature and Natural Resaources-Invasive Species Specialist Group (IUCN-ISSG) listed *C. taxifolia* in world's 100 worst invasive species (Dra et al. 2018). Considering its unchecked multiplication and global invasion, there was a call for its rapid eradication (Jousson et al. 2000).

This alga is mostly recognized as an obnoxious invader, which has over-shadowed its pharmacological worth. Evidently, *C. taxifolia* protects itself by producing toxic secondary metabolites, thus, deters predators or herbivores from feeding (Marić et al. 2017). Secondary metabolites like caulerpenyne (CYN), 10,11-epoxycaulerpenyne, taxifolials obtained from *C. taxifolia* exhibit unique structural properties and have demonstrated varied toxicity against bacteria, viruses, sea urchin, and human cancer cells (Barbier et al. 2001; Gross and König 2006; Lemée et al. 1993). Cancer is posing a challenge to pharmaceutical industry, with lung and breast cancer as two of the most leading causes of death worldwide. Cancer complexity, limited antidotes, less efficient drugs, drug resistance, and higher costs incite hunt for novel and effective cancer drugs at a greater pace (Harvey et al. 2015). It is hypothesized that invasive potential of *C. taxifolia* can be utilized against cancer. In this study, we attempted to evaluate anti-oxidant, anti-cancer activities of methanolic extract of *C. taxifolia* (CTE). The ability to induce reactive oxygen species (ROS), and mitochondrial membrane potential (MMP) perturbations was also endeavored.

Results

Phycochemicals of CTE disrupt anti-oxidant machinery, alter ROS and modulate MMP in cancer cells

CTE with 8 ± 1% yield was obtained and presence of terpenes, alkaloids, tannins, and flavonoids was observed. There are no previous reports of flavonoids or tannins from the genus *Caulerpa*, unlike other algae. Our results suggest the presence of other unreported or novel metabolites in this alga. The effect of CTE on oxidative stress was studied spectrophotometrically by dihydroethedium (DHE) assay in MDA-MB-231 cells (Fig. 1). A dose dependent increase of up to 6-folds in ROS was observed in comparison to untreated control cells. CTE also demonstrated a concentration-dependent increase in glutathione reductase (GR), superoxide dismutase (SOD) and catalase activity. A 2-fold increase in GR activity from 10.72 to 21.44 nmol/min/mL, 1.7-fold increase in SOD activity from 2.0 to 3.49 U/mL and 1.8-fold change in catalase activity from 37.51 to 69.26 U/min/g FW was recorded with respect to control (Fig. 2). The results demonstrated a dose-dependent increase in MMP, of up to 40% at highest treatment (0.5 μg/μL) of CTE, after 24 h treatment (Fig. 3).

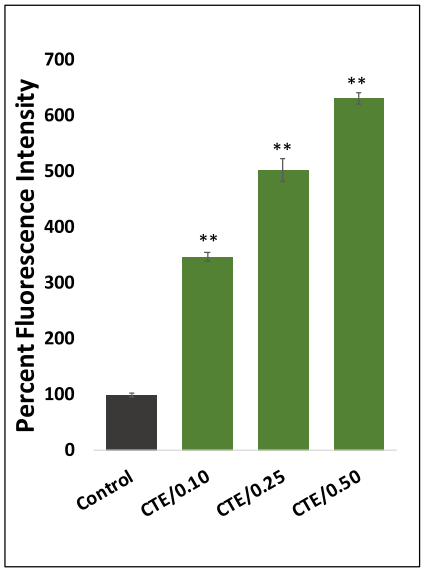

Fig. 1 DHE Assay: Intracellular ROS levels as determined in MDA-MB-231 cells by DHE. CTE concentrations are expressed in μg/μL and results are expressed in terms of mean percent DHE fluorescence intensity w.r.t. control. (*$P < 0.05$, **$P < 0.01$)

CTE exhibits anti-cancer potential by causing G_1/S phase cell cycle arrest

The cytotoxicity of CTE was tested on MDA-MB-231, T-47D, H1299 cells, and hPBMCs (human peripheral blood mononuclear cells) for the first time by MTT (3–4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The percent cell viability of hPBMCs, MDA-MB-231, T-47D, and H1299 cells, after 48 h treatment with 0.1, 0.25 and 0.5 μg/μL of CTE is depicted in Fig. 1. CTE exhibits growth promoting effect on hPBMCs, but inhibits growth of MDA-MB-231, T-47D, and H1299 cells with IC_{50} 0.19 ± 1, 0.27 ± 1, and 0.43 ± 1 μg/μL, respectively (Fig. 4). The observed cytotoxicity was significantly ($P < 0.05$; $P < 0.01$) different from their respective untreated controls. Most pronounced anti-proliferative effect was observed in MDA-MB-231 cells. CTE treatment also revealed cell cycle arrest at G1 phase in MDA-MB-231 cells (Fig. 5). There was dose (0.1 to 0.3 μg/ml) as well as time (24 and 48 h) dependent increase in cell populations in G1 phase while S and G2 cell populations declined.

Discussion

Phycochemicals present in extract result in disruption of anti-oxidant machinery in cancer cells

The phycochemicals detected in the study are previously accounted for various bioactivities like anti-microbial,

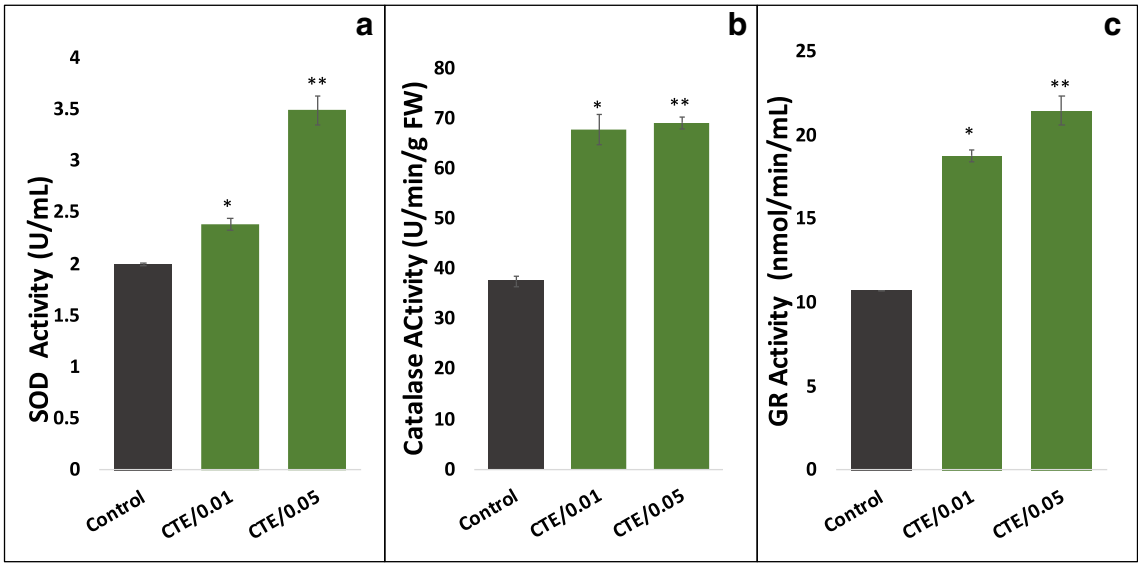

Fig. 2 Anti-oxidant enzyme assays: A) SOD assay, B) Catalase Assay, and C) GR Assay. The enzyme assays were conducted on MDA-MB-231 cells with CTE concentrations 0.01 and 0.05 μg/μL, in triplicates. Results are depicted in terms of relative enzyme activities. (*P < 0.05, **P < 0.01)

insecticidal, anti-trypanosomal, anti-depressants, anti-inflammatory, anti-oxidant and anti-tumour activity (Kochanowska-Karamyan and Hamann 2010; Otoguro et al. 2011; Paduch et al. 2007; Roleira et al. 2015). A sesquiterpenoid, CYN, is the major metabolite obtained from this species. Although it is also reported in other species of the genus, but maximum yield is obtained from *C. taxifolia*. CYN exhibits anti-microbial, neurotoxic, phytotoxic, anti-mitotic, and anti-proliferative activity (Barbier et al. 2001; Tejada et al. 2016). Besides, alkaloids like caulerpin, caulersin and racemosin C, with potent anti-cancer activity are reported from other *Caulerpa* species, but not from *C. taxifolia* (Yang et al. 2015). Secondary metabolites are reported to contribute to cancer cell death by inducing intracellular ROS, as a consequence of aggravated mitochondrial metabolism (Ghasemzadeh and Ghasemzadeh 2011). Cancer cells already have enhanced basal oxidative stress, making them sensitive to treatments inducing ROS and disrupting the cellular homeostasis. Induction of oxidative stress is proposed as an anti-cancer drug therapy in several studies (Farooqi et al. 2015; Noh et al. 2015). This study reports an elevated ROS with concomitant increase in anti-oxidative enzyme machinery. However, the ROS levels seem to surpass the defense system and cause an imbalance, thereby resulting in cell death. Similar results are reported by ethanolic extracts of *Gracilaria tenuistipitata* (red marine alga), which increases ROS, decreases GSH and causes apoptosis in Ca9–22 cells (oral squamous cancer) (Yeh et al. 2012). A sesequiterpene, costunolide, is also reported to induce ROS generation and MMP disruption, subsequently leading to apoptosis, in T24 cells (bladder cancer) (Rasul et al. 2013). The anti-oxidant enzyme machinery of cells acts as a first line of defense against free radicals, thereby, maintaining a cellular homeostasis of ROS and anti-oxidants. An increased enzyme activity in response to CTE might be indicating an immediate defense strategy against elevated levels of ROS like superoxide, and peroxide. Alternatively, elevated enzyme activity can be a consequence of increased expression of the said enzymes, and can be deciphered by analyzing mRNA profile of these enzymes (Schumacker 2015). There are no previous reports of effect of *C. taxifolia* on anti-oxidant enzymes in cancer, however, *Caulerpa* species themselves are known to possess excellent anti-oxidant system that can be attributed to the unrestrained growth and highly adaptable nature of invasive strains (Cavas and Yurdakoc 2005). Crude and

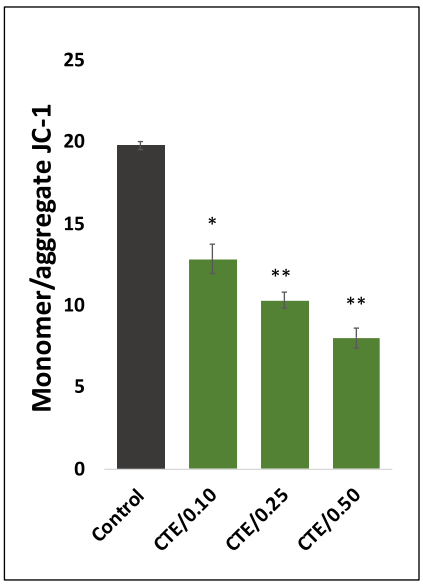

Fig. 3 JC-1 Assay: MMP modulations as determined by JC-1 in MDA-MB-231 cells. Dose-dependent mitochondrial membrane hyperpolarization is depicted by CTE. Results are expressed as mean monomer/aggregate JC-1 ratio. Concentrations are expressed in μg/μL. (*P < 0.05, **P < 0.01)

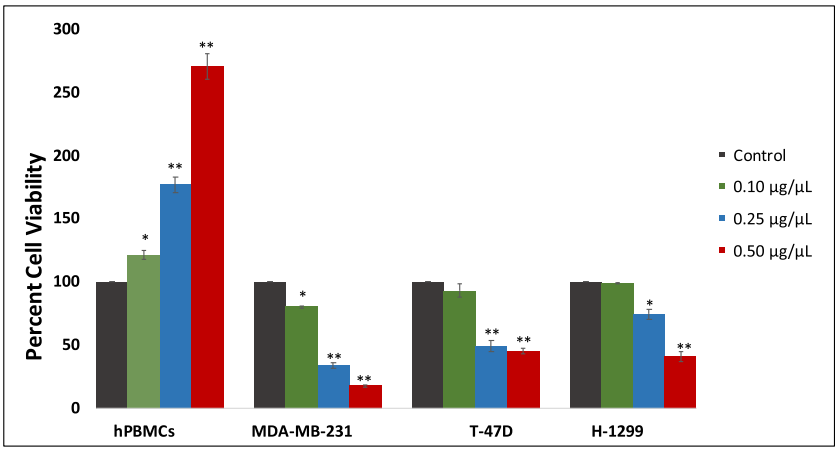

Fig. 4 MTT Assay: Cell viability as determined by MTT in hPBMCs, MDA-MB-231, T-47D and H-1299 cells after 48 h CTE treatment. Results are expressed as mean percentage of cell viability. Concentrations are expressed in µg/µL. (*P < 0.05, **P < 0.01)

semi-purified fractions of *C. Mexicana* and *C. cupressoides* are reported to possess anti-inflammatory activity which is also associated with their anti-oxidant effects (Lee et al. 2013).

Altered ROS hyperpolarizes the mitochondrial membrane potential after treatment

MMP is a crucial determinant for cell's survival and mitochondrial hyperpolarization or depolarization results in loss of cellular integrity. Hyperpolarization of mitochondrial membrane by CTE could be a cause of mitochondrial superoxide production as suggested by DHE assay (discussed previously). Superoxide dependent cytotoxicity and elevated MMP is previously reported by curcumin in HepG2 cells (human hepatoma) (Cao et al. 2007). MMP modulations are usually accompanied by release of cytochrome c and Ca^{2+} influx, which further initiate a cascade of signaling events leading to mitochondria-mediated apoptosis (Weinberg and Chandel 2015). Similar results were obtained by proapoptotic protein Mcl-1S (myeloid leukemia factor-1S), a B cell lymphoma-2 (Bcl-2) family protein, that induced reduction of anti-apoptotic Mcl-1 L, with concurrent induction of mitochondrial hyperpolarization and Ca^{2+} influx (Morciano et al. 2016). An FDA-approved drug, Ivermectin, also causes transient mitochondrial membrane hyperpolarization, followed by a sudden depolarization and cell death in 4 T1.2 cells Balb/c (triple negative breast cancer cells) (Draganov et al. 2015). Another study reports MMP modulation by pepsin digested *C. microphysa* extract, followed by modulation of several pro and anti-apoptotic signals suggesting that *C. microphysa* mediates via mitochondrial pathway of apoptosis (Chou et al. 2014).

CTE exhibits anti-cancer potential by causing G_1/S phase cell cycle arrest

Earlier reports reveal inhibition of cell proliferation in *Paracentrotus lividus* (sea urchin) eggs by CYN, 10,11-epoxycaulerpenyne and taxifolial A obtained from *C. taxifolia* with IC_{50} 16 ± 2 µg/mL, 20 ± 1 µg/mL, and 28 ± 1 µg/mL, respectively. CYN and 10,11-epoxycaulerpenyne also displayed inhibitory effect on BHK21/C13 cells (hamster fibroblast) with IC_{50} 15 ± 2 µg/mL, and 11 ± 2 µg/mL, respectively (Lemée et al. 1993). In another study, CYN is reported to exhibit anti-proliferative effect on 8 cancer cells, having most pronounced effect on colorectal cells with IC_{50} 6.1and 7.7 µM (Fischel et al. 1995). In SK-N-SH cells (neuroblastoma), growth inhibitory activity of CYN was observed with IC_{50} 10 ± 2 µM with concomitant microtubule modulations (Barbier et al. 2001). Cytotoxicity of CYN is concentration-dependent, and 2–24 h treatment modules exhibit similar cytotoxicity curves (Fischel et al. 1995). The present study, reveals selective cytotoxicity of CTE to cancer cells and growth promoting effect on hPBMCs, suggesting a potent in vitro anti-cancer activity

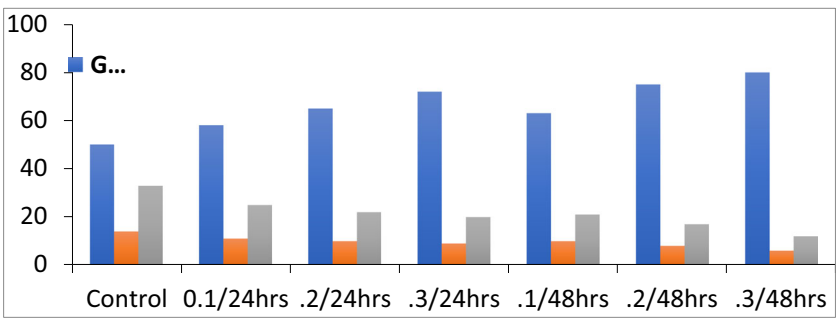

Fig. 5 Cell Cycle Analysis: Cell cycle progression studied by PI staining MDA-MB-231cells after 24 and 48 h at 3 different concentrations

of *C. taxifolia* against breast cancer and lung cancer. No previous reports of cell cycle modulations by CTE exist, although a few reports of other *Caulerpa* species have done rounds, claiming interference in DNA replication and cell cycle arrest at a metaphase like stage in sea urchin embryos by CYN (Pesando et al. 1996). Another study reports induction of G_0/G_1 phase arrest in HL60 cells (promyelocytic leukaemia) by methanolic extract of *C. racemosa* (Lakmal et al. 2014). Likewise, *C. microphysa* extract blocks cell cycle at G_0/G_1 phase by downregulating the expression of cyclin D-E, CDK2, CDK6 and upregulation of p21, p27, p53 and subsequently causes apoptosis in WEHI cells (Chou et al. 2014). Results from the present study showed cell cycle arrest by *C. taxifolia* which might be associated with apoptosis. The anti-proliferative activity, ROS induction and modulations of anti-oxidant enzyme defense system, with concomitant MMP perturbations, and cell cycle arrest by CTE suggest potential of *C. taxifolia* in cancer therapeutics. The purification and characterization of active metabolites is in progress and subsequent evaluation of *in vivo* activity shall be attempted.

Conclusion

The anti-proliferative activity, ROS induction and modulations of anti-oxidant enzyme defense system with concomitant MMP perturbations and cell cycle arrest by CTE suggest potential of *C. taxifolia* in cancer therapeutics. Further research to gain more understanding about the signalling pathways is suggested.

Materials and methods

Sampling

C. taxifolia specimen was collected from coasts of Tenneti, Andhra Pradesh, India in the month of January. Fresh thalli were washed and shade dried, followed by crushing to coarse powder using grinder. The dried powder was stored at room temperature until further use.

Extract preparation

The solvent extract of *C. taxifolia* was prepared by homogenizing the powdered sample with methanol for 24 h with continuous shaking (Ryu et al. 2013). Cell debris were removed and supernatant was separated after centrifugation at 5000 rpm for 5 min at 4 °C. CTE was concentrated under reduced pressure and dissolved in dimethylsulfoxide (DMSO) for further analyses.

Phycochemical screening

Qualitative analyses were done to detect the presence of various phycochemicals like alkaloids, tannins, flavonoids, cardiac glycosides and anthraquinones by the methods described previously (Mehdinezhad et al. 2016).

Cells and chemicals utilized

NCI-H1299 (lung carcinoma), T-47D (Breast cancer) and MDA-MB-231 (Breast cancer) cells were purchased from National Centre for Cell Science (NCCS), Pune. T-47D were cultured in RPMI 1640 (Roswell Park Memorial Institute) (Gibco by Life Technlogies, USA) while MDA-MB-231 and NCI-H1299 were cultured in DMEM (Dulbecco's modified eagle medium), high glucose (Gibco by Life Technlogies, USA) supplemented with 10% fetal bovine serum (FBS) (HiMedia Laboratories) at 37 °C, 5% CO_2 and 95% relative humidity. Seed stocks were prepared in DMSO and stored at −80 °C.

DHE assay

Intracellular ROS levels were monitored in MDA-MB-231 cells after 0.1, 0.25 and 0.5 μg/μL of CTE treatments for 24 h. (Joshi et al. 2016). The cells were washed twice with 1X PBS and the fluorescence was measured using a microplate reader (BioTek™ Synergy™ H1), after staining with 10 μM DHE at 535/635 nm (excitation/emission).

Superoxide dismutase assay

Cu-Zn SOD activity was assayed by pyrogallol method, in a reaction mixture constituting 50 μL 0.1 M Tris, 16 μL 6 mM EDTA, 34 μL 6 mM pyrogallol prepared in 0.1 N HCl and 5 μL cell lysate and 0.01 or 0.05 μg/μL CTE (Marklund and Marklund 1974). The experiment was performed in triplicates and absorbance was recorded at 420 nm.

Catalase assay

Catalase activity was measured using method described by Aebi 1984, which records the disappearance of peroxide spectrophotometrically at 240 nm (Aebi 1984). In a reaction mixture, 0.059 M H_2O_2 prepared in 0.05 M phosphate buffer (pH 7.0), enzyme extract and CTE were taken and mixed well at room temperature followed by immediate absorbance measurement at 240 nm for 3 min.

Glutathione reductase

Glutathione reductase (GR) or glutathione sulfhydryl reductase (GSR) activity was assayed by monitoring oxidation of

NADPH to $NADP^+$ at 340 nm, in a reaction mixture constituting 50 μL assay buffer, 5 μL 20 mM GSSG, 5 μL 2 mM NADPH, 5 μL cell lysate, 35 μL double distilled water and 0.01 or 0.05 μg/μL CTE (Carlberg and Mannervik 1985).

JC-1 assay

The changes in mitochondrial transmembrane potential in T-47D cells were determined using 5,5′,6,6′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) probe with a microplate reader (Alex et al. 2014). MDA-MB-231 cells were grown in 96 well plates at a density of 10,000 cells/well and treated with 0.10, 0.25 and 0.5 μg/μL CTE for 24 h at 37 °C. The cells were washed with 1X PBS with subsequent staining by 20 μM JC-1 for 30 min at 37 °C. The fluorescence was recorded at 485/535 and 550/600 nm using microplate reader.

Cell proliferation

NCI-H1299, T-47D, MDA-MB-231 and human peripheral blood mononuclear cells (hBMCs) were seeded at a density of 10,000 cells per well, in 96-flat well culture plates. The cell monolayers were allowed to grow in 10% FBS supplemented media until 24 h, after which the cells were subjected to serum starvation for growth phase synchronization. The medium was replenished after 12 h and cells were treated with CTE at concentrations of 0.1, 0.25 and 0.5 μg/μL, in triplicates. After 48 h, cells were washed with 1X phosphate buffer saline (PBS) and assayed with 0.5 mg/mL MTT prepared in PBS. The plates were re-incubated for 4 h in dark and later, supernatant was discarded and formazan crystals were dissolved in 100 μL DMSO with constant shaking for 30 min. The absorbance was recorded at 570 nm on microplate reader (Joshi et al. 2017).

Cell cycle

Cell cycle assay was performed on MDA-MB-231 cells following protocol described previously (Joshi et al. 2017). MDA-MB-231 cells were grown in 96 well plates at a density of 10,000 cells/ well. The cells were treated with 0.25 μg/μL CTE for 48 h at 37 °C, after which cells were washed with 1X PBS. Cells were harvested and subjected to chilled ethanol followed by incubation at −20 °C for 4 h. After incubation, cells were treated with ribonuclease A (100 μg/mL) to ensure RNA degradation, if any. Then the cells were centrifuged and washed with 1X PBS. After washing, cells were stained with 50 μg/mL propidium iodide (PI) and incubated for 30 min at room temperature in dark and then, analyzed on flow cytometer (BD Accuri).

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Authors' contributions RM and SB conducted all the lab experiments. UPY did cell cycle analysis. SS designed the experiments and all authors contributed in writing the manuscript. The final manuscript is read and approved by all authors.

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Availability of data and material All data generated or analyzed during this study are included in this published article.

Compliance with ethical standards

Ethics approval and consent to participate Not Applicable.

Consent for publication Not Applicable.

Competing interests The authors declare that they have no competing interests.

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