Anti-brain cancer activity of chloroform and hexane extracts of *Tinospora cordifolia* Miers: an *in vitro* perspective

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KEY WORDS

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ABSTRACT

Background: Plants have been suggested as safest source of therapeutic agents, with multi targeted mode of action and least side effects. *Tinospora cordifolia,* commonly known as Guduchi in India, is one of the most highly valued herbs in Ayurvedic medicine. It possesses potential anti-cancer, anti-inflammatory, hepatoprotective, anti-diabetic, immune-stimulatory and various other beneficial activities.

Purpose: The present study was aimed to investigate the differentiation inducing potential of chloroform and hexane extracts of *T. cordifolia* using U87MG glioblastoma and IMR-32 neuroblastoma cell lines as model system.

Results: Chloroform (Chl-TCE) and hexane (Hex-TCE) extracts significantly reduced the rate of proliferation and induced cell differentiation as evidenced by MTT assay and immunostaining for GFAP and MAP-2 in glioblastoma and neuroblastoma, respectively. Further these extracts increased the expression of stress markers HSP70 and Mortalin and induced senescence. Chloroform and hexane extracts also inhibited the migration of U87MG glioblastoma and IMR-32 neuroblastoma as indicated by wound scratch assay and supported by reduced expression of NCAM. Furthermore these extracts are not toxic to normal cells as they showed no inhibitory effects on primary astrocytic and neuronal cultures.

Conclusions: The present study suggests that chloroform and hexane extracts of *T. cordifolia* retard the rate of proliferation, induce differentiation and inhibit migration of human glioblastomas and neuroblastomas, thus may act as potential phytotherapeutic intervention in treatment of neural cancers.

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Introduction

One of the several hypotheses about origin of neural cancers is accumulation of mutations in pre-cancer cell which regularly divides to form nervous system during embryogenesis [1]. Glioblastoma is highly aggressive and invasive primary malignant brain tumor in adults [2], whereas, neuroblastoma is most common extracranial solid cancer of childhood and infancy [3]. Complexity and heterogeneity of the underlying oncogenic pathways of these neural cancers render them nonresponsive to conventional treatment therapies like radiotherapy, chemotherapy, surgery and immunotherapy. Currently differentiation based therapy and herbal remedies are in much discussion for cure of cancers. Plants have been suggested as safest source of drugs with multimodal actions and least side effects [4]. As a therapeutic modality for cancer cells, differentiation based therapy aims to reactivate original endogenous differentiation programs and to redirect cells into specific lineage [5]. Although treatment of malignant tumors with this approach is quite appealing but development of differentiation inducing agents with minimal side effects and maximal target specificity has been limited.

Tinospora cordifolia, commonly known as "Guduchi" is an Indian shrub belonging to family menispermaceae and is used as important medicine of Indian traditional medicine system since times immemorial. *T. cordifolia* is a large, glabrous, deciduous climbing shrub which is distributed throughout the Indian tropical subcontinent and China, ascending to high altitude of 300 meters [6]. It finds a special mention for its use in tribal or folk medicine in different parts of the country. *T. cordifolia* has been referred as "Heavenly elixir" and "Nectar of immortality" in ancient Ayurvedic literature and is being used for treatment of various ailments [7]. It is also known as "Amrita" due to its adaptogenic and immuno-modulatory properties [6]. *T. cordifolia* has been traditionally used for treatment of fever, gout, arthritis, dysentery, diarrhea, diabetes, anemia, asthma and psoriasis [8–9]. This plant contains glycosides such as tinocordiside, tinocordifolioside, cordiosides and palmitosides, steroids such as β-sitosterol, ecdysterone, alkaloids such as palmatine, berberine, magnoflorine, 11-hydroxymustakone, some diterpenoid lactones and aliphatic compounds as active constituents responsible for its medicinal properties. Various extracts of Giloy have been reported to possess anti-oxidative, anti-diabetic, anxi-

olytic, anti-depressant and chemo-preventive activities [10]. Formulations of *T. cordifolia* extract with turmeric are effective in preventing hepatotoxicity [11]. Some recent studies have shown anti-feedent activity of clerodane glycosides isolated from chloroform extract of *T. Cordifolia* along with anti-cancer activity [12–13].

Differentiation inducing and anti-cancer potential of 50% ethanolic extract of *T. cordifolia* (TCE) on neural cancers has been reported recently from our lab [14–15]. The TCE was further fractionated using solvents of increasing polarity i.e. hexane, chloroform, ethyl acetate and butanol. The current study was designed to investigate anti-cancer potential of Chloroform (Chl-TCE), Hexane (Hex-TCE), Ethyl acetate (EA-TCE) and Butanol (B-TCE) extracts of *T. cordifolia*. U87MG, grade IV human glioblastoma and IMR-32, human neuroblastoma cell lines, used as model system in this study are well reported cell lines for *in vitro* study of neural cancers. Initial MTT screening showed that out of four fractions only Chl-TCE and Hex-TCE had anti-proliferative activity, so these two fractions were used for detailed study of molecular markers. Differentiation of glioblastoma has been characterized by expression of Glial fibrillary acidic protein (GFAP), while expression of Microtubule associated protein (MAP-2) characterizes differentiation of neuroblastoma [16–17]. GFAP expression studies not only provide information about brain physiology but are also useful to understand neurological disorders [18]. MAP-2 is expressed in dendrites of terminally differentiated post mitotic neurons and it's *in vitro* expression induces stabilization of microtubule bundles [19]. Further, the investigation of senescence inducing potential was tested by studying heat shock proteins HSP70 and Mortalin. HSP70, a stress response protein plays an important role in cellular differentiation and proliferation, whereas, Mortalin, a senescence marker is implicated in control of cell proliferation and apoptosis inhibition [20–21]. The anti-migratory potential was checked by evaluating the expression of neural cell adhesion molecule (NCAM) which plays an important role in preventing dissemination of malignant tumors by modulating neurite outgrowth and matrix adhesion [22]. Further, to evaluate whether these extracts specifically kill cancer cells or are also toxic to normal cells, primary astrocytes and hippocampal neurons from 0–2 day old rat pup brains were also exposed to these extracts.

Methods

Plant material and preparation of Chloroform and Hexane extracts

50% aqueous ethanolic extract (TCE) was obtained from Indian Institute of Integrative Medicine, Jammu, India. Briefly, it was prepared by dissolving 5 g of dried *T. cordifolia* stem powder in 100 mL of aqueous ethanol (50:50), which was incubated overnight, followed by filtration and drying. Dried TCE was weighed and then reconstituted in distilled water to obtain concentrated paste. n-Hexane was added to concentrated TCE, mixed by shaking and separated with the help of separating funnel. This procedure was repeated thrice. Obtained hexane fraction was evaporated to dryness using rotary evaporator (Buchi Labortechnik, Switzerland) and designated as Hex-TCE. To the remaining concentrate, chloroform was added and mixed. Chloroform fraction obtained was dried and designated as Chl-TCE. Similarly, EA-TCE and B-TCE were obtained from the concentrated TCE after Chl-TCE. Dried Hex-TCE and Chl-TCE were reconstituted in DMSO to prepare stocks which were further diluted in complete DMEM containing 10% FBS to obtain required concentrations.

Cell Culture and Treatment

Human U87MG glioma and IMR-32 neuroblastoma cell lines were obtained from National Centre for Cell Science, Pune (India). Primary astrocytic culture was established by sacrificing 0–2 day old albino Wistar rat pups, dissecting out the whole brain, chopping into few small pieces after removal of meninges followed by trypsinization and obtaining single cell suspension. The single cell suspension was seeded into culture flask containing DMEM (Sigma Aldrich, USA) and maintained in CO_2 incubator. Medium was changed on every 3rd day and astrocytic population was separated from microglial cells by mild trypsinization [23]. For primary neuronal culture, only hippocampus was removed from pup brain, chopped into 2–3 pieces, trypsinized and single cell suspension was obtained, which was directly seeded into 24-well plate in neurobasal medium (Life Technologies, USA). Neurobasal medium is specific medium for neuronal growth and maturation. Both the cell lines and primary astrocytic cultures were maintained on DMEM (Sigma Aldrich, USA) containing 10% FBS (Biological Industries, Israel) and 1X PSN antibiotic (Life Technologies, USA) in a humidified environment with 5% CO_2. Cells were trypsinisized, subcultured and seeded into 24 or 96 well plates or 90 mm^2 Petri dishes (Corning, Sigma Aldrich, USA) according to the experiment. After 24 hours, cells were given treatment with a range of different concentrations of both the extracts initially (for 48 hours (U87MG) and 72 hours (IMR-32)) and then 10 µg/mL of Chl-TCE and 15 µg/mL of Hex-TCE were selected for further experiments using U87MG and IMR-32 cell lines. Control cultures were given only media change.

All animal experimental protocols were approved by Institutional Animal Ethical Committee, Guru Nanak Dev University, Amritsar registered to "Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India" (Registration no. 226/CPCSEA) (permission number 226/CPCSEA/2015/17) and performed in accordance with the relevant guidelines of 'Animal Care and Use' laid down by the same committee.

Cell Proliferation and Morphological studies

Chl-TCE and Hex-TCE were tested for cytotoxicity and anti-proliferative activity on U87MG and IMR-32 using the

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) salt (Sigma Aldrich, USA). Cells were seeded in 96 well plates at the seeding density of 5,000 cells/mL and kept in humidified CO_2 Incubator. After 24 hours, U87MG cells were given treatment for 48 hours with different concentrations of Hex-TCE and Chl-TCE (1–50 µg/mL). Similarly, IMR-32 cells were given treatment for 72 hours (IMR-32) with different concentrations of Hex-TCE and Chl-TCE (1–40 µg/mL). After completion of treatment, cells were incubated with MTT containing serum less media (0.5% w/v) for 2 hours in CO_2 incubator. Purple formazan crystals obtained from MTT due to mitochondrial dehydrogenase activity were then dissolved and measured spectrophotometrically at 594 nm. Changes in cell morphology after treatment with extracts were studied by phase contrast microscopy (Nikon TE2000).

Immunostaining

U87MG glioblastoma and IMR-32 neuroblastoma cells were seeded in 24 well plates at seeding density of 10,000 cells/mL and treated with Chl-TCE (10 µg/mL) and Hex-TCE (15 µg/mL). Primary astrocytes and hippocampal neurons were seeded at 20,000 cells/mL plating density followed by same treatment regimen. After completion of treatment regimen, the control and treated cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.3% Triton X-100 in PBS. Cells were blocked with BSA and then incubated with mouse anti-α-Tubulin (1:500), mouse anti-HSP70 (1:500), mouse anti-Mortalin (1:500), mouse anti-MAP-2 (1:250), mouse anti-NCAM (1:500) and rabbit anti-GFAP (1:500), in a moist chamber for 24 hours at 4 °C. All primary antibodies were obtained from Sigma Aldrich, USA and were diluted in 0.1% PBST. After three washings with 0.1% PBST, cells were incubated with secondary antibody (1:500) (Alexa Fluor 488/543 goat anti-mouse IgG, Alexa fluor 488 goat anti-rabbit, Life Technologies, USA) for two hours at room temperature followed by incubation with DAPI (Sigma Aldrich, USA), nuclear stain and then coverslips were mounted using antifading mounting medium, Fluoromount (Sigma Aldrich, USA). Images were captured using Nikon A1R Confocal Laser Microscope and analysis was done using NIS elements AR analysis software version 4.11.00 (Nikon, Japan).

Protein assay and Western blotting

Cells seeded (10,000 cells/mL) and treated in 90 mm^2 Petri dishes were harvested using 1 mM EDTA-PBS. Harvested cells were homogenized in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% Sodium deoxycholate, 0.1% SDS, 1.0% NP-40). Supernatant was collected and protein content was estimated by Bradford method spectrophotometrically. 30 µg Protein samples were then resolved in 10% and 7% SDS-PAGE gels, transferred to PVDF membrane (Hybond-P GE healthcare, USA) using the semidry Novablot system (Amersham Pharmacia, USA). Blots were probed with primary mouse monoclonal antibodies anti-α-Tubulin (1:5000), anti-GFAP (1:3000), anti-HSP70 (1:2500), anti-Mortalin (1:1000), anti-MAP-2 (1:2500) and anti-NCAM (1:2000) diluted in 5% skimmed milk for overnight at 4 °C. Immunoreactive protein bands were visualized after incubation with goat anti-mouse HRP conjugated secondary antibody (Merck Millipore, USA) for 2 hours at room temperature and development of blot with ECL Western blot detection system (GE Healthcare, USA). Blots were developed and antibody labeling intensity was quantified by using Image Quant LAS 4000 (GE Healthcare, USA).

Wound scratch assay

To check anti-migratory potential of Chl-TCE and Hex-TCE, cells were seeded at high density (30,000 cells/mL) and grown to achieve confluency. A straight scratch was given with microtip on all the coverslips containing confluent monolayer of cells followed by change of medium and treatment with extracts. Images were captured at zero hour, 8 hours (for U87MG) and 24 hours (for IMR-32) of treatment and gap closure was calculated after image analysis by Image Pro Plus software version 4.5.1 from the Media Cybernetics.

Statistical analysis

All the experiments were performed in triplicates and values were represented as mean ± SEM. Results were analyzed using one way ANOVA (Holm-Sidak post hoc test) performed by Sigma stat software (Version 3.5) for windows. Values ($p < 0.05$) were considered as statistically significant.

Results

Chl-TCE and Hex-TCE reduced the rate of proliferation in U87MG and IMR-32 cell lines

U87MG glioblastoma and IMR-32 neuroblastoma cell lines were treated with different concentrations of Chl-TCE and Hex-TCE. Decrease in cell number and changes in morphology were observed with increasing concentration of both Chl-TCE and Hex-TCE. Further dose dependent cell number decrease was confirmed by MTT assay (Fig. 1). IC_{50} of Chl-TCE and Hex-TCE for U87-MG was 25 µg/mL and 50 µg/mL (Fig. 1a), respectively and 32.5 µg/mL and 35 µg/mL for IMR-32 (Fig. 1b), respectively. Phase contrast micrographs showed reduced cell number and highly differentiated morphology of cells at 10 µg/mL of Chl-TCE and 15 µg/mL of Hex-TCE in both U87MG and IMR-32 cell lines (Fig. 1c). Phase contrast and MTT data was further supported by DAPI staining showing reduced cell number in treated groups as compared to control control (Fig. 1 d). These concentrations were then selected for further studies on both the cell lines. Same concentrations of both the extracts were also tested on primary astrocytic and hippocampal neuronal cell culture to elucidate whether these extracts are toxic to normal cells or not. Astrocytes showed no decrease in cell number along with normal morphology as compared to control cultures (Fig. 2a). Primary hippocampal

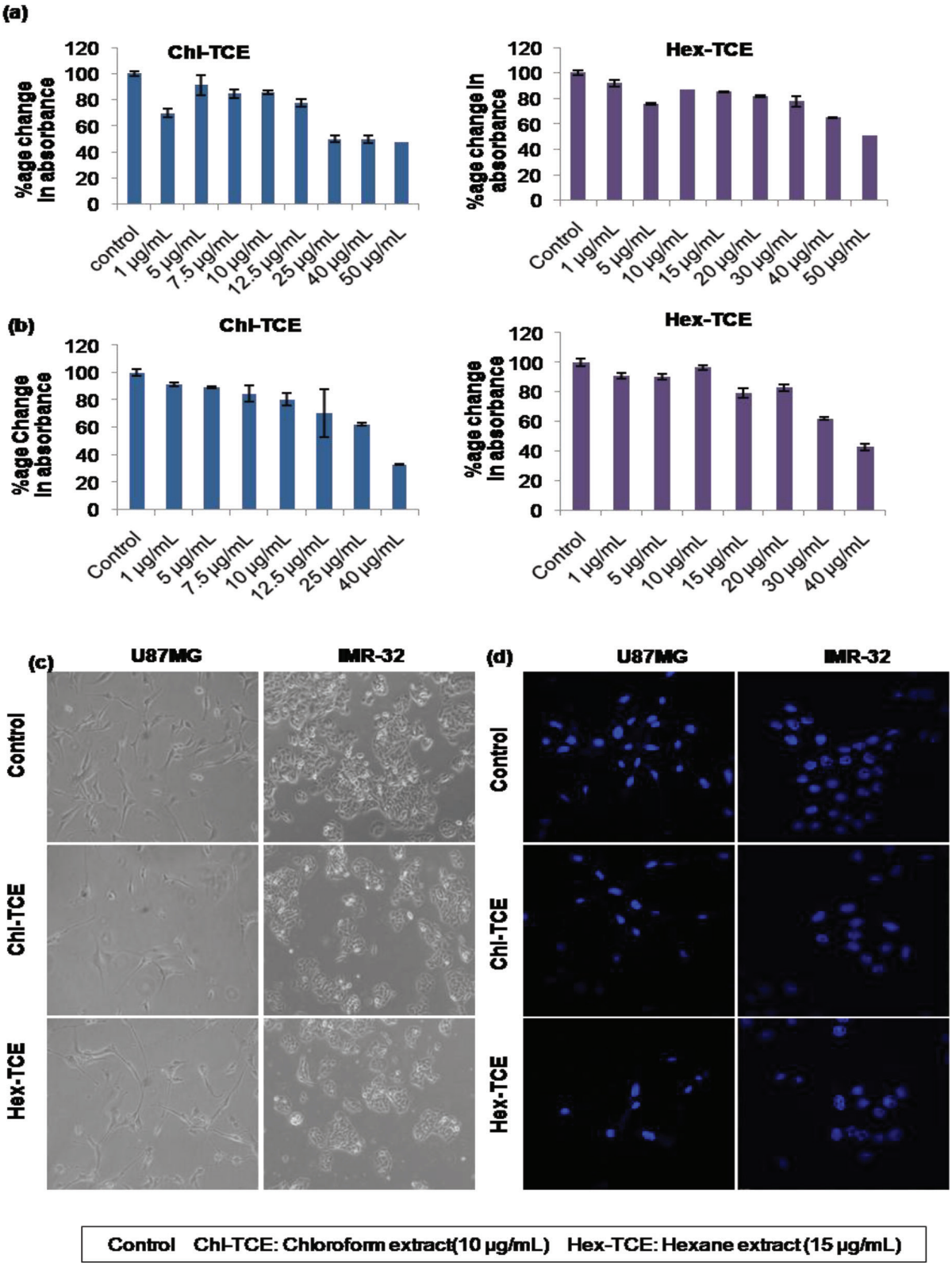

Fig. 1: Chl-TCE and Hex-TCE inhibited cell proliferation. MTT assay showing dose dependent decrease in cell number after treatment with these extracts. Histograms represent percentage change in absorbance at various concentrations of chloroform and hexane extracts in U87MG **(a)** and IMR-32 **(b)**. **(c)** Phase contrast images of U87MG and IMR-32 control and treated cells captured at 10X objective. **(d)** Confocal images of U87MG and IMR-32 control and treated cells stained with nuclear stain DAPI, captured at 60X objective.

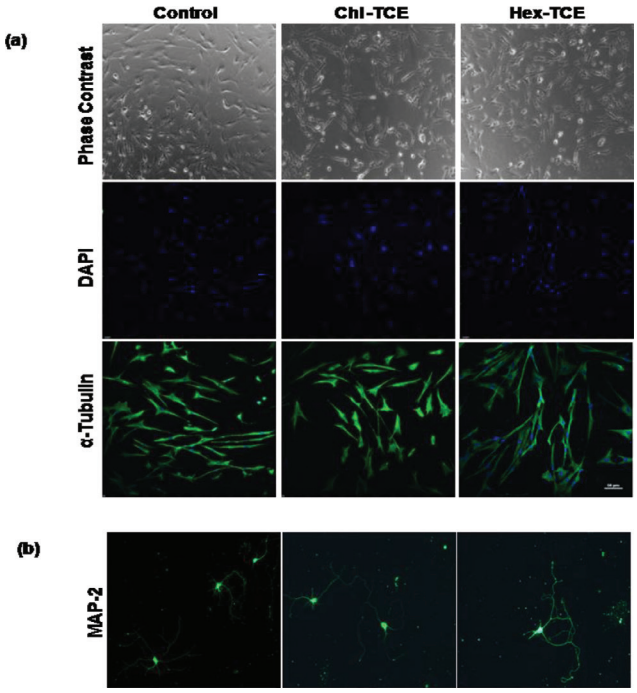

Fig. 2: Chl-TCE and Hex-TCE don't have negative effects on normal glial and neuronal cells. **(a)** Phase contrast, α-Tubulin immunostained and DAPI stained confocal images of primary astrocyte cultures treated with these extracts exhibit almost comparative cell number and similar morphology to control. **(b)** Confocal images of primary hippocampal neurons immunostained for MAP-2 after treatment with Chl-TCE and Hex-TCE. Confocal images were captured at 40X objective (Scale bar: 50 µm).

neurons treated with Chl-TCE and Hex-TCE also showed control like morphology and comparable MAP-2 intensity (Fig. 2b). This preliminary data suggested that activity of these extracts was cancer cell specific and posed no harmful effects to normal cells.

Chl-TCE and Hex-TCE induced differentiation in U87MG and IMR-32 cell lines

α-Tubulin immunostaining of U87MG showed cells with longer stellate processes and short cell body in both Chl-TCE and Hex-TCE treated cells (Fig. 3a, upper panel). To further confirm cell differentiation, expression of GFAP, a glial cell differentiation marker was studied both by immunostaining and Western blotting. A significant increase was observed in GFAP expression in both the Chl-TCE and Hex-TCE treated groups ($p < 0.05$) (Fig. 3a, lower panel). The immunostaining data was further supported by Western blotting results (Fig. 3c). Similarly α-Tubulin immunostaining of IMR-32 cells also showed highly differentiated cell morphology after treatment with Chl-TCE and Hex-TCE as compared to control cultures (Fig. 3b, upper panel). MAP-2 expression was studied to confirm differentiation inducing potential of these extracts (Fig. 3b, lower panel). Western blotting results showed a statistically significant increase in MAP-2 expression ($p < 0.05$) in Chl-TCE treated group, whereas, its expression in Hex-TCE treated group was not statistically significant (Fig. 3d).

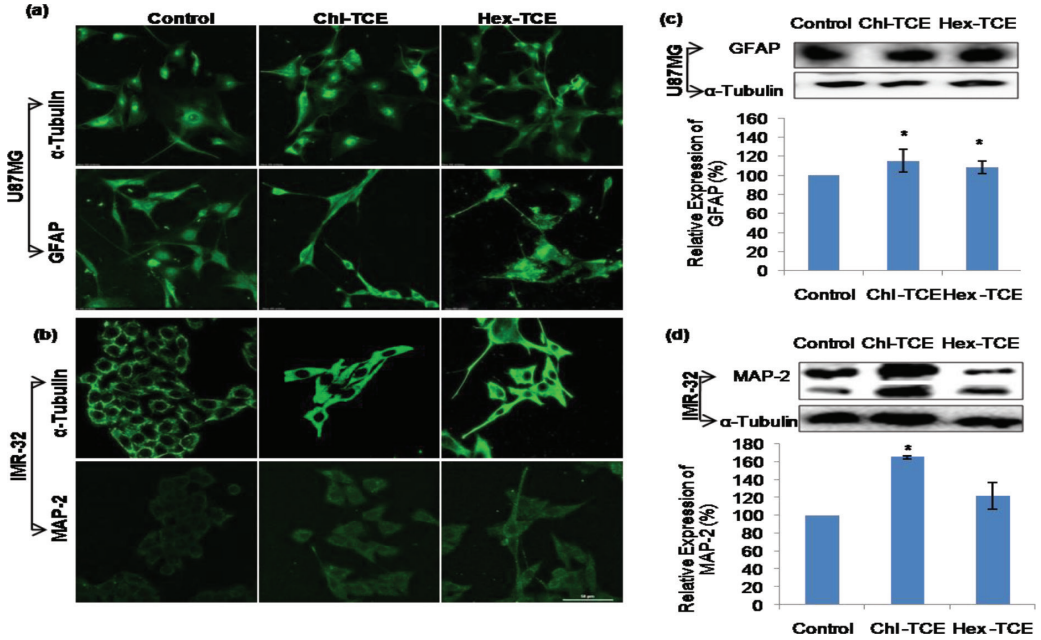

Fig. 3: Chl-TCE and Hex-TCE induced differentiation in U87MG and IMR-32. Confocal images of U87MG **(a)** and IMR-32 **(b)** showing immunostaining of α-tubulin and GFAP. Representative Western blot hybridization signals for GFAP in U87MG **(c)** and IMR-32 **(d)**. Histograms represent the densitometric analysis i.e. percentage in GFAP expression in treated cells as compared to control. Values are representative of mean ± SEM. '*' (*P < 0.05*) *represents statistical significant difference between control and treated groups.* Confocal images were captured at 60X objective (Scale bar: 50 µm).

Chl-TCE and Hex-TCE increased the expression of stress markers and induced senescence

To study the effect of Chl-TCE and Hex-TCE on stress and senescence in U87MG and IMR-32 cell lines, we studied the expression of HSP70 and Mortalin. HSP70 is an ATP dependent essential uncoating enzyme, induction of which is important in neuronal differentiation and neurite extension, whereas, Mortalin is a mitochondrial heat shock protein and is well established as a senescence marker. Expression of HSP70 and Mortalin was upregulated with treatment of both the extracts on U87MG and IMR-32 cell line (Fig. 4a, 4b). A characteristic pancytoplasmic expression of Mortalin was observed in IMR-32 cells treated with Chl-TCE and Hex-TCE, whereas, perinuclear expression was observed in control IMR-32 cells (Fig. 4b, lower panel). Western blotting results also supported the immunostaining data (Fig. 4c, 4d) as total cellular expression of HSP70 and Mortalin was found to be significantly enhanced in U87MG on treatment with both the extracts ($p < 0.05$) (Fig. 4c). Upregulated expression of HSP70 and Mortalin was also observed in treated IMR-32 cells, and Hex-TCE specifically showed more pronounced increase in expression of these markers (Fig. 4d). HSP-70 and Mortalin belong to same family of stress proteins and their enhanced expression may be correlated with induction of senescence.

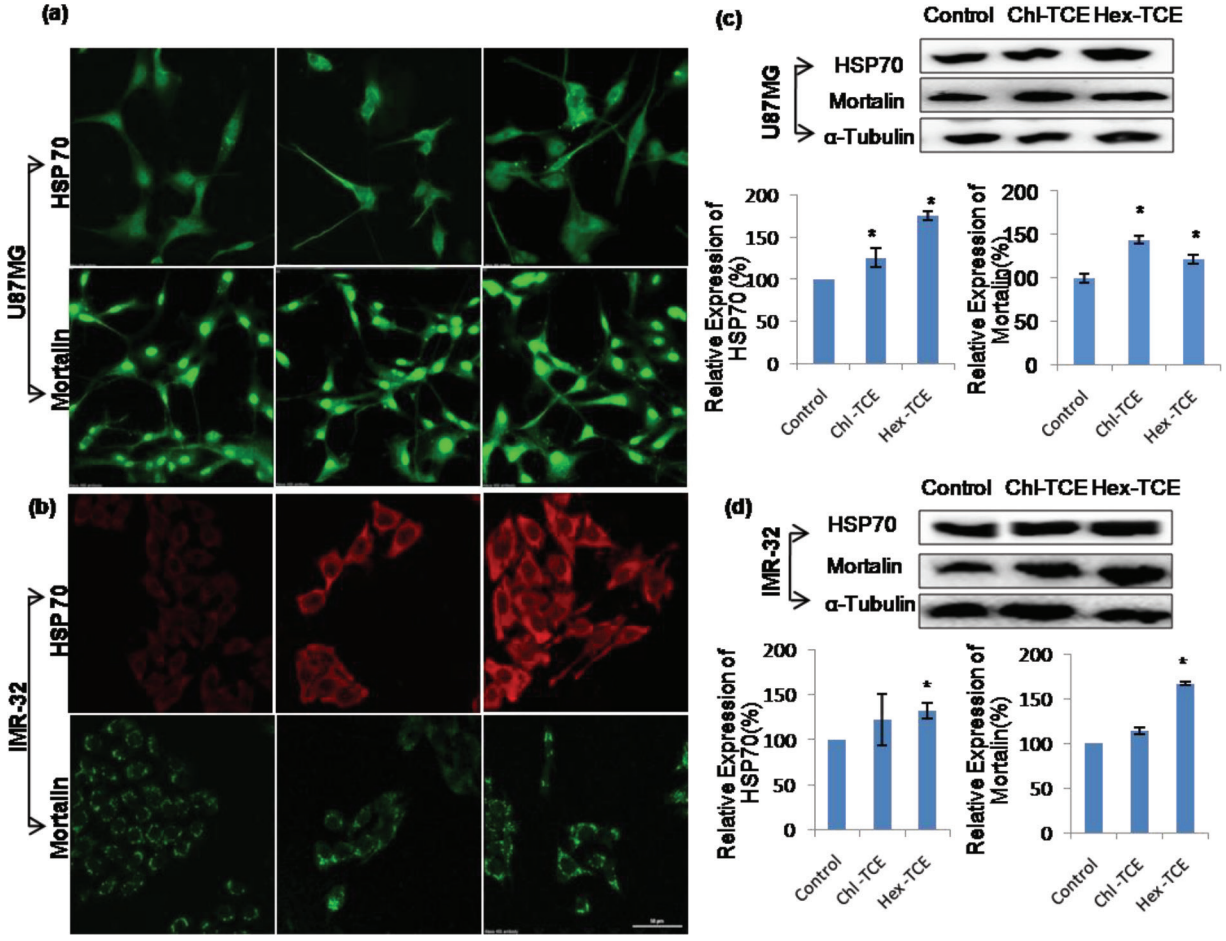

Fig. 4: U87MG and IMR-32 cells treated with Chl-TCE and Hex-TCE undergo senescence. Confocal images of U87MG **(a)** and IMR-32 **(b)** showing immunostaining of HSP70 and Mortalin. Representative Western blot hybridization signals for HSP70 and Mortalin in U87MG **(c)** and IMR-32 **(d)**. Histograms represent the densitometric analysis i.e. percentage in HSP70 and GFAP expression in treated cells as compared to control. Values are representative of mean ± SEM. '*' ($P < 0.05$) *represents statistical significant difference between control and treated groups.* Confocal images were captured at 60X objective (Scale bar: 50 μm).

Chl-TCE and Hex-TCE inhibited migration in U87MG and IMR-32 cell lines

Both the U87MG and IMR-32 are reported to have invasion properties. To further explore anti-migratory potential of these extracts, wound scratch assay was carried out and percent gap closure (area of gap repopulated by migrating cells) was measured after mentioned time duration for both cell lines. When scratched U87MG cells were treated with Chl-TCE and Hex-TCE, highly reduced migration (0.87% and 2.07% gap closure in Chl-TCE and Hex-TCE respectively) was observed in treated cells as compared to control cells (taking gap closure as 100% in control) after 8 hours (Fig. 5a, left panel). Similarly, In case of IMR-32 control cells, scratch was invaded and colonized

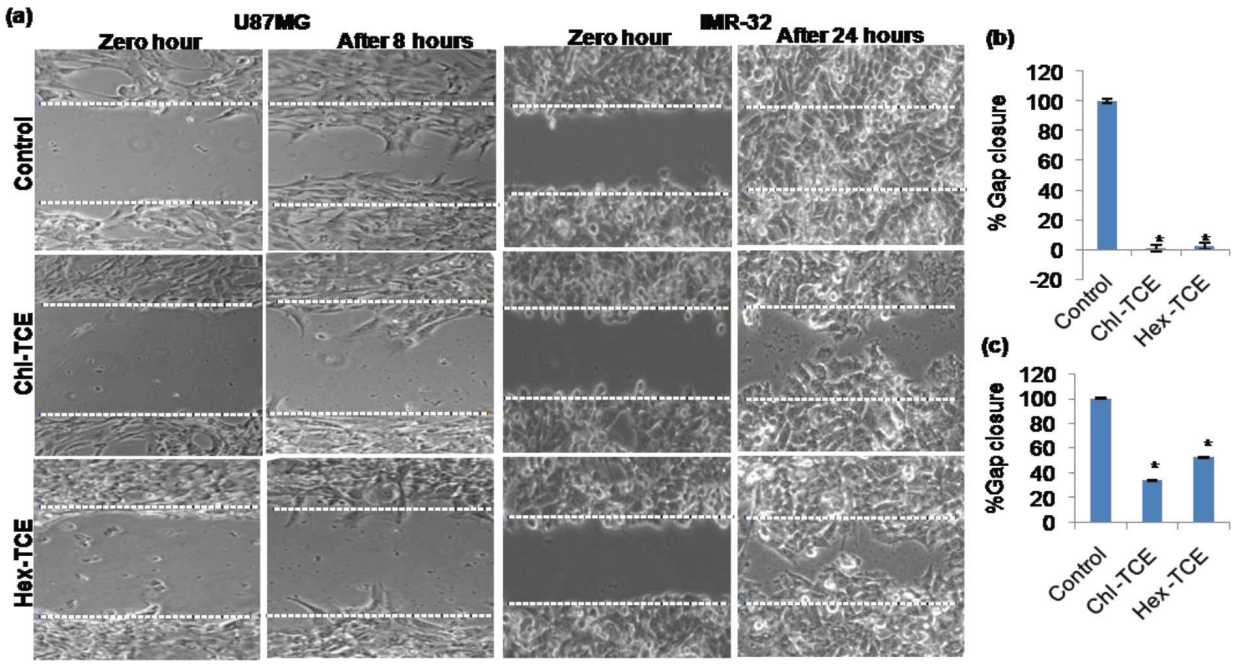

Fig. 5: Chl-TCE and Hex-TCE inhibited migration. **(a)** Phase contrast micrographs providing a comparative overview of migration in control and treated U87MG and IMR-32 cells. Images show the width of scratch at zero hour and after 8 hours for U87MG, after 24 hours for IMR-32. Histograms represent percentage gap closure in control, chloroform and hexane extract treated U87MG **(b)** and IMR-32 **(c)**. Values are representative of mean ± SEM. '*' (*P < 0.001*) *represents statistical significant difference between control and treated groups.* Phase contrast images were captured at 10X objective.

(taking gap closure 100% in control) after 24 hours, whereas, only 33.42% and 52.17% gap closure was observed in Chl-TCE and Hex-TCE treated groups, respectively (Fig. 5a, right panel). Thus significantly reduced migration rate ($p < 0.001$) was observed for both the cell lines after treatment with extracts (Fig. 5b, 5c). Further these cells were immunostained for neural cell adhesion molecule (NCAM), a marker of plasticity (Fig. 6a). Reduced expression of NCAM was observed in Chl-TCE and Hex-TCE treated groups as compared to control. Immunostaining data was also supported by Western blotting data with significant reduction ($p < 0.05$) of NCAM expression in Hex-TCE treated group in both U87MG and IMR-32 cell lines (Fig. 6b).

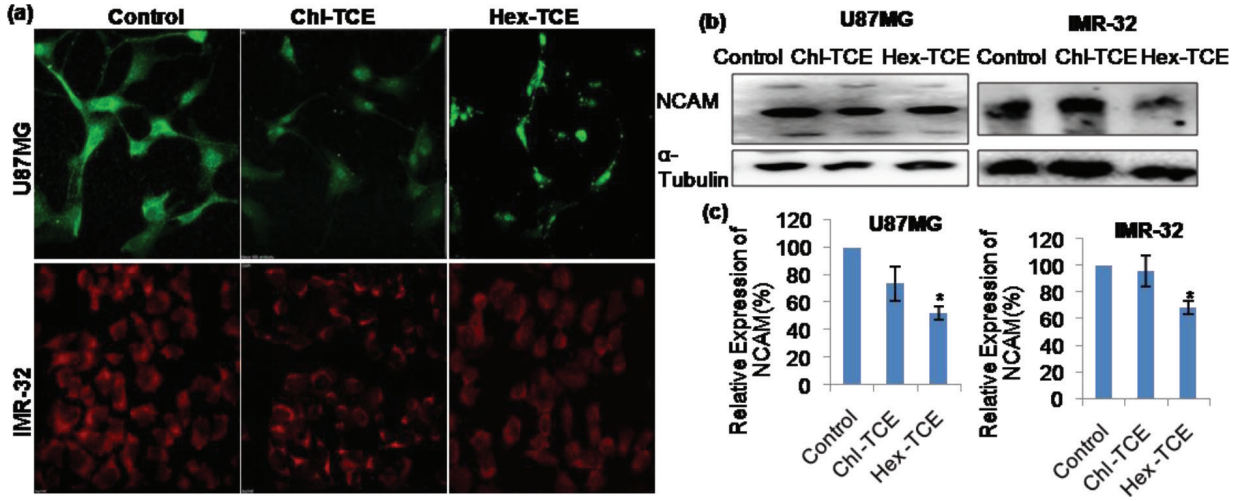

Fig. 6: Reduced expression of NCAM supported anti migratory property of Chl-TCE and Hex-TCE. **(a)** Confocal images of control and treated U87MG and IMR-32 cells showing immunostaining for NCAM. **(b)** Representative Western blot hybridization signals and **(c)** histograms show reduced expression of NCAM in treated groups as compared to control. Values are representative of mean ± SEM. '*' (*P < 0.05*) *represent statistical significant difference between control and treated groups.* Confocal images were captured at 60X objective (Scale bar: 50 μm).

Discussion

The present study was aimed to elucidate the anti-cancer activity of chloroform (Chl-TCE) and hexane extracts (Hex-TCE) of *T. cordifolia*. Previous studies from our lab have reported the differentiation inducing potential and anti-cancer activity of 50% ethanolic extract of *T. cordifolia* (TCE) [9–10]. TCE was further fractionated by chloroform, hexane, ethyl acetate and butanol to explore the nature of expected active compounds and to reduce the effective dose on glioblastoma and neuroblastoma cell lines. Chloroform and hexane fractions were found to possess anti-proliferative activity at very low doses (10 µg/ml of Chl-TCE and 15 µg/ml of Hex-TCE) as compared to effective doses of TCE (250 µg/ml and 350 µg/ml) for both the cell lines [9–10], whereas, ethylacetate and butanol fraction did not show anti-cancer activity (Supplementary Fig. 1). The phytochemical constituents responsible for activity of *T. cordifolia* have been reported to be alkaloids, glycosides, diterpene lactones, steroids and aliphatic compounds out of which non polar alkaloids and diterpenoid lactones possess anti-cancer activity [24]. Majority of fatty acids, alkaloids and diterpenoid lactones dissolve in hexane and chloroform solvent when TCE is fractionated with hexane, chloroform, ethyl acetate and butanol solvents having lower to higher polarity. The highly polar glycosides are reported to possess immunomodulatory and neuroprotective activities [20, 25–26]. We have generated some preliminary data which shows that polar butanol fraction containing these compounds enhance proliferation of normal (data not shown) as well as cancerous cells and recently reported neuroprotective activity of this fraction [27].

Reduction in effective dose of Chl-TCE and Hex-TCE may be due to higher concentration of non-polar alkaloids, diterpenoid lactones and fatty acids in chloroform and hexane fractions. Some reported alkaloids of *T. cordifolia* are palmatine, berberine, choline, magnoflorine, timbetrine, jatorrhizine, isocolumbine etc. Palmatine has been reported to possess anti-cancer activity against skin carcinomas induced by 7,12-dimethylbenz(a)anthracene (DMBA) in Swiss albino mice [28]. Berberine has been reported to be a part of many Chinese traditional medicines for cancer. It inhibits the growth of cancer causing microorganisms, inhibit oncogenes and the enzymes related to carcinogenesis [29–30]. Tinocordin, columbin, 8-hydroxycolumbin, 10-hydroxycolumbin are diterpeniod lactones reported in Chloroform extract of *T. cordifolia* [12]. Another diterpenoid epoxy clerodane from chloroform: methanol extraction of alcoholic extract of *T. cordifolia* has been reported to possess anti-cancer activity against hepatocellular carcinoma induced by diethylnitrosamine [24]. Glycosides have been reported to be present in butanol fraction of *T. cordifolia* extract [31–32]. Combination of proliferation inhibiting and enhancing compounds in crude extract (TCE) may be the reason of higher effective dose (250 µg/mL and 350 µg/mL) which has been earlier reported from our lab [14–15].

Treatment of U87MG and IMR-32 cell lines with Chl-TCE and Hex-TCE resulted in significant decrease in their cell number in a dose dependent manner. MTT assay is a widely used assay to measure anti-proliferative activity as the absorbance is linearly related to the number of metabolically active cells in the culture. The phase contrast images also clearly showed reduction in cell number in treated cultures. Elongated and very fine processes were observed in phase contrast images of extract treated U87MG cells, whereas, elongated cells with emerging processes were observed in IMR-32 cells (Fig 1c). This data suggests that treatment with Chl-TCE and Hex-TCE induced differentiation like morphology in both the cell types. Combined microscopic observations and MTT data results were used to select the effective doses of both the extracts for these neural origin cancer cell lines. Differentiation induction by these extracts suggests that both the extracts have potential to revert cancer cells to the normal differentiated cells. However, selected concentrations were not toxic to primary astrocytic cells as suggested by similar morphology and cell number to control cells, indicating their specificity for cancerous cells (Fig. 2a). Primary hippocampal neuronal cultures treated with Chl-TCE and Hex-TCE also showed morphology as well as intensity of MAP-2 immunostaining similar to control cultures suggesting no adverse effects on normal neuronal population as well (Fig. 2b). Their differentiation inducing potential was further confirmed by enhanced expression of cytoskeletal markers of glial and neuronal cells, GFAP and MAP-2, respectively. GFAP, a biochemical marker of astrocytic differentiation determines shape of astrocytes [17]. Enhanced expression of GFAP also suggests anti-tumor activity of these extracts as glioma cells injected with GFAP cDNA have been reported to show significantly reduced tumor growth [33]. The other cytoskeletal marker, MAP-2 has been reported to be expressed in dendrites of postmitotic neurons and its induction in melanomas leads to microtubule destabilization, cell cycle arrest and delayed or suppressed tumor progression [19]. Since expression of MAP-2 is considered as hallmark of neuronal development and differentiation, significantly enhanced expression of MAP-2 also suggests that Chl-TCE and Hex-TCE induced neuronal differentiation [16].

The hallmark of differentiation based therapy is that cancer cells after undergoing differentiation enter into senescence, so further senescence inducing potential of these extracts was also confirmed by studying expression of homeostasis and senescence marker proteins-HSP70 and Mortalin. Chl-TCE and Hex-TCE were found to enhance expression of these markers in both U87MG and IMR-32 cell lines. HSP70 is a molecular chaperone that modulates glial as well as neuronal cell differentiation [20, 34]. Upregulated HSP70 expression in differentiated cells has been supported by various reports suggesting the induction of HSP70 by anti-cancer/anti-neoplastic compounds such as berberine [34], vincristine, paclitaxel [35] and curcumin [36]. Berberine induced differentiation in neuro2a neuroblastoma cell by suppressing Transforming growth factor β, modulating ERK signaling pathway and inhibited cancer stemness, which was accompanied by upregulated expression of HSP70 [34]. Mortalin has been reported to be expressed at perinuclear locations in transformed tumor cells, whereas, this protein is expressed in

pancytoplasmic region in normal cells [21]. In IMR-32 cells, redistribution of perinuclear Mortalin to pancytoplasmic locations after treatment with Chl-TCE and Hex-TCE may indicate activation of senescence pathway by both the extracts.

To further ascertain the tumor suppressing activity of Chl-TCE and Hex TCE, the anti-invasive and anti-migratory efficacies of these extract were also studied. A scratch created on confluent monolayer mimics migration of cells *in vivo* and provides information about migration rate of cells and the effect of extract treatments on their migration rate [37]. Maximum migration of cells to scratched area (gap closure 100%) in control of both the cell lines and significantly reduced migration as depicted from smaller gap closure in Chl-TCE and Hex-TCE treated U87MG and IMR-32 cells suggest that both extracts retard the rate of migration. This may be due to controlled proliferation and induction of cell differentiation by these extracts. Anti-migratory properties of Chl-TCE and Hex-TCE were further supported by reduced expression of NCAM in both the cell lines after treatment. Enhanced NCAM expression is reported to induce migration by epithelial cell delamination and loss of adherens junctions and promoting tumor invasion [38–39].

An important observation made from expression of differentiation markers GFAP and MAP-2 and wound scratch assay was that both anti-migratory and differentiation inducing potential of Chl-TCE fraction were more pronounced than Hex-TCE in both the cell lines. On the other hand, Hex-TCE was observed to inhibit proliferation and inducing senescence more effectively in both the cell lines. Differential behavior of these extracts may be attributed to different active compounds present in these non polar fractions. A detailed study is required to characterize the active compounds present in these extracts.

Conclusion

In conclusion, the current data suggests that chloroform and hexane extracts of *T. cordifolia* (Chl-TCE and Hex-TCE) retard the rate of proliferation and migration, induce differentiation and senescence by targeting different cellular pathways in both the U87MG glioblastoma and IMR-32 neuroblastoma cell lines, and thus may act as potential phytotherapeutic intervention in treatment of neural cancers.

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Authorship contribution

Experiments were designed and conceived by AS and GK. Experiments were performed by AS, SKS and RM. Data was analyzed by GK and AS. Lab facilities/ materials/ reagents/ analysis tools were provided and contributed by GK. Manuscript was written and approved by GK and AS.

Disclosures and compliance with ethical standards

Ethical statement

All animal experimental protocols were approved by Institutional Animal Ethical Committee, Guru Nanak Dev University, Amritsar registered to "Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), GOI" (Registration no. 226/CPCSEA) (permission number 226/CPCSEA/2015/17) and performed in accordance with the relevant guidelines of 'Animal Care and Use' laid down by the same committee.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary file:
Anti-brain cancer activity of chloroform and hexane extracts of *Tinospora cordifolia* Miers: an *in vitro* perspective.

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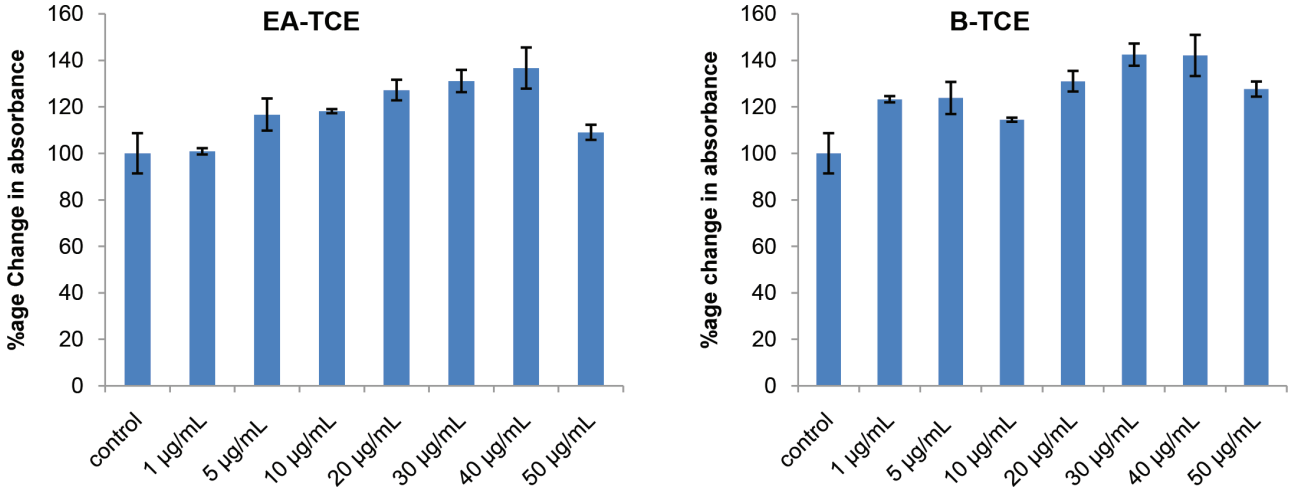

Fig. S1: **MTT assay:** Histograms presenting percentage change in absorbance after treatment with different concentrations (1-50µg/ml) of EA-TCE and B-TCE, suggesting dose dependent effect of these extracts on cell number of C6 glioma.