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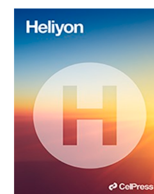

Research article

Calotropis procera: A double edged sword against glioblastoma, inhibiting glioblastoma cell line growth by targeting histone deacetylases (HDAC) and angiogenesis

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A B S T R A C T

Despite substantial investments in anti-glioblastoma (GBM) drug discovery over the last decade, progress is limited to preclinical stages, with clinical studies frequently encountering obstacles. Angiogenic and histone deacetylase inhibitors (HDACi) have shown profound results in preclinical studies. Investigating a multicomponent anti-cancer remedy that disrupts the tumor angiogenic blood vessels and simultaneously disrupts HDACs, while inducing minimal side effects, is critically needed. The crude extracts derived from medicinal plants serve as a renewable reservoir of anti-tumor drugs, exhibiting reduced toxicity compared to chemically synthesized formulations. *Calotropis procera* is a traditional medicinal plant, and its anticancer potential against many cancer cell lines has been reported, however its antiangiogenic and HDAC inhibitory action is largely unknown. The anticancer activity of methanol leaf extract of *C. procera* was tested in three types of human glioblastoma cell lines. Wild-type and transgenic zebrafish embryos were used to evaluate developmental toxicity and angiogenic activity. A human angiogenic antibody array was used to profile angiogenic proteins in the U251 GM cell line. A real-time reverse transcriptase polymerase chain reaction (RT PCR) assay was used to detect the differential expression of eleven HDAC genes in U251 cells treated with *C. procera* extract. The extract significantly reduced the proliferation of all three types of GBM cell lines and the cytotoxicity was found to be more pronounced in U251 GM cells, with an IC_{50} value of 2.63 ± 0.23 μg/ml, possibly by arresting the cell cycle at the G2/M transition. The extract did not exhibit toxic effects in zebrafish embryos, even at concentrations as high as 1000 μg/ml. The extract also inhibited angiogenic blood vessel formation in the transgenic zebrafish model in a dose-dependent manner. The results from the angiogenic antibody array have suggested novel angiogenesis targets that can be utilized to treat GBM. Real-time RT PCR analysis has shown that *C. procrea* extract caused an upregulation of HDAC5, 7, and 10, while the mRNA of HDAC1, 2, 3 and 8 (Class I HDACs), and HDAC4, 6, and 9 (Class II) were downregulated in U251 GM cells. The cytotoxicity of the *C. procera* extract on GBM cell lines could be due to its dual action by regulation of both tumor angiogenesis and histone deacetylases enzymes. Through this study, the *C. procera* leaf extract has been suggested as an effective remedy to treat GBM with minimal toxicity. In addition, various

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novel angiogenic and HDAC targets has been identified which could be helpful in designing better therapeutic strategies to manage glioblastoma multiforme in human patients.

1. Introduction

Glioblastoma multiform (GBM) is one of the major tumours of the central nervous system, accounting for more than 70 % of all brain and nervous system-related cancers [1]. In Saudi Arabia, brain and central nervous system tumours rank 15th among all occurring cancers, with an incidence rate of 5.39 new cases per 100,000 population [2]. This rate is higher than in other countries based on population density [3].

There are limitations to the efficacy of monotherapy to treat GBM due to toxicities, poor pharmacokinetics, and mis-regulated growth and survival pathways in GBM. Thus, it is rational to design a combinational treatment modality, representing an attractive approach to enhance care in patients with GBM. This study addresses the fundamental aspects of these needs. So far, two methods have shown promising results in treating GBM: epigenetic modulation via inhibiting histone deacetylases (HDACs) and targeting tumor angiogenesis.

Anti-angiogenesis therapy targeting VEGF has become the most effective technique for treating GBM [4]. Anti-angiogenic therapy drugs have been shown to improve the efficacy of conventional therapies in preclinical investigations. Several anti-angiogenic medicines have been tested in clinical trials as alternatives to standard cancer treatments [5,6]. Preclinical trial data also have suggested that these anti-angiogenic therapy drugs have improved the efficacy of conventional treatments through mechanisms other than blood vessel normalization in glioblastoma patients [7]. So far, “Bevacizumab” (an antibody against VEGF-A) has shown meaningful effectiveness in glioblastoma in controlled clinical trials [8]. However, several toxicities associated with Bevacizumab have been reported in glioblastoma patients [9]. These toxicities can be attributed to the inhibition of VEGF, impeding some vital functions associated with VEGF, such as blood vessel integrity, vascular homeostasis, clotting, wound healing, and kidney filtration, depending on VEGF activation [10]. Hence, more research is needed to explore other pro-angiogenesis targets besides VEGF to reduce toxicities. Crude extracts isolated from medicinal plants target multiple anticancer pathways, and are less toxic than synthetic anti-angiogenic molecules [11]. Hence, such crude extracts may offer the possibility of better GBM management.

Epigenetic regulation via histone modification are generally reversible and thus are attractive therapeutic targets. Histone deacetylase inhibitors (HDACi) are being developed for anticancer therapy. Several HDAC inhibitors have shown exceptional anti-glioblastoma properties by targeting numerous anticancer pathways, and many of them are in clinical trials, intended to be used as mono or combined therapy against GBM [12–14]. HDACi have been shown as potent anti-GBM medications in preclinical trials [15]. However, due to unwanted side effects and toxicities, most of these inhibitors could not be developed into therapeutics. HDACs are histone-modifying enzymes consisting of a large family of proteins; 18 different isotypes have been identified in mammalian cells. Surprisingly, different HDAC isoforms regulate specific cellular functions, and their expression is cell-type-dependent [16]. To design a better therapeutic strategy, finding the isoform-specific regulation of HDAC in glioblastoma is critical.

Zebrafish transgenic line TG (fli1:EGFP) is routinely used as in vivo angiogenic model to evaluate the angiogenic activity of crude extracts from medicinal plants [17,18]. The medicinal plant *C. procera* has been traditionally employed to treat various illnesses, including cancer [19]. *C. procera* is a member of the *Asclepidaceae* botanical family and is distributed natively across regions of Southeast Asia and Africa. The leaves of *C. procera* possess medicinal properties that make them helpful in treating various ailments such as sinus fistula, snake bite, rheumatism, burn injuries, mumps, and body pain [20,21]. The anticancer potential of *C. procera* has been reported in many human cancer cell lines. However, the angiogenic protein profiling and HDAC modulatory activity of this plant in glioblastoma cell lines are being reported for the first time through this study.

The cytotoxicity of *C. procera* leaf extract was evaluated in three GBM cell lines, namely U251GM, U87 GM and A1235. The angiogenic protein profiling was achieved using a human angiogenic antibody array. Real-time RT PCR was conducted to determine differential expressions of 11 HDAC isoforms in U251 cells treated with *C. procera* extract. The developmental toxicity and angiogenic activity of *C. procera* were evaluated in wild-type and transgenic zebrafish embryos.

2. Material and methods

2.1. Chemicals and reagents

The chemicals and solvents used in this study were all of molecular biology grade, including Methanol (cat # 322415 Sigma Aldrich Germany), SU 5614 (Item No. 17410, Cayman Chemical 1180, Mi 48,108 · USA), and Trichostatin A (Cat. No. 1406 Tocris Bioscience Minneapolis, Minnesota, 55,413, United States).

2.2. Preparation of plant extract

The *C. Procera* plant was collected in March 2022 from Wadi Hanifa (Riyadh, Saudi Arabia). The plant was authenticated in the herbarium of the Botany Department, King Saud University, Riyadh, Saudi Arabia. The plant was washed thoroughly with running tap water and then distilled water to remove all dirt and contamination. The leaves were removed, dried under shade and ground to powder. Sixty (60) grams of powder was used for extraction using the Soxhlet extraction method, essentially the same as reported

previously [17]. The extract was dissolved in molecular biology grade methanol to make a stock concentration of 10 mg/ml 50, 150, 300, and 500 μg/ml working concentration was diluted in embryos water to expose the zebrafish embryos. While extract was diluted in cell culture medium to prepare working concentration (0.5.1.0,2.5,5.0,7.0,10.0 μg/ml) to treat GBM cell lines.

C. procera is potentially a toxic plant, hence in order to assure safety, all procedures including drying, grinding, and extract preparation were done under a fume hood while wearing protective clothing and masks at all times.

2.3. Glioblastoma cell line culture

Three glioblastoma cell lines derived from human patients are used in this study. These cell lines are.

i) U87 MG American type culture collection (ATCC cat # HTB-14),
ii) U-251 MG (formerly known as U-373 MG cat # 09063001, European Collection of Authenticated Cell Cultures (ECACC)), and
iii) A1235 glioblastoma cell line originated from Aaronson S.A.; Mt. Sinai Medical Center; New York; USA [22].

U-87 MG (Uppsala 87 Malignant Glioma) is a commercially available epithelial cell line. In 1966, researchers at Uppsala University isolated this cell line from a 44-year-old male glioblastoma patient [23]. U87 MG harbors wild type tumor protein p53 (TP53) [24]. The U-251 cell line was originally derived in the Wallenberg laboratory in Uppsala, Sweden. The cell line was obtained from a male patient diagnosed with malignant astrocytoma [25]. This cell line contains mutated TP53 [24]. U87 MG and U251 cell lines were kindly provided by Mana M Alshehri, King Abdullah International Medical Research Center, King Saud Bin Abdulaziz University for Health Sciences, King Abdulaziz Medical City, Ministry of National Guard Health Affairs, Riyadh, Saudi Arabia.

The cell lines were cultured using Dulbecco's Modified Eagle Medium (DMEM), with supplementation of 10 % fetal bovine serum (FBS) and 1 % antibiotics essentially same as reported previously [26]. As these cells were intended for genomic studies (RT PCR and Western Blotting), thus all cell lines were screened for possible mycoplasma contamination and subsequently treated with mycoplasma elimination kit using the LookOut® Mycoplasma Elimination Kit (Cat # MP0030 Sigma-Aldrich), following the instruction manual.

2.4. Cell viability assays

The cells were grown in a 96-well plate in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum. Fifty thousand cells were seeded in each well with 200 μl of the media and incubated overnight at 37 °C in 5%CO_2 to let them adhere to the cell culture plate. The cells were treated with different concentrations (0.5, 1.0. 2.5. 5.0, 7.0, 10.0 μg/mL) of plant extract for 48 h.

The GBM cell lines were also treated also with Trichostatin A (TSA; HDAC inhibitor) to have comparative antiproliferation activity between *C. procera* and TSA (an HDAC inhibitor). Trichostatin A (Cat. No. 1406 Tocris Bioscience Minneapolis, Minnesota, 55,413, United States) was dissolved in DMSO to make stock concentration of 5 mM. Cells were exposed to serial dilution (1.0, 1.5, 3.0, and 5.0 μM) of TSA for 48 h.

The cell viability was assessed by CyQUANT™ MTT Cell Viability Assay (Cat # 13,154 Invitrogen ThermoFisher Scientific, Boston, Massachusetts USA) according to the manufacturer's instructions. The optical density was measured by a microtiter plate reader at 540 nm.

2.5. Cell cycle analysis

Cells were washed with PBS, then fixed in 70 % ice-cold ethanol t Cell cycle analysis in fix cells were done by using FxCycle™ PI/RNase Staining Solution (Catalog number: F10797 Thermofisher USA) by following the instruction provided by manufacturer. Cells were treated with Ribonuclease A, stained with propidium iodide, then analyzed on a BD FACSCalibur. Demarcation of cell cycle phases was performed using BD CellQuest software.

2.6. Differential expression of histone deacetylase (HDAC) genes in GB cell line

Real-time Reverse Transcriptase Polymerase chain reaction (qRT-PCR) was conducted to assess the relative expression of various HDAC isoforms in the U 251 MG cell line. This cell line was chosen based on the cell viability results by plant extract treatment. The U251 cells showed maximum level of sensitivity upon *C. procera* treatment. The cells were treated with IC $_{50}$ value (2.5 μg/mL) of plant extract. Total RNA from target samples and control cell lines was extracted with the QIAamp RNA mini kit (QIAGEN) according to the manufacturer's instructions. The cDNA was prepared with the Reverse Transcription System (Promega). Relative qRT-PCR for the expression was performed with SYBR Green on Applied Biosystems 7500 Fast Real-Time PCR System as reported [27].

The primers used to amply various isoforms of HDAC are tabulated as follows.

Gene Name	Forward Primer	Reverse Primer
HDAC1	CCAAGTACCACAGCGATGAC	TGGACAGTCCTCACCAACG
HDAC2	CAGACAAGCGGATAGCTTG	GGTTGCTGAGCTGTTCTGA
HDAC3	GAGCTGGACACCCTATGAA	ACTCTTGGTGAAGCCTTGc

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Gene Name	Forward Primer	Reverse Primer
HDAC4	CAAGCACCCCTCGTCACAG	TGCTGCTGGAACTGCTGC
HDAC5	GGGAACCATCCTTGGAAATC	GAACTGGGCATGGCTCTTG
HDAC6	CCGGAGGGTCCTTATCGTAG	GCGGTGGATGGAGAAATAGA
HDAC7	GGAACAGCAGCGACTGGC	GTCACCGCAGGAGCACTG
HDAC8	CCTAAAGTGGCCTCCATGG	CCGTCAATCAGGCATTGG
HDAC9	GAACTCTAAGCCAGATGGGG	GCCCACAGGAACTTCTGACT
HDAC10	CCTCTTAGATGGGATGCTGG	CCGAACAGCCACATCCAG
HDAC11	GCACACGAGGCGCTATCTTA	AAGGAAGTTGGGGAGGAAGA

2.7. Angiogenic protein profiler array

The Proteome Profiler Human Angiogenesis Array Kit (catalogue no. ARY007, R&D Systems) was used to assess the regulation of angiogenic proteins in U251 cell line upon treatment with *C. procera* leaf extract. Human Angiogenesis Array contain four nitrocellulose membranes each containing 55 different capture antibodies printed in duplicate. The U 251 cells were cultured in duplicate in 6 well cell culture plates. The cells were treated with *C. procera* leaf extract (2.5 μg/ml; IC_{50} value), Trichostatin A (1 μM), and Semaxanib (1.5 μM; IC_{50} value determined by conducting MTT cell viability assay). Each treatment was done separately. Mock (methanol 0.5 % V/V) treated cells served as negative control. The cells were treated for 48 h, and the total proteins were isolated using Mammalian Cell Lysis Kit (MCL1 Sigma-Aldrich). Protein isolates were quantified using Qubit Fluorometers (ThermoFisher Scientific USA) by following manufacturer instructions. Proteins were aliquoted and stored at −70 0 °C until use. The buffer and reagents in human angiogenesis array kit were re-constituted as illustrated in kit manual. The membranes were blocked in array buffer (provided in the kit) for 1 h at room temperature. 0.5 ml of array buffer were added to 1 ml of protein samples and then 15 μl of reconstituted antibody cocktail was added to each protein samples and incubated at room temperature for 1 h. The blocking buffer was removed from the membrane and membranes were incubated with antibody cocktail overnight at 4 °C on a rocking platform shaker. The membranes were washed three times for 10 min each with washing buffer (supplied in the kit). The membranes were incubated with diluted Streptavidin-HRP (supplied in the kit) for 30 min at room temperature. The washing solution was replaced with Chemi Reagent Mix and membrane were immediately exposed to X-ray film for 1–10 min. In order to have un-biased results, the membrane from treated and control samples were placed side by side during exposure.

The differential protein expression was analyzed by measuring the difference in pixel intensity of each protein spot in treated versus control samples. The pixel densities of the developed x-ray film were gathered then analyzed through MATLab using the protein Array tool developed by Ref. [28]. The pixel intensities were converted to graph using the Protein array tool.

2.8. In vivo developmental toxicity and angiogenesis assay

2.8.1. Animal

Zebrafish drug screening assays were used to evaluate the in vivo toxicity of the *C. procera* leaf extract. The Wildtype and Tg (fli1:EGFP) animal were obtained from the zebrafish international resource center (University of Oregon USA). The fish were maintained following local and international regulations regarding use of laboratory animals, as well as the zebrafish book [29].

2.8.2. Ethical/institutional review board (IRB) approval

This study does not involve the use of any human subjects. Zebrafish larvae less than of 5 days post fertilization were used which do not require IRB approval, according to European legislation (EU Directive, 2010/63/EU) described by Ref. [30].

2.8.3. Embryo treatment

Zebrafish embryos were collected by natural pairwise breeding of adult fish. The embryos were washed with distilled water, screened under a stereo microscope (Olympus) and any dead or unfertilized eggs were removed. The synchronous stage embryos were raised until shield stage (6 hpf; hours post fertilization) and were exposed to serial dilution (0.5–1000 μg/mL) of the plant extract in sterile 35 mm glass Petri dish in embryo medium. The embryos were examined under a dissecting microscope for lethality and the teratogenic profile was recorded and photographed using a Zeiss cell culture microscope. Almost 30 embryos were used for each type of treatment. The experiment was repeated three times using embryos from different batches.

2.8.3.1. In vivo anti-angiogenic activity assessment. The Tg (fli1:EGFP) embryos were treated with plant extract as indicated above. The anti-angiogenic activity of plant extract was quantified in zebrafish embryos by counting the number of inter-segmental blood vessels (isv) and sub-intestinal veins (siv) in control and treated embryos. At the end of treatment, larvae (72 h post fertilization) were photographed using Zeiss Observer Z1 fluorescence microscope with an Axiocam MRc5 camera. The number of angiogenic blood vessels were counted from 10 larvae at 72 h post fertilization, and anti-angiogenic activity was presented by the average value ± standard deviation.

The % anti-angiogenic activity was calculated by following equation.

$$\% \ anti - angiogenic\ activity = \frac{Average\ \#\ of\ isv\ \&\ SIV\ in\ 10\ treated\ embryos}{Average\ \#\ of\ isv\ \&\ SIV\ in\ 10\ control\ embryos} \times 100$$

ISV; intersegmental blood vessels, SIV; sub-intestinal vein.

2.9. Statistical analysis

The IC_{50} (in case of cell lines) and LC_{50} (zebrafish embryos) were calculated using Probit analysis as described previously [17]. Student's *t*-test was used to evaluate differences between two groups and one-way ANOVA to analyze differences among different groups. The 95 % confidence (p-value 0.05) was considered statistically significant between the two groups. Each experiment was repeated at least three times and data are presented as mean ± standard deviation (SD).

3. Results

3.1. Methanolic leaf extract of C. procera suppressed the proliferation of glioblastoma cell lines

Three human glioblastoma cell lines, namely U87 MG (WT p53), U51MG (mutant p53), and A1235 were used to check the cytotoxic activity of methanolic leaves extract of *C. procera*. The *C. procera* inhibited the viability of all tested GBM cell lines. The 50 % inhibitory concentration (IC_{50}) are shown in Table 1, and dose dependent effect on cell mortality is shown in Fig. 1. The cytotoxicity of the leaf extract was found to be more pronounced in U251 cells, with an IC_{50} value of 2.63 ± 0.23 μg/ml. This was followed by U87 cells (IC_{50} value of 3.23 ± 0.25 μg/ml) then A1235 cells (IC_{50} value of 4.90 ± 1.032 μg/ml). Trichostatin A (TSA), a known HDACi [31] was used as a positive control. The TSA inhibited the proliferation of GBM cell lines in a dose-dependent manner (Fig. 2). The IC_{50} values of TSA were 3.03 ± 0.34, 0.89 ± 0.58, and 2.94 ± 0.94 μM/ml for U251, U87, and A1235 cell lines respectively.

3.2. The methanol extract of leaves of C.procera induce cell cycle arrest in GBM cell lines

The human glioblastoma cell lines (U251, U87, and A1235) were treated with *C. procera* leaves methanol extract with a concentration equal to IC_{50} values for 24 h. The effect on cell cycle progression was monitored by flow cytometry, and the results are shown in Fig. 3. The *C. procera* methanolic extract of leaves induced a G2/M cell cycle arrest in all cell lines. The percent of G2/M cells in control U 251 cells was 18.5 (Fig. 3A), whereas the number of cells in the G2/M phase was increased to 28.4 % upon treatment with *C. procera* extract (Fig. 3B). Similarly, there were 26.6 % cells in the G2/M phase of mock-treated U87 cells (Fig. 3C), which increased to 32.8 % after treating the cells with *C. procera* leaves extract for 24 h (Fig. 3D). The percentage of A1235 cells in G2/M has risen from 21.2 % I (Fig. 3E) to 39.1 % (Fig. 3F) after treating the cell with *C. procera* extracts. All these results indicated that *C. procera* methanol extracts of leaves induced G2/M cell arrest in treated GBM cell lines.

Three types of glioblastoma cell lines were treated with methanol leaf extract of *C. procera* for 48 h and processed for flowcytometry analysis to detect the effect of extract on cell cycle progression. **A)** Mock control U251 cells **B)** U251 cells treated with 2.5 μg/ml of *C. procera,* **C)** Mock U87 cells **D)** U87 cells treated with 3.0 μg/ml of *C. procrea*, **E)** Control mock A1235 **F)** A 1235 cells treated with 5.0 μg/ml of *C. procera* leaf extract. The treatment dose was chosen base on the IC_{50} values as determined by cell viability assay.

3.3. In vivo developmental toxicity testing of C. procera in zebrafish embryos

Wild-type zebrafish embryos were exposed to serial dilution (50,100,150, 300, 500, and 1000 μg/ml) of methanol extract of leaves of *C. procera* from the zygote stage until 72hpf. The results are shown in Fig. 4. The developmental toxicity criteria such as lethality, developmental delay, hatching rate, hatching time, and teratological effects were monitored in mock (0.05 % methanol V/V) and *C. procera* methanol leaves extract treated embryos. The observation revealed that the plant extract did not induce any toxicity in exposed embryos and the morphological characteristics were identical to mock (0.05 % methanol) treated embryos. No lethality was observed in the treated zebrafish embryos even at the highest concentration used (1000 μg/ml) except severe developmental delay (Fig. 4 F).

Representative microphotograph of live zebrafish larvae at 72hpf. **A)** Mock control (0.05 % v/v methanol) larvae showed normal pigmentation and embryonic development at 72hpf. **B, C, D, E)** WT zebrafish larvae exposed to 50, 150, 300, and 500 μg/ml C. *procera* leaf extract. Zebrafish developmental staging criteria by Ref. [32] was adopted to check whether the extract induced any developmental delay. Mock (methanol) and *C. procera* extract-treated embryos had protruding mouths (black arrows) and pectoral fins,

Table 1
Cytotoxicity evaluation of *C.procera in* Glioblastoma cell lines.

Sample	IC50 values (μg/ml)		
	U251	**U87**	**A1235**
C. procera	2.63 ± 0.23	3.23 ± 0.25	4.90 ± 1.03
TSA	3.03 ± 0.34[a]	0.89 ± 0.58[a]	2.94 ± 0.94[a]

[a] IC_{50} in μM (TSA as pure compound).

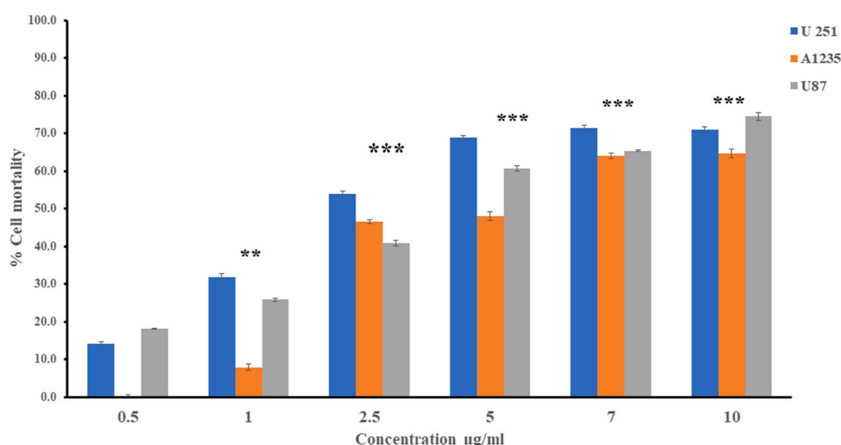

Fig. 1. *C. procera* leaf extract inhibited the growth of Glioblastoma cell lines Three Glioblastoma cell lines (U87 GM, U251 GM and A1235) were treated with serial dilutions (0.5.1.0,2.5,5.0,7.0,10.0 μg/ml) of *C. procera* methanol extract for 48 h. The cell viability was evaluated by MTT cell viability assay. The bars represent the average value of three replicates and error bars are ± standard deviation between the replicates * denotes the degree of statistic difference between plant extract treated and mock control (methanol 0.5 % V/V) cells., **p value = 0.01, ***p value = 0.001.

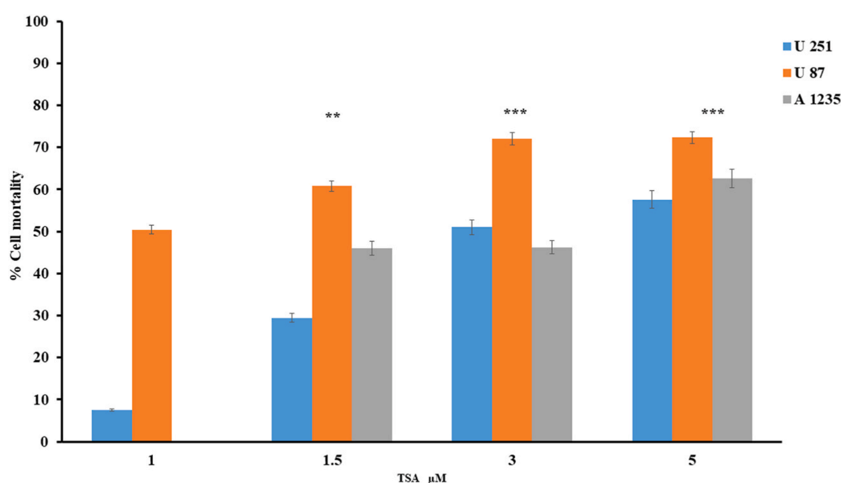

Fig. 2. The antiproliferation potential of Trichostatin A (TSA) in GBM cell lines TSA was used as positive control to compare the effect of *C. procera*, and TSA on cell viability of Glioblastoma cell lines. Three Glioblastoma cell lines (U87 GM, U251 GM and A1235) were treated with serial dilution (1.0, 1.5, 3.0 and 5 μg/ml) of TSA for 48 h X-values represents the concentration of extract and Y-values are percent cell mortality compared with control (mock treated cells). The bars represent the average value of three replicates and error bars are ± standard deviation between the replicates * denotes the degree of statistic difference between plant extract treated and mock treated cell lines. **p value = 0.01, ***p value = 0.001.

showing that there is no development delay induced by the extract. **F)** WT zebrafish embryos exposed to 1000 μg/ml of *C. procera* extract induced severe levels of developmental delay and retarded growth in exposed embryos. The embryos showed severe development delay. The embryos were at in 14–15 somite stage (16 hpf) after 3 days.

3.4. In vivo anti-angiogenic activity of C.procera extract in Tg (fli1:EGFP) embryos

The embryos from TG (flil1:EGFP) were exposed to serial dilution (100, 300, 500 μg/ml) of *C. procera* extract at the shield stage (6 h pdf), and the effect on angiogenesis blood vessel formation was evaluated at 72hpf (the stage at which intersegmental and sub-intestinal vein have completely developed in zebrafish larvae). The developmental toxicity screening (previous section) in wild type zebrafish embryos has indicated that 300 and 500 μg/ml doses did not induce toxicity; and hence transgenic zebrafish embryos were treated with 300 and 500 μg/ml, in order to get dose dependent effect on angiogenesis. The embryos remained exposed to *C. procera* extract up to 72 h. The mock (methanol solvent only) and plant extract treated larvae were photographed using a fluorescent microscope and the number of angiogenic blood vessels were counted. The results are presented in Fig. 5 and Table 2. As indicated in Fig. 5 (A & A′), the angiogenic blood vessels including inter-segmental vessels (isv), and sub-intestinal vein formation and development was not affected in mock (methanol) control embryos. The SIV in control larvae are formed as a network of 10–12 small blood vessels

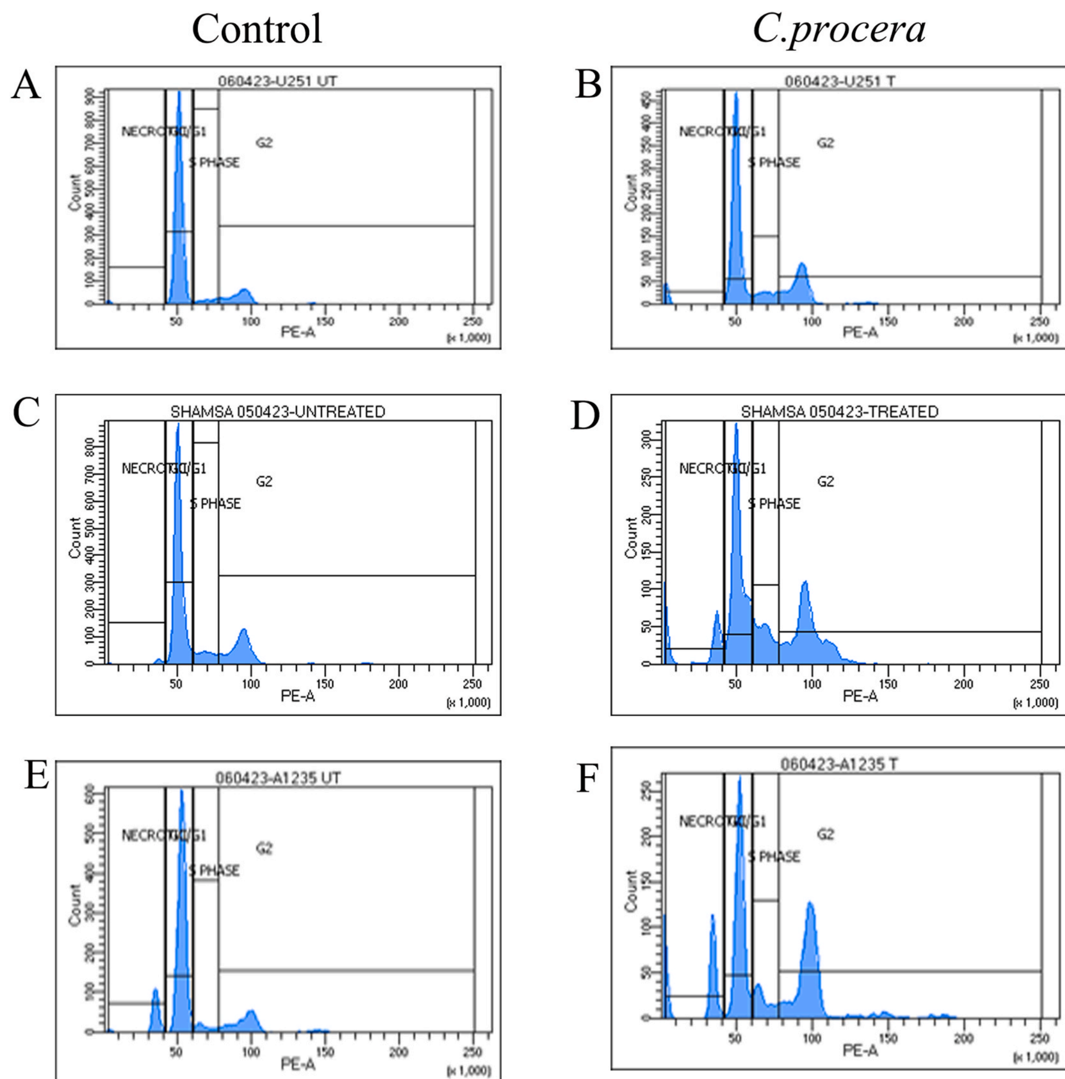

Fig. 3. *C. procera* methanol extract of leaves induced cell cycle arrest in GBM Cells.

as shape of a basket on the yolk region (white arrow in 5A′). *C. procera* leaf extract induced anti-angiogenic activity in a dose-dependent manner in Tg (fli1:EGFP) embryos, generally the sub-intestinal veins were observed to be more affected as compared to inter-segmental blood vessels, that could be due to the fact, that the *C. procera* take longer time to exert the activity. The ISV developed at around 48 hpf, but the sprouting of SIV are completed at 72hpf, hence the antiangiogenic effect on the development of ISV in treated embryos are less pronounced. The embryos treated with 100 μg/ml of leaf extract have shown weakest level of antiangiogenic activity as only 5 ± 1.3 % inter-segmental blood vessels (isv), and 15 ± 1.5 % sub-intestinal vein (siv) were affected in embryos treated with 100 μg/ml extract (Fig. 5B & B′), however, the SIV in treated embryos were much smaller in size and were not connected to each other. A significant (70 ± 0.1.8; p value 0.01) reduction in ISV were seen in zebrafish embryos treated with 300 μg/ml extract. The same embryos were completely devoid of SIV (Fig. 5C &C′). However, at higher concentration (500 μg/ml), *C. procera* leaf extract exhibited the maximum level of antiangiogenic activity by inhibiting 100 % of ISV and SIV (Fig. 7 D and D′).

Zebrafish angiogenesis transgenic line Tg (fli1:EGFP) were treated with serial dilution of *C. procera* leaf extract or solvent (methanol 0.5 % V/V) for 3 days. The images of live embryos at 72 hpf were recorded using Zeiss fluorescent microscope under appropriate filter. A & A′) Mock control (A′ is the same embryo at higher magnification). B-D) TG (flli1:EGFP) larvae at 72hpf treated with 100, 300 and 500 μg/ml C. *procera* leaf extract. The extract inhibited the formation of angiogenic blood vessels in a dose dependent manner and the majority of angiogenic blood vessels (both ISV and SIV) did not form in embryos treated with 500 μg/ml extract. White arrows indicate the angiogenic blood vessels. isv (inter-segmental blood vessels), siv (sub-intestinal vein). B′-D′ same embryos at higher magnification.

3.4.1. Semaxanib and trichostatin a induced severe developmental abnormalities in zebrafish embryos

Tg (fli1:EGFP) embryos were treated with 1 μM Semaxanib and TSA (20 μM) as a positive control. The data is presented in Fig. 6. No

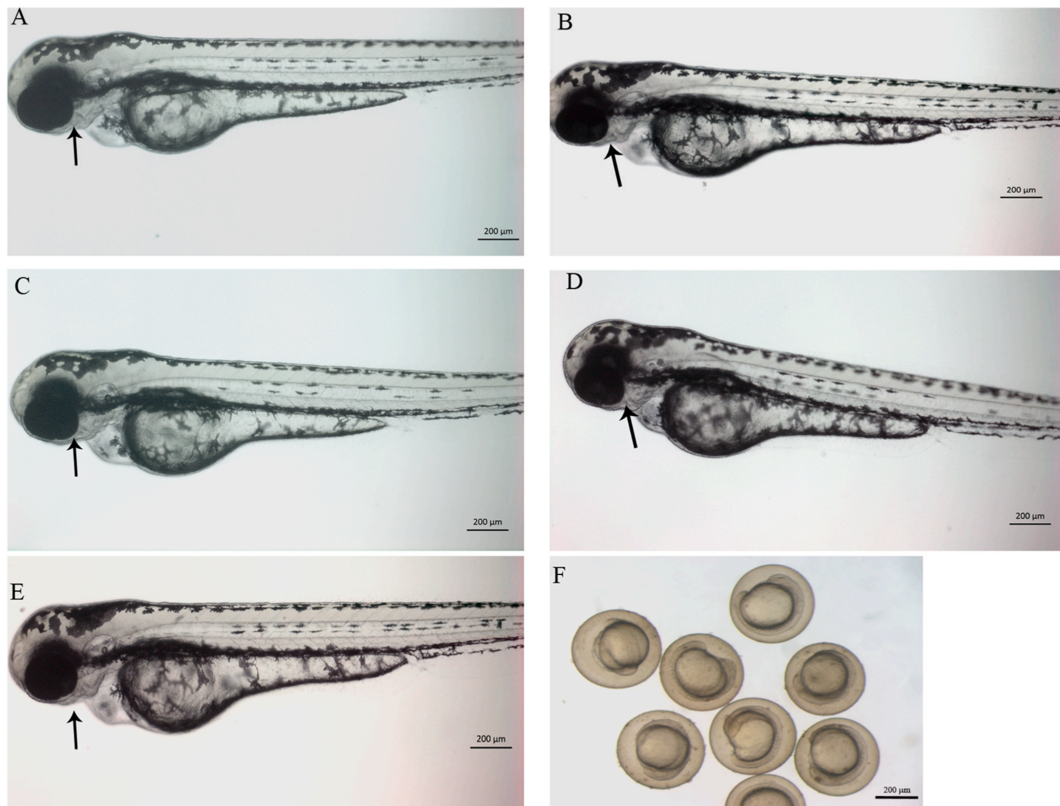

Fig. 4. In vivo developmental toxicity screening of *C. procera* methanol extract of leaves in zebrafish embryos.

teratogenic effect and the abnormal embryonic growth were observed in control (mock treated) larvae shown in bright field (Fig. 6A) moreover, the control larvae did not show any defects in blood vessel development. The inter-segmental blood vessels (isv) branched from the dorsal aorta and connected to dorsal longitudinal anastomotic vessel (DLAV) and similarly the sub-intestinal vein (SIV) number and development was not affected (Fig. 6 B& C). Semaxanib (1 μM) interrupted ninety percent (90 %) of intersegmental blood vessels and 100 % of the sub-intestinal vein in treated transgenic embryos (Fig. 6E and F). However, Semaxanib induced severe embryonic abnormalities in treated embryos, as shown in Fig. 6D. Severe developmental delay, cardiac edema, cardiac hypertrophy, and curved bodies were the results of Semaxanib treatment in zebrafish embryos. The treated embryos did not survive beyond 120 hpf. TSA was chosen as a positive control to represent the comparative effect between *C. procera* as HDAC inhibitor and a known HDAC inhibitor on zebrafish development and angiogenesis. As shown in Fig. 8 (G –I), TSA also inhibited the formation of intersegmental and sub-intestinal blood vessels. The intersegmental blood vessels did not grow enough to connect to the dorsal longitudinal anastomotic vessel (DLAV). Moreover, TSA also induced severe embryonic abnormalities in treated embryos, represented by severe body curvature, cardiac edema, cardiac hypertrophy, and lack of pigmentation (Fig. 6D).

Photomicrograph of live Tg (fli1:EGFP) larvae at 72hpf. **A)** Control (mock treated) larvae shown in bright field at 72hpf. It is evident from this image that methanol (as solvent 0 did not induce any teratogenic effect and the larvae development was not affected. **B &C** shows the image of larvae taken by fluorescent microscope under FITC filter under different magnification. The blood vessels are shown by green color. The control larvae did not show any defects in blood vessel development. The inter-segmental blood vessels (isv) branched from the dorsal aorta and connected to dorsal longitudinal anastomotic vessel (DLAV). The sub-intestinal vein (siv) are branched and arrange as basket shape on yolk sac. **D)** The bright field image of live Tg (fli1:EGFP) larvae at 72hpf treated by semaxanib (1 μM). The semaxanib induced severe level of heart defects such as cardiac edema (CE), and cardiac hypertrophy. The body of treated larvae were curved. **E & F)** The semaxanib interrupted the development of inter-segmental vessels (isv). Majority of isv fail to form, and those which were formed did not develop such that to connect to DLAV. The sub-intestinal vein did not form in 100 % ($n = 35$) of treated larvae. **G)** The brightfield image of Tg (fli1:EGF) larvae at 3dpf treated with trichostatin A (TSA). The teratogenic defects induced by TSA were very severe. The larvae showed sever developmental delay and body curvature, heart defects and much smaller body size. These embryos could not survive beyond 3dpf. **H&I)** The TSA at 20 μM significantly interrupted the formation of inter-segmental (isv) and sub-intestinal (siv) angiogenic blood vessels formation and development.

3.5. Angiogenic protein profiling of U251 cells by C. procera extract

The U251 cells were treated with *C. procera* leaves methanol extract for 24 h, and then total proteins were isolated, and

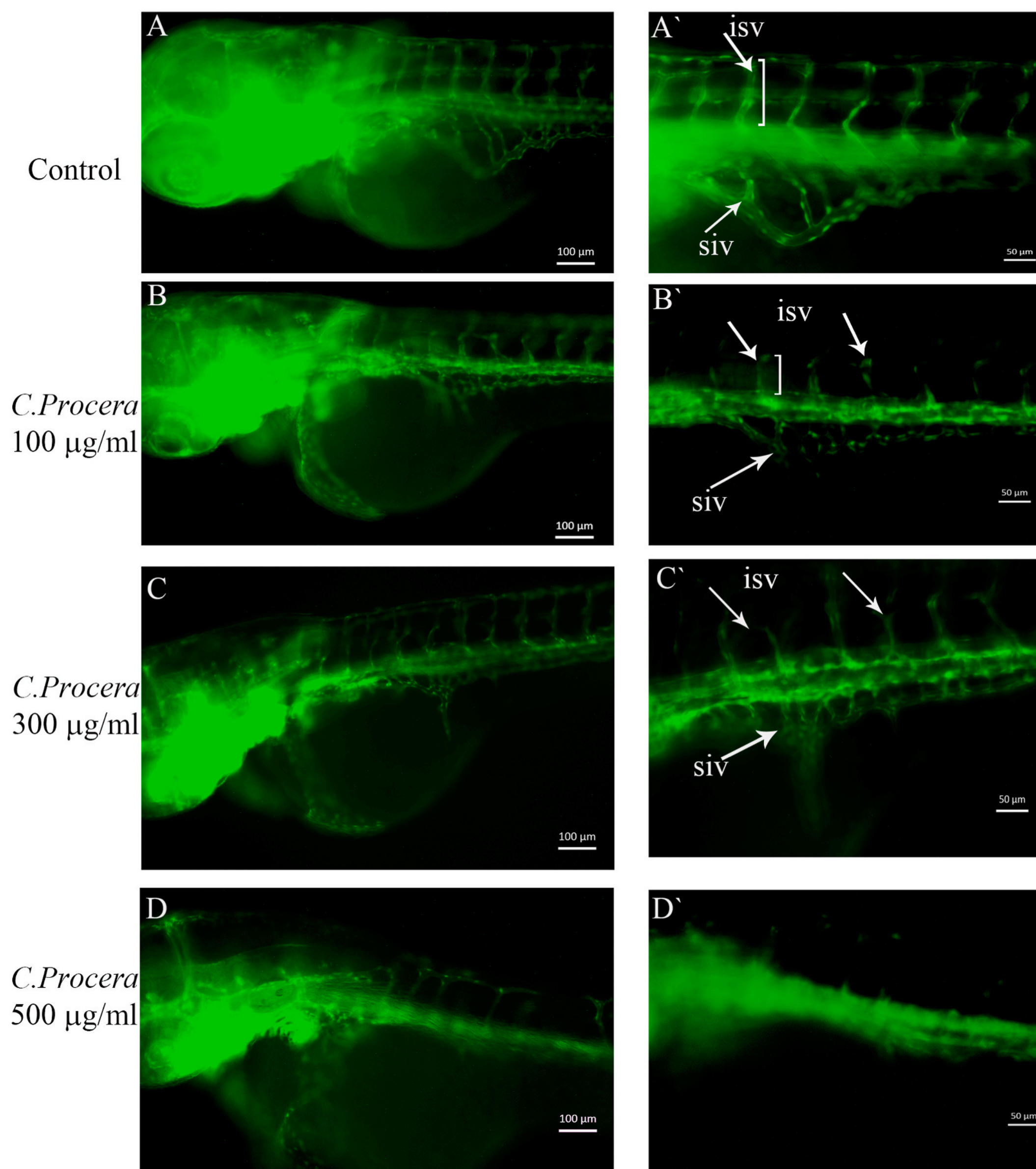

Fig. 5. In vivo anti-angiogenesis activity of *C. procera* leaf extract in Tg (fli1:EGFP) embryos at 72hpf.

Table 2
Anti-angiogenic activity of *C.procera* in Tg (fli1:EGFP) model at 72hpf.

Concentration (μg/ml)	% Anti-angiogenic activity (% blood vessels inhibited)	
	ISV[a]	**SIV**[a]
Control	0	0
100 μg/ml	5 ± 1.3	15 ± 1.5
300 μg/ml	70 ± 0.1.8	100 ± 0.0
500 μg/ml	100 ± 0.0	100 ± 0.0

[a] Mean values from 10 larvae ± standard deviation.

angiogenesis protein profiling was done using a human angiogenesis antibody array. DMSO (0.5 % V/V) treated cells were used as negative control. In order to identify common angiogenic proteins targets between *C. procera* leaf extract, a known angiogenesis inhibitor, and HDAC inhibitor, the U251 GM cells were also treated TSA (potent HDAC and angiogenesis inhibitor) [33], and semaxanib (potent VEGF inhibitor) [34]. The comparative angiogenesis protein profiling of U251 cells is presented in Fig. 7 A-D. The result clearly

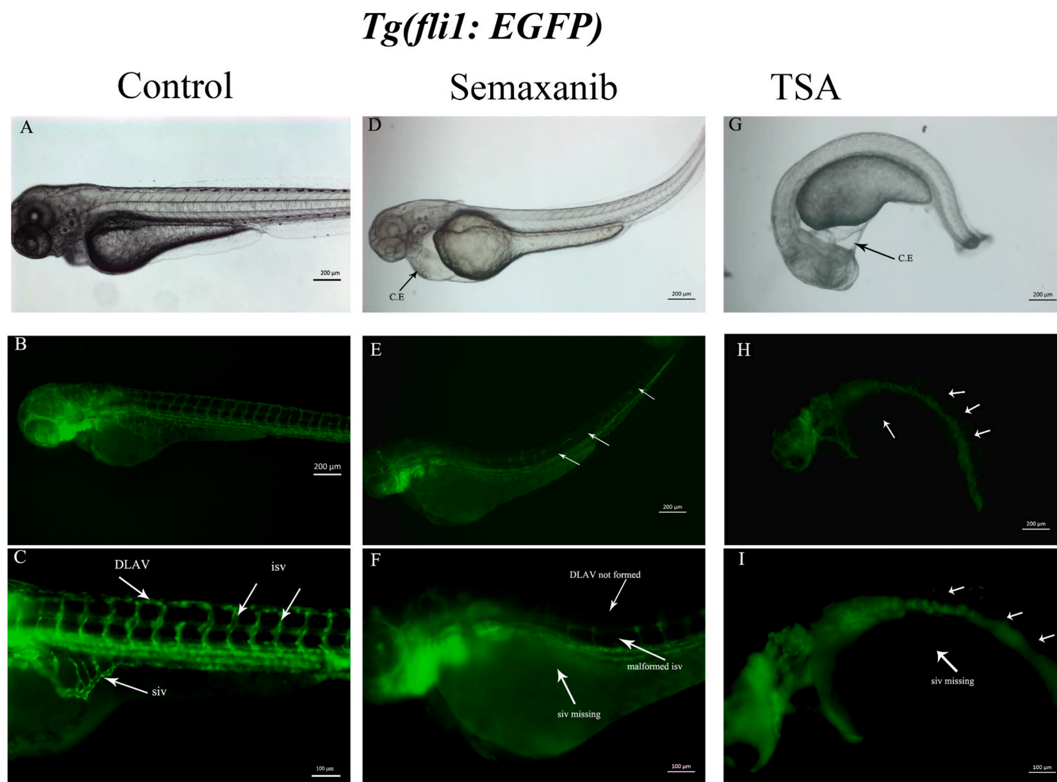

Fig. 6. The semaxanib and Trichostatin A exert an anti-angiogenic effect and also induce several embryonic abnormities in Tg (fli1:EGF) embryos.

shows that *C. procera* leaves extract regulated multiple angiogenesis related proteins in U251 cells same like TSA and semaxanib. The *C. procera* extract downregulated the expression of pro-angiogenic proteins, such as VEGF, Activin A, Coagulation Factor III, Serpin E1, FGF basic, uPA, Endothelin, and insulin-like growth factor binding protein two (IGFBP-2**)**. The ADAMTS1, angiogenin, angiopoietin-1, endothelial growth factor (EGF), and various forms of fibroblast growth factor (FGF), VEGF, were significantly affected by TSA. The semaxanib downregulated the expression of VEGF C, TIMP4, IL8, MMP8, FGF, CXCL1,6, and angiogenin.

The U251 GB cells were exposed to *C. procera* leaf extract (2 μg/ml) for 48 h. Total protein was isolated and processed for angiogenic antibody array. The difference in protein expression between control and treated groups was analyzed by measuring the pixel intensities of images by MTLAB protein array software. **A)** Control methanol treated cells. **B)** *C. procera* extract 2 μg/ml **C)** Trichostatin A (3.0 μM), **D)** Semaxanib (1 μM). The protein array coordinates and graphs (in higher magnification) are presented in supplementary material (Supp Fig. 1 and protein array coordinates).

3.6. C. procera methanol extract of leaf affected the mRNA expression of histone deacetylase (HDAC) belonging to class I, II and VI in glioblastoma cells

Real-time RT PCR was used to evaluate the effect of *C. procera* extract on the expression of different isoforms of HDAC family members in U251 GM cells. The 11 human HDAC isoforms belonging to different HDAC classes were chosen for this study. As shown in Fig. 8, the mRNA of HDAC1,2,3 and 8 (Class I HDAC) were downregulated several folds in treated cells as compared to control cells. Similarly, the HDAC 6 and 9 (class II b) were also downregulated. The expression of HDAC11, which belongs to class IV, was upregulated (4.5 fold) in treated U251 cells. HDAC5 and HDAC7 were also upregulated by 1.6 and 2.7-fold, respectively.

U251 cells were treated with 2.5 μg/ml of methanol leaves extract of *C. procera* for 24 h. HDAC mRNA was analyzed by real-time RT PCR. The error bars represent the Standard deviation of Ct values between three replicates.

4. Discussion

Despite substantial investments in anti-GBM drug discovery over the last decade, progress has been limited to preclinical stages, with clinical studies frequently encountering obstacles [35]. Anti-angiogenesis therapy targeting VEGF was believed to be the most effective treatment for GBM, due the extensive vascularization nature of GBM [4]. Moreover, various HDAC inhibitors have shown anti-proliferative activities in preclinical studies [13,36]. However, recent research data has indicated that monotherapies targeting VEGF and pan HDAC inhibitors have caused unwanted side effects in GBM patients.

The leaf methanol extract of *C. procera* showed significant anticancer activity with minimal IC_{50} values against three types of GBM

Fig. 7. Antiangiogenic protein profiling related to C. procera in GBM cells.

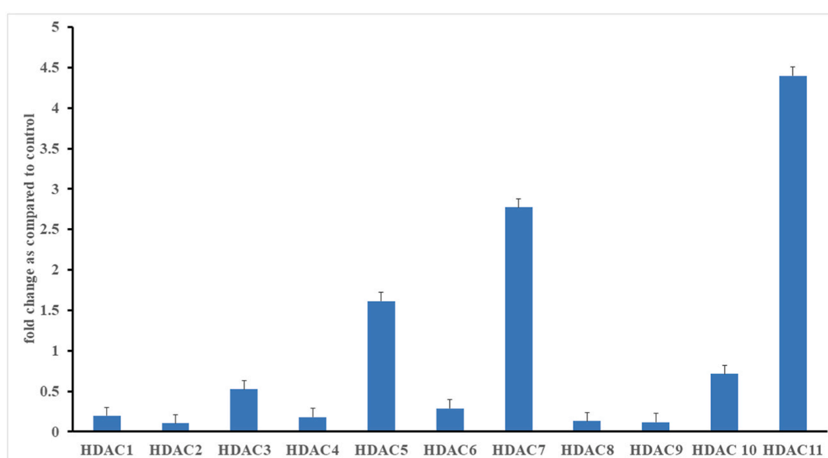

Fig. 8. Differential expression of HDAC isoforms regulation by *C. procera* in U251 cell line.

cell lines in this study. The scientific literature regarding the anticancer potential of *C. procera* against glioblastoma is scarce. The cytotoxicity of calotropin in A172 and U251 glioblastoma cells has been reported quite recently [37]. Calotropin is a pharmacologically active substance discovered in milkweed plants like *C. procera* and *C. gigantea*. However, the crude extract of *C. procera leaf* has

been used in this study, which has advantage over single compound. Crude extrct contain multiple bio-actively ingredients which are responsible to exerted a synergetic effect by targeting multiple anticancer pathways. The cytotoxicty of *C. procera* leaf extract was more profound in U 251 cells which harbour the TP53 mutation, while U87 contain wild-type TP53 [24]. Glioblastoma with tumor protein p53 (TP53) mutations have unfavourable prognosis and showed resistance to traditional chemoradiotherapy. Hence, targeting TP53 presents a promising strategy to address the poor therapeutic response in glioblastoma [24]. The preferential cytotoxicity of *C. procera* leaves crude extract towards U251 cells (this study) and Calotropin [37] indicates its beneficial therapeutic effect towards TP53 mutated glioblastoma.

The anti-cancer property of *C. procera* against many cancers has been shown by in vitro and in vivo studies, for example hepatocarcinogenesis [38,39], human skin melanoma cells (SK-MEL-2) [40], human breast carcimona cell line MDA-MB-435 [41].

A G2/M cell cycle arrest was observed in treated U251 GM, U87GM and A1235 GBM cell lines in this study which are in agreement with other published studies in which G2/M cell cycle arrest by *C. procera* were reported in colon, breast, melanoma and liver cancers [40,42,43]. The perentage of U251 GM, U87GM and A1235 GBM cells arrested in G2/M phase in (this study) were in accordance with published results in colon, breast, melanoma and liver cancers.

Regarding the safety profile, the leaf methanol extract of *C. procera* did not induce lethality in zebrafish embryos up to 1000 μg/ml concentration. A developmental delay was observed in those embryhos which were treated with 100 μg/ml. The embryos were at 14–15 somite stage even after 3 days, which usually appeared after 16 hpf [32]. There are contradicting reports regarding the safety profile of *C. procera* in various experimental animals. The majority of the literature indicated this plant as safe while some have reported toxic effects in tested animals. The *C. procera* leaves ethanol extract was reported to be non-toxic up to a dose of 5000 mg/kg body in rodents [44,45]. Severe genotoxicity and hepatotoxicity by *C. procera* latex in rats and sheep have been reported by another study [46].

The use of angiogenic inhibitors has demonstrated encouraging outcomes in treating solid tumours. Various natural products have been tested in clicinal trials to target the angiogenesis process [47]. The angiogneic activity of *C. procera* has been documented previously by chicken egg chorioallantoic membrane (CAM), and Sponge implantation in mice [48], however this is the first study reporting the anti-angiogenic activity of *C. procera* in zebrafish embryos. The main advantage of using zebrafish embryos as an angiogenic model is that the effect of testing compounds or extracts can be evaluated in live embryo, and embryonic development is considered an alternative of tumor developmental processes. Whereas the in CAM and Songe implantation assays, the angiogensis is induced ectopically, which is not a natural process and hence results from CAM and Songe implantation assays require re-evaluation. Semaxinib is a non-selective inhibitor of receptor tyrosine kinases, and is known to have a high affinity binding with VEGF receptors. Semaxanib has demonstrated efficacy in clinical trials for treating acute myeloid leukaemia and colorectal carcinoma [49]. Trichostatin A (TSA), which is a known HDAC inhibitor has been reported to block angiogenesis in various models of cancer progression including GBM [50]. Both Semaxinib and TSA blocked angiogenic blood vessels in developing zebrafish embryos more efficiently than *C. procera* leaf extract, however, they also induced severe toxicity and high levels of mortality. We have not come across any study in which a comparative antiangioenic activity of *C. procera* extract has been compared with known angiogeneis inhibitor or HDAC inhibitor, and hence this study has advantage over other published resutls as this study indicate the safety prolife of three chemotherpeutic choices using in vivo-developmental toxicity assays.

The angiogenic protein profiling in U251 GM cells treated with *C. procera* leaf extract was compared with angiogenic protein profiling of Semaxanib and TSA as positive controls. Beside identifying the angiogenic protein regulated by *C. procera*, the results from this assay also suggested novel angiogenesis targets that can be utilized to treat GBM. Several angiogenesis proteins explicitly regulated by *C. procera* extract in U251 cells were identified. The *C. procera* extract downregulated the expression of VEGF, Activin A, Coagulation Factor III, Serpin E1, FGF basic, uPA, Endothelin, and insulin-like growth factor binding protein (IGFBP-2). The function of these angiogenic proteins has been demonstrated in several cancers. As it is difficult to discuss the function of all proteins, we will limit our litrature review to those proteins that were found to be downregulated in U251 cells as compared to control cells by *C. procra* treatment.

A study conducted by Zhang et al. [51] has observed significantly elevated levels of Activin A in glioblastomas compared to normal brain tissue, which resulted in a significant increase in the proliferative index of U87 cells as compared to the control group. The potential involvement of the imbalance between Activin A and follistatin has been suggested in the pathogenesis of glioblastoma. The activation of Activin A initiates angiogenesis by regulating the expression of VEGFA, and overexpression of Activin A has been found to be correlated with an unfavourable prognosis in cases of oral squamous cell carcinoma [52]. Transcription factor (TF), which is alternatively referred to as coagulation factor III, thromboplastin, or CD142, is a transmembrane glycoprotein with a molecular weight of 47 kDa [53]. The role of TF in glioblastoma angiogenesis or treatment has yet to be discovered. One study has reported higher TF expression in glioblastoma in tumor biopsies obtained from patients [54]. It is now well-known that tissue factor, aside from its function as a procoagulant activator, plays a significant role in various tumor-associated mechanisms, including angiogenesis [55] and also it is suggested that, therapeutics that specifically target transcription factors may have wide clinical applications in tumor treatment [56]. These proteins can be evaluated as efficient anticancer target for other solid cancers.

Another therapeutic stratagey which has produced meaningful results in GBM is the use of HDAC inhibitors [57]. Several medicinal plants and natural products possess HDAC inhibitory activity [58]. Eighteen (18) mammalian isoforms of HDAC have been cloned so far and each has been attributed to distinct types of cellular function and cancer types [59]. Class I HDACs comprises of HDAC1, 2, 3 and 8, whereas HDAC4, 5, 6, 7, 9, and 10 belong to HDAC Class II [60]. The differential expression of HDAC isoform analysis between glioblastomas and low-grade astrocytomas and normal brain tissue in a cohort of forty-three microdissected tumor samples obtained from patients and 11 normal cerebral tissue samples has shown a significant downregulation of class II and IV HDACs in, and upregulation of HDAC9 protein level in GBM as compared to noraml brain tissues [61]. Similarly the overexpression of HDAC1/2, along

with downregulation of HDAC11 in cancerous GBM tissues has been reported [62].

The results from the real time RT PCR in this study have indicated that *C. procrea* extract caused an upregulation in class II and IV HDACs (HDAC 5, 7 and 10), and downregulation in HDAC4, HDAC6, and HDAC9 mRNA in U251GM cells, which indicated that *C. procera* extract does not merely act as HDAC inhbitor, rahter it's functions was a regulator of HDAC expression. It does so, by downregulating those HDAC isoforms which were upregulated in glioblatoma tumor, as compared to normal brain, and enhanbced the expression of those HDAC isoforms which are depleted in Glioblastoma tissue. This could be due to the presence of multiple bioactive molecules in the crude extract, and a synergetic action of these molecules target multiple anticancer pathways. The chemical characterization of the leaf extract of *C. procera* has indicated the presence of angiogneic regulator and biologically active compounds (data will be reoprted elsewhere).

HDAC11 belongs to class IV of HDAC, and is the least studied member of the HDAC family. We have observed over-expression of HDAC11 in U 251 GM cells which were treated by *C. procera* leaf extract. Quite recently, one study has linked the low expression of HDAC11 to poor prognosis, and proposed HDAC11 as potential biomarker in GBM patients [62].

The main strength of this study is that C. procera leaf extract shows the toxicity specifically towards glioblastoma cells and did not induce toxicity in zebrafish embryos. This indicates that the extract is not toxic to normal (non cancer) cells. Most of the currently used anti-cancer chemotherapies not only kill the tumor cells but also destroyed adjacent normal cells, and these medications are not favorable for cancer treatment. A drug or formulation which induce toxicity selectively in tumor cells with minimal or least toxicity towards non cancer cells is highly desirable. The second strong point for this study is the data presented in this study shows that C. procera leaf extract did not work exclusively as "Pan HDAC inhibitor" but rather, it regulated the expression of HDAC isoforms and on angiogenesis. It had a positive effect on anti-angiogenic proteins expression and simultaneously, downregulated the pro-angiogenic proteins. The third point is that this extract possesses the quality to target two important anti-cancer targets i.e. epigenetic regulation via HDAC regulation and angiogenesis, in single formulation which eliminated the need to apply multiple chemicals for such purpose. The weakness of this study is that further experimentation is required to evaluate the efficacy of the extract in in-vivo models of GBM to determine its potential as a therapeutic agent in humans.

5. Conclusion

A dual therapy or combined therapy approach has demonstrated favorable outcomes in both clinical and preclinical settings, resulting in notable benefits for cancer patients. Glioblastoma has emerged as a significant global health concern, necessitating increased focus from researchers and clinicians to develop more effective therapeutic strategies. The extract derived from the medicinal plant *C. procera* leaves has demonstrated the ability to impede growth in three distinct GBM cell lines. This inhibitory effect is achieved through targeting multiple anti-cancer pathways, regulation of apoptosis, cell cycle arrest and modulation of tumor angiogenesis and epigenetic mechanism via HDAC enzyme activity.

Overall, this study suggests the *C. procera* leaf extract as an effective remedy to treat GBM with reduced toxicity. In addition, various novel angiogenic and HDAC targets have been identified which could be helpful in designing better therapeutic strategies to manage GBM in human patients.

Author contribution statement

Almohannad Baabbad: Writing – review & editing, Writing – original draft, Methodology. Mohammad Ahmed Wadaan: Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Conceptualization. Muhammad Farooq khan: Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. Anas Alazami: Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization. Shamsa Hilal Saleh Alanazi: Writing – review & editing, Writing – original draft, Methodology, Data curation

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24406.

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