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Anti-proliferating effect of *Ocimum sanctum* and *Centella asiatica* plant extract on growth of human glioblastoma cells: An *in vitro* study

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Glioblastoma is aggressive brain tumour with poor prognosis with conventional chemotherapy, hence there is need to find alternative targets for developing newer treatment. Advent of new treatment methods involving medicinal plants have shown to reduced Cancer mortalities and prevents development of drug resistance for chemotherapy. Present study aimed at investigates the anti-proliferating activity of two promising medicinal plants, *Ocimum sanctum* and *Centella asiatica*. We studied the effect of their plant extract on U87MG Glioblastoma cells proliferation, survival effect and apoptosis. Cytotoxic activity was assessed, after the plant extract treatment on U87MG using MTT assay with dose of 1 mg/mL to 25 mg/mL and apoptosis assess was done using Annexin V assay with the three dose (1.5 mg/mL, 2 mg/mL and 2.5 mg/mL). Survivin gene expression was studied using QRT-PCR (Rotar gene Q, Qiagen) has a marker of proliferation. *Ocimum sanctum* and *Centella asiatica* treatment of U87MG cells with dosage of 1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL showed increase in mean apoptotic cells 2.8 %, 4.9%, 10 % and 3.1%, 5.8% and 7.2%, respectively, compared to untreated U87MG cells. Survivin gene analysis of U87MG cells showed down-regulation in gene expression and differences was significant in comparison to untreated control group with both the plant extract, *Centella asiatica* showed more down-regulation (97% with 2.5 mg/mL) than *Ocimum sanctum*. *Ocimum sanctum* and *Centella asiatica* exhibited promising anti-proliferating activity and induces apoptosis by down regulation of survivin gene expression.

Keywords: Anticancer, Cytotoxicity, Eugenol, Medicinal plant, Survivin, U87MG

Cancer is a disease characterized by uncontrolled cell growth caused by genetic and epigenetic alteration leading to reprograming of the cell causing loss of control over cell division[1]. Cancer cells have certain key properties like inefficient apoptosis of cancer cells which is main contributing factor of accumulation of genetic aberrations. Inefficient apoptosis is caused either due to defect in p53 pathway or over-expression of Inhibitors of apoptosis proteins (IAP) family members. IAP family proteins are important for development of gradual resistance against cancer treatment[2]. Development of resistance to cancer chemotherapy is one of the reasons for poor prognosis. Glioblastoma (GBM) is the second most common brain tumor and most aggressive tumors of the brain gilia cells[3,4]. About 3 per 100,000 people develop the GBM per year[5]. GBM carries worst 10 year survival rate of 0.7% forcing the oncologist to administered palliative treatment which includes surgery, chemotherapy and radiotherapy to improve quality of life and to achieve a longer survival time[6]. These conventional treatment methods are not always effective and they also exert severe side effects[7], therefore, alternative treatment methods need to be explored along with improved understanding of molecular and cellular biology of GBM to develop new targeted treatments methods to treat GBM effectively. New treatment methods involving medicinal plants have shown promising results in reducing Cancer mortalities and controlling cancer symptoms. Most of the existing medicine are derived

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Abbreviations: DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate-buffered saline; FITC, Fluorescein isothiocyanate; GBM, Glioblastoma multiforme; IAP, Inhibitors of apoptosis proteins; MAMC, Maulana Azad Medical College; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NK cells, Natural Killer cells; Q-PCR, quantitative PCR

from plants source and accounts for more than 50% of natural sources, thus investigating plants provides for effective medicines in cancer treatment[8]. Medicinal plants are documented for their immunomodulating properties of NK cells and enhancing their anti-malignant effects[9]. Medicinal plants from India such as *Ocimum sanctum* are known to have chemicals like Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), which are largely researched for their therapeutic use[10]. Another medicinal plant *Centella asiatica* also contain various biologically active substances with potential therapeutic application in humans and it is widely distributed throughout India, growing in moist places up to an altitude of 1800 M[11]. Hence the present study was designed to investigate the potential anti-malignant effect of *Ocimum sanctum*, and *Centella asiatica* plant extracts using U87MG glioma cells.

Material and Method

Present study is an *in vitro* study of anti-tumor activity of plant extracts (*Ocimum sanctum* and *Centella asiatica*) on GBM tumor cells (U87MG cells). The study was conducted in the Department of Biochemistry, in collaboration with the Department of Pathology, Maulana Azad Medical College and associated Lok Nayak Hospital, New Delhi, from March 2015 to May 2016. This study was approved by the Institutional Ethical Committee, Maulana Azad Medical College (F.No./I mEC/MAMC/2011).

Study was conducted on two groups, Group 1[control group]: U87MG cells maintained under standard cell culture technique without treatment with Plant extract and Group 2 [treatment group]: tumor cells maintained under standard cell culture technique with aqueous extract of *Ocimum sanctum*/*Centella asiatica*.

Preparation of aqueous extract of medicinal plant

Fresh and healthy leaves of *Ocimum sanctum* and *Centella asiatica* were collected from MAMC's garden as well as different parts of India. The leaves were washed thoroughly with distilled water. The leaves were then dabbed in absolute alcohol. The leaves were dried in hot air oven at 60°C (± 2°C) till they became crispy. The dried leaves were grounded to fine powder with mortar and pestle. The prepared powder of the leaves was weighed and 900 mg of powder was mixed in 30 mL of deionised water to prepare stock solution (30 mg/mL). The contents were mixed thoroughly using magnetic stirrer till all the particles dissolved. The liquid was then poured into a 50 mL centrifuge tube and was centrifuged at 1500 rpm for 10 min. The supernatant was then collected in a different tube and was stored at –20°C until further use. While using, the extracts was filtered using syringe filter (size: 0.22 µM) to prevent contamination to the cell culture.

Cell cultures

U87MG cell line was purchased from the National Centre for Cell Science, Pune. U87MG was maintained in Dulbecco's Modified Eagle's Medium containing 10% Fetal Bovine Serum, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 250 ng/mL Amphotericin, 250 µg/mL Gentamycin and 2 mM L-glutamine. Cell lines were maintained at 37°C in a humid atmosphere containing 5% CO_2. On attaining 70-80% confluence, sub culturing was done after cell counting using a double Neubaer ruled metalized counting chamber and viability testing with the help of Trypan blue assay.

MTT assay [Cytotoxic Assay]

Cytotoxicity effect of *Ocimum sanctum* and *Centella asiatica* was determined by using of MTT assay. Equal number of cells (1×10^4 cells/mL) were seeded in 96 well plates in form of pentaplicates for MTT assay and allowed to grow for overnight in a CO_2 incubator at 37°C to reach 70%-80% growth. After incubation for 24 h at 37°C, cells were treated with *Ocimum sanctum* and *Centella asiatica* at different concentrations of 1, 5, 10, 15, 20, 25 mg/mL (The concentration of aqueous extract were obtained from the studies conducted by Soloway E *et al.*, Kuang W *et al.*, Elias ST *et al.*[12-14] and incubated for 48 h at 37°C. The plates were taken out of the incubator and inverted microscope was used to study the morphology of the cells. The bleached media was removed from each well and 100 µl of MTT (1 mg/mL) solution (Sigma-Aldrich, USA) diluted in DPBS was added to each well followed by incubation at 37°C for 4 h, removal of supernatant and 100 µL of DMSO was added to each well. The plate was incubated in dark for 60 min and the absorbance was measured using a micro-titer plate reader (Bio-Rad) at 570 nm. The percent cell death was calculated as follows:

$$\text{Cell death \%} = \left(\frac{\text{OD of Control}}{\text{OD of treatment sample}} \right) \times \frac{100}{\text{OD of Control}}$$

Early apoptosis detection by Annexin V assay and surviving gene expression

Treatment of both the plant extracts were given for 48 h to U87MG cell line as described above and then processed to examine the percent of early apoptosis using Fluorochrome-conjugated Annexin V [Annexin V-FITC Apoptosis Detection Kit (Biolegan)] and Survivin gene expression using Rotar gene Q-PCR. Equal number of cell were seeded in T 75 cm^2 flask (1×10^5 cell/mL) and then treated with 1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL dose of plant extracts and one control were taken without treatment. Cells were harvested and treated with 5 µL of Annexin V-FITC and 5 µL of propidium iodine in 100 µL of cell suspension. The cells were incubated at room temperature for 15 min and stained cells were analysed by flow cytometry. For Survivin gene expression, RNA extraction from tumor cell line after plant extracts treatment was done using of TRI Reagent^R (Sigma Aldrich) according to the manufacture's protocol. RNA quality was checked by agarose gel electrophoresis and quantified using NanoDrop technology (Washington, DE, USA) and gene expression is analysed using Rotar Gene Q-PCR (Qiagen Inc). Complimentary DNA (cDNA) synthesis was performed by reverse transcribing 100 ng/µL RNA using Verso cDNA synthesis kit (Thermo Scientific, EU) according to the instructions provided and Quantitative Real Time PCR (qRT-PCR) was performed using Rotor–gene Q (Qiagen) analyzer for studying Survivin gene expression. The relative expression was calculated using $2^{-(\Delta\Delta Ct)}$ method. Each experiment was performed in triplicate each.

Primers sequence for survivin gene and beta actin gene were designed using Primer 3 software. The primer sequences used were: Survivin gene Forward 5′CAGATTTGAATCGCGGGACCC3′ and reverse-5′ CCAAGTCTGGCTCGTTCTCAG3′.

β-actin Forward-5′CGACAACGGCTCCGGCATG TGC3′ and

β-actin Reverse-5′GTCACCGGAGTCCATCACG ATGC3′.

The β-actin gene was used as reference housekeeping for normalisation.

Statistical analysis

Statistical analysis was performed using Graph Pad prism. All the quantitative data were analysed by student t test and one-way ANOVA followed by post hoc-tukey was applied to determine the statistical difference among the groups. Data were expressed as mean ± SD. p value <0.05 was considered statistically significant.

Results

Cytotoxic effect of aqueous extracts from *Ocimum sanctum* and *Centella asiatica* were measured on U87MG cells using MTT assay. The effects of treatment were evaluated in vitro to identify the potential cytotoxic activity of the extract in a six dose assay testing where lower and upper concentrations of 1 and 25 mg/mL was used on U87MG cells. Bioactivity of *Ocimum sanctum* and *Centella asiatica* were determined based on the inhibition within the test range on the growth of the treated cells as compared to the controls in three individual experiments (Table 1).

MTT assay, demonstrated potential cytotoxic activity of *Ocimum sanctum* and *Centella asiatica* extracts on U87MG cell line, exhibiting significant inhibition in cell growth compared to untreated control cells with 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL dose of plant extract of *Ocimum sanctum* and *Centella asiatica* showed significant inhibition with all test dose from 1 to 25 mg/mL. More than fifty percent inhibition is seen with dose of 25 mg/mL and 20 mg/mL, respectively, with *Ocimum sanctum* and *Centella asiatica*.

Morphology changes of U87MG cells was observed under inverted microscope after plant extracts treatment at 25 mg/mL and 20 mg/mL dose concentration for 48 h. Treated cells were become more rounded up, shrunken in size, membrane bulge (bleb) and detached from the monolayer surface of the wells (Fig. 1), with decreasing cell number of cells when compared to the untreated control.

Table 1 — Percent inhibition of crude plant extracts in U87MG cells

Plant	Dosage of plant extract					
	1 mg/mL	5 mg/mL	10 mg/mL	15 mg/mL	20 mg/mL	25 mg/mL
Ocimum sanctum	3.8 % ±1.6	12.6% ±1.2*	16.1 % ±2*	25.7% ±2.7*	41.8% ±6.6*	65.3% ±8.9*
Centella asiatica	21.8% ±2.2*	25.5% ±0.93*	32.8% ±5.5*	41.9% ±8.8*	61.5% ±9.8*	70% ±2.1*

The cytotoxic effect is expressed in terms of the percent inhibition of cells growth relative to the untreated control after 48 h exposure to the extracts. Results were expressed as mean ± SD of pentaplicate determination and experiment was repeated thrice. There was a significant difference in cell inhibition in extracts-treated cultures compared with untreated-control in U87MG cells (*P <0.001) and p value was calculated by one way ANOVA test (with Tukey's post hock test)

Effect of *Ocimum sanctum* and *Centella asiatica* on U87MG cells apoptosis

The effects of different treatment dosage of plant extracts (1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL) were evaluated for apoptosis in U87MG cell line. *Ocimum sanctum* extract showed statistically differences in apoptosis with different treatment doses (2.8% apoptosis with 1.5 mg/mL dose (P <0.001), 4.9% apoptosis with 2.0 mg/mL (P <0.0001), and 10.3% apoptosis with 2.5 mg/mL (P <0.0001). The difference was statistically significant compared to control group (Fig. 2A).

Effect of different treatment doses of *Centella asiatica* on U87MG cells compared to control group showed 3.1% apoptosis (P <0.001), 5.8% apoptosis (P <0.0001) and 7.2% apoptosis (P <0.0001) with 1.5 mg/mL, 2.0 mg/mL and 2.5 mg/mL, respectively, and the difference among the group compared to control was found to be statistically significant (Fig. 2B).

Effect of *Ocimum sanctum* and *Centella asiatica* on survivin expression in U87MG cells

Survivin gene expression was analysed in U87MG cells after treatment with doses of 1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL of *Ocimum sanctum* and *Centella asiatica* extract. We observed reduction in expression of survivin gene among the different dosage groups. *Ocimum sanctum* extract of 1.5 mg/mL showed 17% fold reduction, 2.0 mg/mL treated group showed 38% fold reduction and 2.5 mg/mL treated group showed 68% fold down regulation compared to untreated control and differences was found to be significant (Fig. 3A). *Centella asiatica* extract at dose 1.5 mg/mL showed, showed 73% fold reduction, 2.0 mg/mL dose showed 86% fold reduction and 2.5 mg/mL treated group showed 97% fold down regulation of survivin gene compared to untreated control and the difference was found to be significant (Fig. 3B).

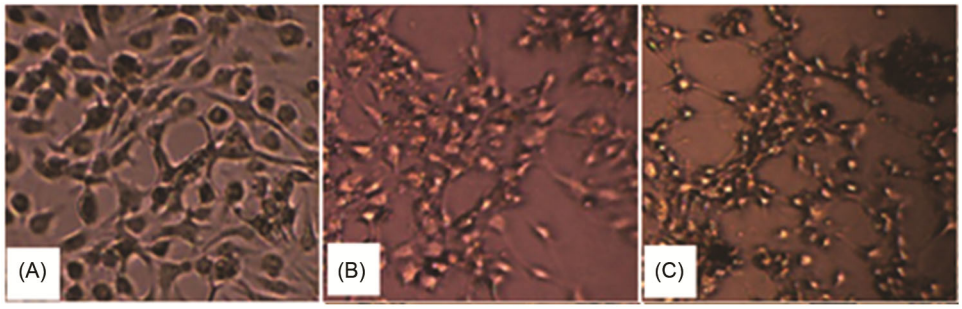

Fig. 1 — Microscopic picture of U87MG cell morphology. U87MG cells morphology under inverted microscope after treatment of plant extracts. (A) Untreated U87MG cells for 48 h; (B) *Ocimum sanctum* treated U87MG cells for 48 h with 25 mg/mL treatment dose; and (C) *Centella asiatica* treated U87MG cells for 48 h with 20 mg/mL treatment dose

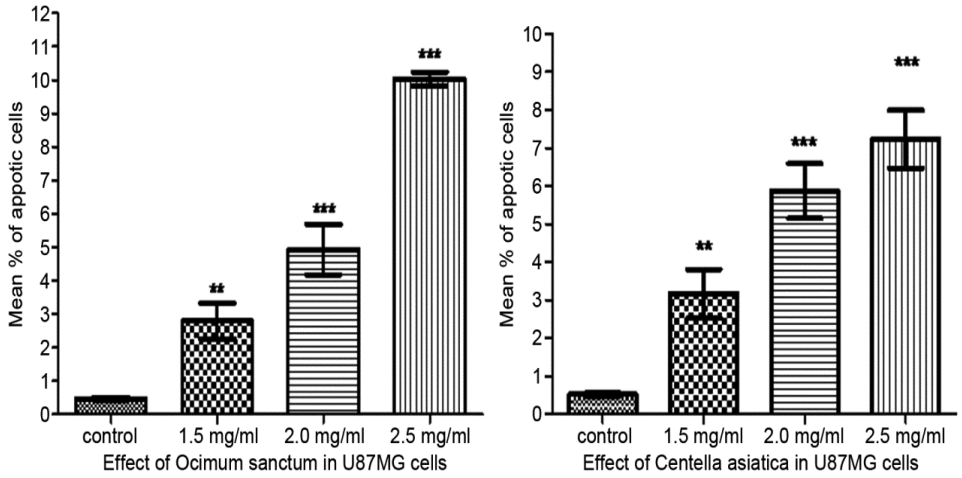

Fig. 2 — Apoptosis cells distribution after different dose of plant extract treatment. The anti-proliferating effect is expressed in terms of mean percentage of apoptotic cells relative to the untreated control after 48 h exposure to the different doses of extracts (A: *Ocimum sanctum* and B: *Centella asiatica*). Results were expressed as mean ± SD and experiment was repeated thrice. p value was calculated by one-way ANOVA test (with Tukey's post-hock test)

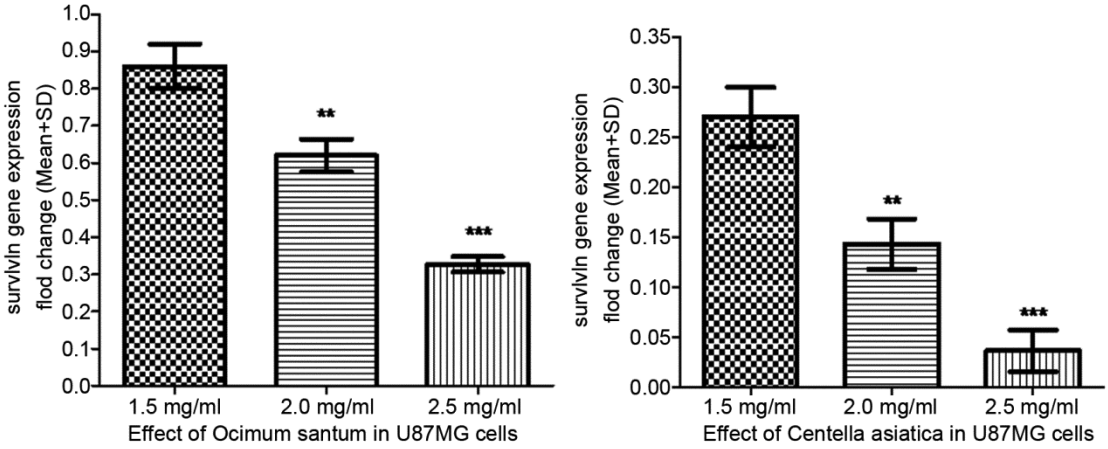

Fig. 3 — Survivin gene expression after different dose of plant extract treatment. Survivin gene expression is expressed in terms of fold change relative to the untreated control after 48 h exposure to the different doses of extracts (A: *Ocimum sanctum* and B: *Centella asiatica*). Results were expressed as mean ± SD and experiment was repeated thrice. There was a significant difference among the group compared to control group (**P <0.001 and *** P <0.0001) and p value was calculated by one-way ANOVA test (with Tukey's post-hock test)

Discussion

Cancer is fourth common cause of death worldwide and is expected to increase over the next 15 years according to WHO[15]. There is need for new anticancer compounds with diverse modes of action with lesser side effect and also to address the need for treating drug resistant cancer. Since most of Chemotherapy drugs have its origin from plant source[16], searching for new anticancer compounds from plants provide a good starting point. Plants contain natural compounds such as polyphenols, flavonoid, taxols *etc*., that are known to reduce the risk of diseases such as cancer, diabetes and neurodegenerative diseases[17,18]. Plant compounds used in traditional medicine are generally considered safe as they are usually consumed as part of the diet like eating apple fruit and drinking orange juices during the first two years of life resulted in a decrease in the incidences of leukaemia in children under the age of 15 year[19]. In our study we used *Ocimum sanctum and Centella asiatica* which is shown to have anti-cancer potential along with other pharmacological actions[20–29] and to study their utility as a potential anticancer treatment option. Here we studied the anti-malignant effect of these plant extracts on growth of human tumor cell line U87MG (glioma cell line).

In the present study, we observed medicinal plant extracts *Ocimum sanctum* and *Centella asiatica* inhibited the proliferation of cancer cells significantly, in a dose-dependent manner in U87MG cells. We observed cytotoxic effect of *Ocimum sanctum* and *Centella asiatica* at dose concentration 25 mg/mL showing 65% cytotoxicity with *O. sanctum* and 70% with *C. asiatica*. The cytotoxic effect was incremental with both plant extract treatment. In present study, we used three doses (1.5 mg/mL, 2 mg/mL and 2.5 mg/mL) which showed effective cytotoxity from our MTT assay experiment. Similar observation was reported by Prachi Shivpuje *et al.*, using *Ocimum sanctum* in oral cancer cell line and reported significant cytotoxic effect of aqueous extract of *O. sanctum* after 48 h of exposure at different dosage[30]. Studies in literature have shown *Centella asiatica* Leaves Extract has anti-tumor activity, decreasing lung tumor nodule in Benzo(a)pyrene-Induced tumour Mice model[31] and *induced* apoptosis in breast cancer cells[28]. We observed anti-proliferating activities of crude aqueous plant extracts of *Ocimum sanctum* and *Centella asiatica* on U87MG cancer cells in apoptosis assay, *O. sanctum* showed maximum apoptosis of 10% at 2.5 mg/mL and *C. asiatica* showed 7.5% apoptosis at the same dose compared to control untreated U87MG cells. Batanai Moyo *et al.*, have reported anti proliferative effect with plant extract (T. welwitschii) on Jurkat T Cells, they found the growth of the Jurkat T cells was significantly reduced compared to untreated cells after 72 h of treatment and showed apoptosis by DNA fragmentation[32].

We further investigated expression of survinin gene after plant extracts treatment in tumor cell lines. Survivin, an inhibitor of apoptosis is a protein that in humans is encoded by the *BIRC5* gene[2]. It is highly expressed in most cancers and is associated with chemotherapy resistance. The survivin protein inhibit

caspase 3 activation, thereby leading to negative regulation of apoptosis or programmed cell death. In cancer cells, over expression of survivin leads to decrease in apoptosis and increase in tumour growth. Current research suggests that survivin might provide a new target for cancer therapy by disruption of survivin induction pathways leading to increase in apoptosis and decrease in tumour growth. In our study, treatment of plant extracts in U87MG cells, showed significant decrease (P <0.001) in survivin gene expression with both *Ocimum sanctum* and *Centella asiatica*, in concentration dependent manner compared to untreated U87MG cells. *Centella asiatica* showed better down-regulation then *Ocimum sanctum* with 97% reduction on treatment with 2.5 mg/mL concentration. Similar studies with other plant extracts have shown to effect survivin gene[33-37]. Study conducted by Guzalnur *et al.* investigated anti-carcinogenic effects of the phenolic-rich plant extract on human cervical cancer cells (SiHa), they observed down-regulation of anti-apoptotic Bcl-2 expression and telomerase and Survivin expression on treatment with plant extract from Savda Munziq plant[37]. Another study conducted by Wen-Hung Wang *et al.*, investigated antioxidant activity and Anti proliferative effect of the Jaboticaba (Myrciaria Cauliflora) seed extracts in oral carcinoma cells and showed water extract of Jaboticaba seeds induces oral cancer cell apoptosis by decreasing the expression of surviving[38]. Eugenol, the active molecule in the plant extract of *Ocimum sanctum* have shown anti cancer effect in other cancers such as lung, colon, gastric, melanoma and breast cancer[39]. Similarly, *Centella asiatica* plant extract has shown effective in Leukemic cells (THP-1 cells) and breast cell (MCF7)[40,41]. Conventional therapy are also known to produce drug resistance[42], hence medicinal plants provide alternatives for developing new drugs to targeting cancers and preventing drug resistance. In present study the major molecule eugenol (allylbenzene) found in *Ocimum sanctum* has shown promising anticancer effect, similarly D Roselin Jenifer *et al*. (2021) have study plant extract for the active molecules that could have potential anticancer effect, and reported phenolic and benzene group have show interaction with matrix metalloproteinases, which are important in cancer progression and metastasis[43].

Conclusion

We presented in this study the use of crude aqueous extracts of a two medicinal plants (*Ocimum sanctum* and *Centella asiatica*) in glioma cell lines and evaluated the anti-malignant effect on the growth of cancer cells through different methods includes MTT assay, Anexin-V assay, Survivin expression assay. In conclusion, this study showed two plant extracts (*Ocimum sanctum* and *Cassia angustifolia*) exhibited promising anti-proliferating activity against human U87MG cells by the decrease of survivin expression which in turn to induce apoptosis. This study might help to design further studies regarding the anti-tumor effects of medicinal plant extracts which may be attributed to its ability to serve as an anti-tumor alternative for cancer treatment with more studies on animal model and other cancer types confirming the effect of this medicinal plant extracts.

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Conflict of interest

All authors declare no conflicts of interest.

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