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Anti-proliferative and apoptosis inducing activity of *Calophyllum inophyllum* L. oil extracts on C6 glioma cell line

Ayşegül Erdemir*

Department of Molecular Biology and Genetics, Faculty of Arts & Science, Yildiz Technical University, Esenler 34220 Istanbul, Turkey.

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***Corresponding Author**
Tel.: +90 212 383 4462
E-mail: aerdemir@yildiz.edu.tr

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Abstract

Glioblastoma multiforme (GBM) is one of the most lethal type of brain tumors. Current treatment methods of GBM including radiotherapy and chemotherapy are not sufficient to combat this disease and there is a great need to develop new treatment approaches. *Calophyllum inophyllum* L. is a polyphenol rich plant with wide biological activities. The aim of this study was to investigate cytotoxic, anti-proliferative and apoptotic activity of *C. inophyllum* oil (CIO) extracts on the C6 glioma cell line to determine its potential use in cancer treatment. Treatment of C6 glioma cells with CIO extracts for 24 and 48 h were resulted in a significant decrease in cell viability with IC_{50} values of 0.22% and 0.082%, respectively. Proliferating cell nuclear antigen labeling of CIO extract treated C6 glioma cells showed cell proliferation decreased to 55.6% and 30.3% for 24 and 48 h. Percentage of apoptotic cells after CIO extract treatment were found to reach 68.8% after 48 h. Analysis of mRNA expression levels of key genes involved in apoptosis pathway showed that CIO extract treatment induces both intrinsic and extrinsic apoptosis pathways on C6 glioma cells. Findings of this study showed that CIO extracts are promising in development of new treatment strategies for glioblastoma.

Introduction

Glioblastoma multiforme (GBM) is one of the most common and malignant primary brain tumor in adults. GBM grows rapidly and has a high rate of invasion and angiogenesis capacity but they often do not metastasize out of the brain. This tumor type carries the highest incident rate among brain and central nervous system tumors with the incident rate of 3.19/100.000 population (Thakkar et al., 2014). On the other hand, GBM has worst prognosis among other types of brain cancers (Hanif et al., 2017) . The median survival for GBM is 15 months and less than 5% of patients survive 5 years post-diagnosis (Delgado-López & Corrales-García, 2016). The standard treatment approach for GBM is very complex, it includes surgical resection of tumor followed by radiotherapy and chemotherapy (Delgado-López & Corrales-García, 2016). Despite optimal treatment, high resistance of tumor cells to chemotherapy and radiotherapy, GBM has very poor survival rate. Since conventional therapies not sufficient to combat this disease there is a great need to develop new treatment approaches.

Phytochemicals are natural plant derived compounds and known to be as a great source for development of many drugs. Many phytochemicals and their derivatives are known to have high antitumor potential for cancer treatment. In addition, it is known that phytochemical-based agents increase treatment efficiency and reduce most of the side effects of drugs

(Choudhari et al., 2020). Polyphenols are one of the most diverse phytochemical groups and exhibits wide range of biological activities including anti-cancer, anti-proliferative and anti-oxidant activities (Upadhyay & Dixit, 2015). *Calophyllum inophyllum* L. (Calophyllaceae) is a polyphenol rich evergreen pantropical tree found in Africa, Asia, and Pacific countries (Dweck & Meadows, 2002). Various parts of *C. inophyllum* plant such as barks, leaves, and fruits are used for different traditional medicinal purposes (Raharivelomanana et al., 2018). *C. inophyllum* contains wide range of phytochemicals such as triterpenoids, steroids, coumarins, and flavonoids (Susanto et al., 2019). Hence, *C. inophyllum* extracts are also known to have anti-oxidant, anti-microbial, anti-viral, anti-inflammatory and anti-proliferative activities (Dweck & Meadows, 2002). Nevertheless, studies carried out until today, mostly focused on the uses of *C. inophyllum* extracts in cosmetic industry and wound healing (Ansel et al., 2016; Raharivelomanana et al., 2018). Considering its wide biological activities *C. inophyllum* has a great potential to be used in pharmaceutical development, especially for use in cancer treatment. However, there are limited numbers of studies in the literature that have investigated the potential use of *C. inophyllum* extracts in cancer treatment (Hsieh et al., 2018; Jaikumar et al., 2016; Shanmugapriya et al., 2017).

Development of most phytochemical-based drugs begins with the *in vitro* investigation of anti-tumor potential of plant extract. In this study, we aimed to investigate cytotoxic, anti-proliferative and apoptotic activity of *C. inophyllum* oil extracts on the C6 glioma cell line in order to determine its potential use in cancer treatment.

Materials and Methods

Materials

Pure cold-press *C. inophyllum* oil (100%) (CIO) extract was purchased from EuropeVital Herbal and Aromatic Oil, Turkey. The C6 glioma cell line and L929 mouse fibroblast cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Proliferating cell nuclear antibody (PCNA) was purchased from Thermo Scientific™, USA. ApopTag® Peroxidase In Situ Apoptosis Detection Kit was obtained from Millipore, USA. Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, USA) was used for RNA isolation. SensiFAST™ cDNA Synthesis Kit Bioline Reagents Ltd., UK) was used for cDNA synthesis. SensiFAST™ SYBR Lo-Rox (Bioline Reagents Ltd, London, UK) kit was used for quantitative real-time polymerase chain reaction (qRT-PCR).

Cell Culture

The C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's Nutrient Mixture F-12 medium (1:1) containing 5% fetal bovine serum (FBS), streptomycin (100 µg/mL), penicillin (100u/ml) and l-glutamine (0.2 mM). L929 fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's Nutrient Mixture F-12 medium (1:1) containing 10% fetal bovine serum (FBS), streptomycin (100 µg/mL), penicillin (100u/ml) and l-glutamine (0.2 mM). Cells were incubated at 37°C in a saturated humidity atmosphere with 5% CO_2.

MTT Cell Viability assay

The cytotoxic effect of CIO extract on C6 glioma and L929 fibroblast cell lines were determined using MTT (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded on a 96 well plate at a density of $1x10^4$/well and incubated overnight. Next day, old media was removed and cells were treated with medium containing various concentrations of CIO and cells were cultured for 24 and 48 h. After 24 and 48 h incubation with CIO extract, supernatants were removed and MTT solution (50 µg/mL) was added on each well for 3 h incubation at 37°C. The amount of purple formazan was determined by dissolving it in 0.1 mL DMSO and measuring the optical density at 570 nm in a microplate reader. Untreated cells were used as control group. The cytotoxic effect of CIO extract against C6 glioma cells was expressed as IC_{50}. Cell viability (%) was calculated by the following equation:

$$Cell\ viability\ \% = \frac{OD\ value\ of\ treated\ cells}{OD\ value\ of\ control\ cells} x\ 100$$

Proliferating cell nuclear antibody (PCNA) cell proliferation assay

The effect of CIO extract treatment on C6 glioma cell proliferation was determined by immunocytochemical detection of the presence of PCNA antibody as previously described (Ersoz et al., 2019). C6 glioma cells were cultured on 24 well plates at a density of $1x10^4$ cells in wells containing coverslips. Cells were incubated until they reach confluency. Then, 24 and 48 h IC_{50} concentration of CIO extracts were applied on C6 glioma cells. After 24 and 48 h incubation, cells were washed with phosphate buffered saline (PBS). Cells were fixated by ice-cold methanol treatment for five minutes. Following fixation step, blocking solution was applied and cells were incubated with PCNA primary antibody (1:300) overnight at 4°C. After overnight incubation cells were washed again and biotinylated secondary antibodies, streptavidin, biotinylated horseradish peroxidase were applied on cells. AEC kit (Invitrogen, Camarillo, USA) was used to stain immunoreactive cells and hematoxylene was used as counterstain. Olympus BX-50 bright field microscope was used to visualize stained and unstained cells. 10 random fields were chosen, and stained/unstained cells were counted to determine the percentage of immunoreactive cells. Percentage immunoreactivity was calculated as follows:

$$immunoreactivity\ \% = \frac{\#\ of\ immunoreactive\ cells}{\#\ of\ total\ cells} \times 100$$

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Immunocytochemical investigation of CIO extracts on C6 glioma cell apoptosis induction was determined by TUNEL assay according to manufacturer's protocol. C6 glioma cells were seeded on coverslips at a density of $1x10_4$ cell per well. Then, 24 and 48 h IC_{50} concentration of CIO extracts were applied on cells. Cells were fixated with ice-cold methanol followed by immediate addition of equilibration buffer. TUNEL reaction mixture (terminal deoxynucleotidyl transferase and reaction buffer with nucleotide mixture) was added on cells and incubated for 1 h at 37°C. Diaminobenzidine (DAB) as the substrate of peroxidase was used to stain the DNA fragments. 10 random fields were selected to count stained/unstained cells. Percentage of apoptotic cells was determined as follows (Ersoz et al., 2019):

$$apoptotic\ cells\ \% = \frac{\#\ of\ apoptotic\ cells}{\#\ of\ total\ cells} \times 100$$

Gene expression analysis by quantitative real-time polymerase chain reaction

C6 glioma cells were treated with IC_{50} concentration of CIO extracts for 48 h. Total RNA isolation from cells was performed using the Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, USA), according to the manufacturer's instructions. After isolation, RNA integrity and concentrations were checked by using NanoDrop UV-Vis spectrophotometer. SensiFAST cDNA synthesis kit (Zymo Research, Irvine, USA) was used to synthesize cDNA from purified RNA and manufacturer's instructions were followed for synthesis. Briefly, 1 µg RNA, 4 µl 5x TransAmp Buffer and 1 µl reverse transcriptase enzyme were mixed and reaction mixture was completed to 20 µl with dH_2O. Then, this mixture was incubated 10 min at 25°C for primer annealing, 30 min at 42°C for reverse transcription and 5 min at 85°C for inactivation step in a thermal cycler. SensiFAST SYBR Lo-Rox kit (Bioline Reagents Ltd, London, UK) was used to detect gene amplification in qRT-PCR. Reactions were carried out using AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, USA). Reaction conditions were 2 min pre-denaturation at 95˚C, denaturation at 95˚C for 5 s, annealing at 63˚C for 10 s and elongation at 72˚C for 15 s for 40 cycles. Primer pairs of target genes and housekeeping β-actin gene were listed in Table 1. The relative gene expressions of all the genes tested were determined by using $2^{-\Delta\Delta Ct}$ method where β-actin gene was used as internal control (Livak & Schmittgen, 2001) (Table 1).

Statistical analysis

Graphpad Prism software version 6 (GraphPad Software, La Jolla, CA) was used for all statistical analysis. Unpaired t-tests were used for comparisons between groups, differences were considered to be significant at the level of $p<0.05$.

Results and Discussion

Determination of C6 glioma cell cfigureytotoxicity

Cytotoxic activity of CIO extracts on C6 glioma cells were determined by MTT assay. L929 fibroblast cells were used to determine CIO extract's cytotoxic activity on normal cells. C6 glioma and L929 fibroblast cells were treated with different concentrations of (0.02% to 1%) CIO extract for 24 and 48 h. CIO extracts inhibited viability of C6 glioma cells in a dose and time dependent manner. On the other hand, L929 cells treated with various concentrations of CIO extract did not change cell viability for both 24 and 48 h treatment (Figure 1a and 1b). As shown in Figure 1a, 24 h treatment of C6 glioma cells with CIO extracts did not show any significant cytotoxic activity in lower concentrations such as 0.02% and 0.05%. However, increasing concentrations of CIO started to inhibit proliferation of C6 glioma cells. The significant decrease in cell proliferation was observed at 0.125% concentration and IC_{50} was found 0.22% for 24 h treatment with CIO extract. L929 cell viability did not change even in the higher concentrations of CIO treatment (Figure 1a). The inhibitory effect of CIO extracts on C6 glioma cells were higher after 48 h treatment compared to 24 h (Figure 1b). Figure 1b represents inhibitory effect of CIO extracts on C6 glioma cells and L929 fibroblast cells after 48 h treatment. C6 glioma cell viability decreased to 55% after treatment with 0.05% CIO extracts. Cell cytotoxicity of C6 glioma cells were significantly increased at concentrations of 0.05% and above (Figure 1b). Treatment of C6 glioma cells with 1% CIO extract resulted in 11% cell viability.

Table 1. Specific primer sequences used for qRT-PCR

Gene	Forward Primer	Reverse Primer
β -actin	5'-CATGTACGTTGCTATCCAGGC-3'	5'-CTCCTTAATGTCACGCACGAT-3'
Cytochrome-c	5'-CTTTGGGCGGAAGACAGGTC-3'	5'-TTATTGGCGGCTGTGTAAGAG-3'
Caspase-3	5'-AGAGGGGATCGTTGTAGAAGTC-3'	5'-ACAGTCCAGTTCTGTACCACG-3'
Caspase-9	5'-CTCAGACCAGAGATTCGCAAAC-3'	5'-GCATTTCCCCTCAAACTCTCAA-3'
Caspase-8	5'-GTTGTGTGGGGTAATGACAATCT-3'	5'-TCAAAGGTCGTGGTCAAAGCC-3'
Bax	5'-CCCGAGAGGTCTTTTTCCGAG-3'	5'-CCAGCCCATGATGGTTCTGAT-3'
Bcl-2	5'-GGTGGGGTCATGTGTGTGG-3'	5'-CGGTTCAGGTACTCAGTCATCC-3'

IC_{50} concentration for 48 h treatment with CIO extract was calculated as 0.082%. Moreover, treatment with all concentrations of CIO extracts did not show any cytotoxic effect on L929 cells (Figure 1b).

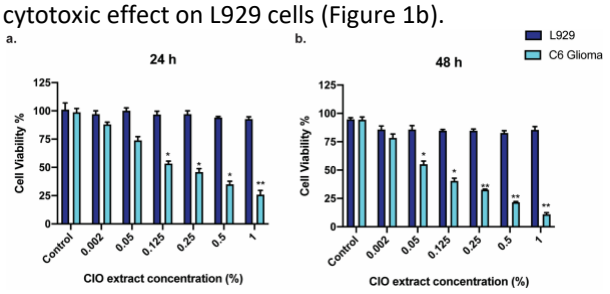

Figure 1. Percentage cell viability of L929 and C6 glioma cells after treatment with CIO extracts for **a)** 24h and **b)** 48 h. Data are expressed as mean ± standard deviation from three independent experiments. **p<0.05, ** p<0.001*.

Biological activity of C. *inophyllum* oil extracts were also previously tested in different studies. A study by Ansel et al. (2016) tested cytotoxic activity of C. *inophyllum* oil extracts cytotoxicty on human keratinocytes (HaCaT) and normal dermal fibroblasts (ECACC). In both cell lines, *C. inophyllum* oil extracts did not show any significant cytotoxic activity in a concentration range from 1% to 0.125%. Our results also revealed that CIO extracts were safe to be used in L929 fibroblast cells in the concentration range of 0.02% to 1%. The cytotoxic activity of CIO extracts was evaluated in different studies but most of these studies were focused on human skin cells (Ansel et al., 2016). However, literature on examining the impact of CIO extract on cancer cells were limited. A study investigating the in vitro anticancer of *C. inophyllum* ethanolic leaf extract on MCF-7 breast cancer cell lines found that IC_{50} of 120 µg/mL strongly inhibits MCF-7 cell growth. The same study was reported that the increase in cytotoxic activity of MCF-7 cells were also dependent on increasing concentrations of C. *inophyllum* ethanolic leaf extract (Léguillier et al., 2015).

CIO extracts inhibits C6 glioma cell proliferation

The effect of CIO extracts on C6 glioma cell proliferation was determined using PCNA immunocytochemical staining. C6 glioma cells were treated with IC_{50} concentrations of CIO extracts for 24 and 48 h. Then, immunostained cells were counted and PCNA positive cell rates were determined (Figure 2). CIO extract treatment significantly reduced C6 glioma cell proliferation both 24 and 48 h treatment. 24 h treatment with CIO extract resulted in 55.6% PCNA positive cells while 48 h treatment decreased PCNA positive cell numbers to 30.3% (Figure 2). Sustained proliferative signaling is one of the key hallmarks of cancer (Hanahan & Weinberg, 2011). Therefore, it is crucial to determine potential anti-cancer agents' effect on cell proliferation. It is known that phytochemicals are polyphenol rich natural compounds that derived from different plants (Upadhyay & Dixit, 2015). *C. inophyllum* is a polyphenol rich plant and contains many different biologically active groups such as flavonoids and coumarins. These chemical groups are individually a great source of potential anti-tumor agents (Itoigawa et al., 2001). On the other hand, studies suggest even minor compounds in oil extracts might have great importance in order to synergistically act and inhibit cancer cell proliferation (Bakkali et al., 2006). Our results revealed that CIO extracts inhibits cell proliferation in a time dependent manner on C6 glioma cells. This inhibition could either be a result of synergistic activity of CIO extract's functional groups or individual activity of flavonoids and coumarins.

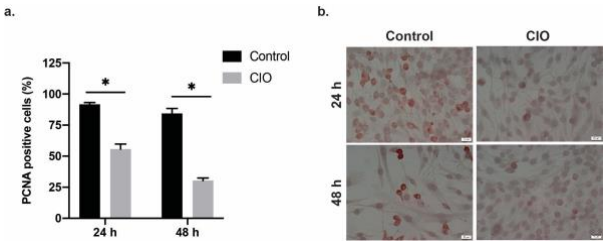

Figure 2. Effect of CIO extracts on proliferation of C6 glioma cells. **a)** PCNA positive cell rate of C6 glioma cells after 24 and 48 h treatment with IC_{50} concentration of CIO extracts. **b)** Bright-field microscope images of immunostained cells. **p<0.05.*

Determination of CIO extract induced apoptotic activity on C6 glioma cells by TUNEL assay

Immunocytochemical detection of CIO extracts apoptotic activity on C6 glioma cells showed by TUNEL assay. TUNEL method detects apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis (Kyrylkova et al., 2012). C6 glioma cells were treated with 24 and 48 h IC_{50} concentrations of CIO extracts. Results showed that CIO extracts induces apoptosis on C6 glioma cells (Figure 3).

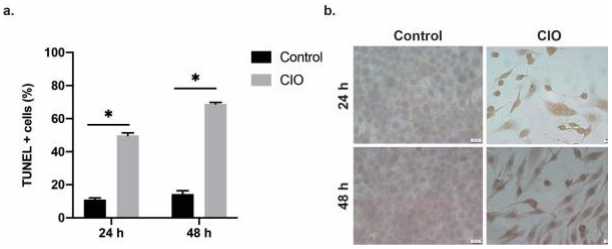

Figure 3. Detection of apoptotic C6 glioma cells treated with CIO extracts for 24 and 48 h. **a)** TUNEL positive labeled cell percentage **b)** Bright-field microscope images of immunostained cells. **p<0.05.*

Both 24 and 48 h incubation of C6 glioma cells with CIO extracts resulted in a significant increase in apoptotic cell number. TUNEL positive stained C6 glioma cell percentage was increased to 49.8% after 24 h treatment with CIO extracts. Moreover, apoptotic C6 glioma cell percentage reached 68.8% after 48 h treatment with CIO extracts. Untreated C6 glioma cells were used as controls. In contrast with CIO extract

treated cells, apoptotic activity did not change in control group for 24 and 48 h. Apoptosis is one of the most important events in cancer progression. Cancer cells are known for their resistance to apoptosis. Therefore, it is important to activate apoptosis in cancer cells in order to prevent tumor growth and cancer progression. Our results demonstrated on a cellular level that CIO extracts induces apoptosis on C6 glioma cells.

Determination of apoptosis related genes expression levels on CIO extract treated C6 glioma cells

TUNEL labeling assay results revealed that CIO extracts induces apoptosis on C6 glioma cells. In order to elucidate molecular base of CIO induced apoptosis induction, expression levels of key genes involved in intrinsic and extrinsic apoptosis pathway were investigated. For this aim, mRNA levels of cytochrome-c, caspase-3, caspase-9, caspase-8, bax and bcl-2 genes of CIO extract treated C6 glioma cells were determined by qRT-PCR. Since TUNEL labeling revealed 48 h incubation with CIO extracts increased apoptotic cell percentage to 68.8%, gene expression levels of apoptosis related genes were determined after cells treated with 48 h IC_{50} concentrations of CIO extracts. Differential gene expression levels were presented in Table 2. Results showed that CIO extract treatment induces both intrinsic and extrinsic apoptosis pathway on C6 glioma cells. Genes that activates intrinsic pathway of apoptosis were showed increased level of expression. Cytochrome-c is an important role to initiate intrinsic apoptosis pathway. Once it released from mitochondria to cytosol, it binds to apoptotic protease activating factor-1 and this event subsequently leads to formation of apoptosome complex (Arnoult et al., 2002). Our results showed that CIO extracts increased cytochrome-c expression 4.1 times than control.

Table 2. Relative mRNA expression levels of apoptosis related genes after CIO extract treatment on C6 glioma cells (*p<0.001*)

Gene	Relative Fold Change
β -actin	1
Cytochrome-c	4.1137
Caspase-3	4.3866
Caspase-9	6.2334
Caspase-8	2.3667
Bax	3.0157
Bcl-2	-0.8218

Release of cytochrome-c from mitochondria depends on Bcl-2 and Bax proteins activity. Bcl-2 protein binds pro-apoptotic Bax to prevent pore formation and release of cytochrome-c from mitochondria (Naseri et al., 2015). Expression level of Bax was 3.01 times higher than control cells. On the contrary, Bcl-2 relative mRNA expression was found to be -0.82 indicating the apoptosis activation and reverse relationship between Bcl-2 and Bax. Moreover, caspase-3 and caspase-9 expressions were also increased 4.3 and 6.2 times, respectively. Activation of caspase-9 also supports that CIO extracts induces intrinsic apoptosis pathway. Despite lack of literature on CIO extracts apoptosis inducing activity on cancer cells, one study established that treatment of DLD-1 human colon cancer cells with pigments from *C. inophyllum* seed oil resulted in apoptosis induction and G2/M cell cycle arrest (Hsieh et al., 2018). A study by Shanmugapriya et al. (2017) also revealed that treatment of MCF-7 breast cancer cells with *C. inophyllum* fruit extracts decreased Bcl-2 expression and increased Bax, cytochrome-c and p53 expressions. Taken together, our results were found to be consistent with the literature and it can be concluded that different extracts of *C. inophyllum* induces apoptosis on various cancer cell lines.

Conclusion

This study was aimed to investigate anti-proliferative and apoptosis inducing activity of CIO extracts on C6 glioma cells. Our results demonstrated that CIO extracts significantly reduced glioma cell viability. Additionally, immunocytochemistry studies showed that cell proliferation decreases, and apoptosis induction was increased after CIO treatment. Apoptosis induction was also confirmed by qRT-PCR analysis. Overall, our results suggest that CIO might have a potential use in cancer treatment. However, further *in vitro* and *in vivo* studies investigating the molecular mechanisms of the anti-cancer activity of CIO extracts are required.

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