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Enhanced cytotoxic potential of *Orthosiphon stamineus* extract in MCF-7 cells through suppression of nucleolin and BCL₂

Renuka Saravanan¹, Brindha Pemaiah², Sriram Sridharan², Mahesh Narayanan¹ and Sivakumar Ramalingam¹

¹Department of Chemistry and Biosciences, SRC, SASTRA University, Kumbakonam 612 001, Tamil Nadu, India;

²Centre for Advanced Research in Indian System of Medicine, SASTRA University, Thanjavur, Tamil Nadu, India.

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Abstract

The aim of the present study was to analyze the anti-cancer effect of *Orthosiphon stamineus* leaf extract on MCF-7 cells. Ethyl acetate extract of the plant was eluted and pooled into 6 fractions based on their R_f values and the fractions were tested for cytotoxicity. Fraction 3 was found effective (IC₅₀ = 28.5 µL/mL) and was subjected to LC-MS analysis which indicated the presence of biochanin, eleutherol and cinnamic acid. Further, cytotoxic effect of the fraction was confirmed in terms of nucleolin, bcl₂, bax and cytochrome c expression in MCF-7 cells through RT-PCR where a marked decrease in nucleolin, bcl₂ and increase in bax and cytochrome c were observed. The presence of eleutherol, a well-known anti-oxidant and anti-cancer agent, has been reported for the first time in *O. stamineus*. The anti-cancer activity of *O. stamineus* may be due to the presence of eleutherol and cinnamic acid.

Introduction

Breast cancer is one of the most frequent malignant tumors (Ferlay et al., 2012). Though there are rapid advancements in the field of medicine still an effective treatment for breast cancer is yet to be found.

Extracts from the plant in the form of pure compounds or standardized extract which is the natural product offer plenty of opportunities to discover new drug due to the availability of diverse chemical (Cosa et al., 2006). Many of the phytochemicals are safe and found to be an alternative which is effective and has less adverse effect. However, to verify the efficacy of bioactive compounds clinical trials are necessary, where some of the medicinal plants like *Catharanthus roseus*, *Pleurotus eous* (Xu et al., 2015), *Taxus baccata* and *Bleekeria vitensis* (Saravanan et al., 2014) were found to have a positive effect on breast cancer.

Orthosiphon stamineus Benth (Family Lamiaceae) is distributed plant all over Africa and Southeast Asia

(Awale et al., 2002). It is a well-known perennial herb and is used in the treatment of various diseases like influenza, jaundice, epilepsy, fever, edema, diabetes and hypertension (Ho et al., 2010).

In the previous studies, the researchers had detected the presence of terpenoids, sterols, carbohydrates, flavonoids, alkaloids, quinones, phenol and coumarins in ethyl acetate extract and flavanoids and alkaloids in methanolic extract of *O. stamineus* leaves (Saravanan et al., 2017).

Biochanin A, a vital isoflavone is a methoxylated isoflavone from red clover. This includes delayed S phase in G2/M phase progression (Ying et al., 2002). It is found to be a powerful agonist of the human aryl-hydrocarbon receptor (Medjakovic and Junbauer, 2008). Cinnamic acid, a natural substances used by humans as an alternative flavoring agent and scents. It is classified under auxin, a plant hormone that helps in regulating cell growth and differentiation (Del et al., 2011). In MCF-7 cells treated with ethyl acetate fraction of *O.*



stamineus, the expression of bcl₂, nucleolin, bax, cytochrome c have been studied.

Materials and Methods

Plant collection and authentication

O. stamineus leaves were collected from Irula Tribal Women's Welfare Society, Thandrai, Tamil Nadu, India. Identification and authentication were confirmed with the help of Flora of Gamble (Manjamalai et al., 2011). Voucher specimen was deposited at the herbarium of Centre for Advanced Research in Indian System of Medicine, SASTRA University, Thanjavur, Tamil Nadu. All the chemicals were purchased from Merck (Germany) were of HPLC reagent grade.

Preparation of plant extract

Fresh leaves of *O. stamineus* were washed in running water, shade dried and pulverized to coarse powder. About 100 g of dried sample was macerated with 150 mL of ethyl acetate separately and kept overnight at room temperature in a shaker. The extract was collected and dried in a vacuum rotavapor at 40°C followed by lyophilization using freeze dryer (Christ Martin, USA) and stored at 4°C till use. The crude ethyl acetate extract was fractionated by column chromatography and further analyzed with identification of molecules using LC-MS/MS.

Column chromatography

Two grams of *O. stamineus* lyophilized sample was dissolved in 5 mL of ethyl acetate and allowed to dry with mixing of silica gel G (60-100 mesh). Slurry were prepared, then packed to the column (15 cm) with the help of *n*-hexane (100%) followed by the mixtures of chloroform, ethyl acetate and ethanol (90:10, 80:20, 70:30 and 50:50) of increasing polarity, to obtain fractions for yellow amorphous powder. About 40 fractions were eluted with different solvents with increasing polarity. All the collected fractions were subjected to TLC analysis, based on TLC profile fractions with similar R_f values were pooled into 6 fractions.

Thin layer chromatograph (TLC) procedure

The TLC developing was set in a twin trough chamber and was examined in various solvent systems, such as ethyl acetate and chloroform in the ratio of 1:1. The fractions were run on silica gel 60 F254, a pre-coated aluminum plate of 0.2 mm thickness. The plates were developed using suitable solvents and visualized with vanillin-sulfuric acid reagent. Retardation factor (R_f) was calculated using the formula.

$$R_f = \frac{\text{Distance travelled by solute (cm)}}{\text{Distance travelled by solvent (cm)}}$$

Among the fractions, F1 to F6 obtained from ethyl

acetate were subjected to cytotoxic effect against MCF-7 cells.

Cell line

The human breast adenocarcinoma cell lines (MCF-7) was obtained from National Centre for Cell Science (NCCS), Pune, India and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO₂, 95% air and 100% relative humidity.

Cell treatment procedure

The monolayer cells were detached with trypsin-EDTA to make single cell suspensions and viable cells were counted. 100 μL of 1 × 10³ cells/well suspension were seeded into 96-well plates and incubated to allow for cell attachment with the conditions mentioned above for 24 hours. Then the cells were treated with different concentrations of the test samples, dissolved in neat dimethyl sulfoxide (DMSO). Following sample addition, the plates were incubated further for 48 hours with the conditions previously mentioned. The medium alone served as control and triplicate was maintained for all concentrations (Mosmann, 1983).

MTT assay

Cytotoxic assay on MCF-7 with different concentration of ethyl acetate fraction was performed by the method described elsewhere (Monks et al., 1991). 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 hours of incubation, 15 μL of MTT (5 mg/mL) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4 hours. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 μL of DMSO and then measured the absorbance at 570 nm using micro plate reader. The %cell inhibition was determined using the following formula.

$$\% \text{Cell inhibition} = (100 - \text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

Nonlinear regression graph was plotted between %cell inhibition and log concentration and IC₅₀ was determined using GraphPad Prism software.

LC-MS/MS

500 μL of F3 was subjected to LC/ESI/MS/MS using UHPLC+ focused (Ultra high performance liquid chromatography) RP liquid chromatography coupled to mass spectrometer (micrOTOF-Q II, Bruker, Germany). Liquid chromatography separations were carried out on a C18 reverse phase column (120 Å, 2.1 × 150 mm

Acclaim 120, UHPLC+ Ultimate 3000 series, Dionex). UV detector was set arbitrarily at 260 nm. A discontinuous gradient elution at a flow rate of 0.2 mL/min was performed using mobile phase A (acetonitrile) and B (water-MilliQ acidified with 0.1% formic acid). Mass spectrometer with ESI ionization at negative mode equipped with HyStar 3.2 software was optimized to detect the exact mass and mass fragmentation pattern of each eluted compound. TIC spectra were acquired and elaborated using the HyStar software data analysis module. MS/MS experiments were carried out by means of auto scanning mode, where the mass spectrometer software made a choice in real time about the selection of ion to fragment based on the intensity of each peaks with a threshold set above 1500 absolute counts. Optimized parameters consisted in collision energy 10 eV, focusing potential of 350 V_{pp} (Voltage per peak), transfer time of 80 μs, pre pulse storage of 5 μs the instrument was operated in the negative ion mode with a capillary voltage of 3.5 KV, capillary temperature was 280°C, sheath gas (N₂) flow rate was 6 L/min and the data were acquired in the AutoMSn scanning modes. Scan range was m/z 50–1500; number of microscan was set at 3 (Arun et al., 2014).

RT-PCR

Expression of nucleolin, bcl₂, cytochrome c and β-actin in fraction 3 treated MCF-7 cells were determined by semi-quantitative RT-PCR. Total RNA or poly (A) + selected RNA were used as template for RT-PCR and primed with random primers, oligo (dT), or a gene-specific primer (GSP) using a reverse transcriptase.

The MCF-7 cells (1×10^5 cells/group) were treated with no (control) or with different concentration (10, 20, 40 μg) of ethyl extract sample of *O. stamineus*. After 24 hours of treatment, the medium was completely removed from the flask and the total RNA was isolated using Trizol reagent (Sigma). Using the total RNA as template, cDNA was synthesized as per the manufacturer's instruction. The RT-PCR for apoptotic genes was performed in a total volume of 20 μL reaction mixture containing random primer pairs (forward and reverse 0.5 + 0.5 μL concentration) 1.0 μL, 10x reaction buffer containing master mix (25 mM MgCl₂, 10 mM dNTPs, Taq polymerase 2.5 U) 10 μL, cDNA as template 2 μL and remaining volume is nuclease free dH₂O 7 μL. Amplification cycles consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 40 sec and extension at 72°C for 1 min, for a total of 32 cycles followed by final extension at 72°C for 10 min (Paulpandi et al., 2012).

Fluorescence microscopic analysis of apoptotic cell death

Approximately 1 μL of a dye mixture (acridine orange (AO) and ethidium bromide (EtBr) each 100 mg/mL in distilled water) was mixed with 9 mL of cell suspension

(1×10^5 cells/mL) on clean microscope cover slips. The selected cancer and normal cells were collected, washed with phosphate buffered saline (PBS) (pH 7.2) and stained with 1 mL of AO/EtBr. After incubation for 2 min, the cells were washed twice with PBS (5 min each) and visualized under a fluorescence microscope (Nikon Eclipse, Inc, Japan) at 400x magnification with an excitation filter at 480 nm. Likewise the cells were placed on glass coverslip in a 24-well plate and treated with complex for 24 hours. The fixed cells were permeabilised with 0.2% triton X-100 (50 μL) for 10 min at room temperature and incubated for 3 min with 10 μL of DAPI by placing a coverslip over the cells to enable uniform spreading of the stain. The cells were observed under (Nikon Eclipse, Inc, Japan) fluorescent microscope.

Results

Various fractions were obtained from column chromatography with different solvents, in the order of increasing polarity. Totally 40 fractions were eluted, among the fractions F1 to F6 obtained from ethyl acetate were subjected to cytotoxic effect against MCF-7 cells. The IC₅₀ values for the fractions were at 33.0, 85.2, 28.5, 84.7, 67.2 and 56.4 μL/mL respectively (Figure 1). Of the six fractions F3 (IC₅₀ 28.5 μL/mL) represents good cytotoxic effect against MCF-7 cells. Thus, F3 was subjected to LC-MS analysis, presence of isoflavanones- biochanin, eleutherol-naphthofuran, and cinnamic acid which were detected in LC/MS/MS analysis (Figure 2).

Phytochemical investigation on ethyl acetate fraction 3 of the *O. stamineus* led to the identification of a complex mixture of flavonoids predominantly containing biochanin A-O-rhamnoside, eleutherol and cinnamic acid-o-hexoside. Using negative ionization mode these base peaks were identified as deprotonated molecule [M-H]⁻. The retention time and the mass to charge ratios (m/z) of the base peaks and its respective fragmented peaks

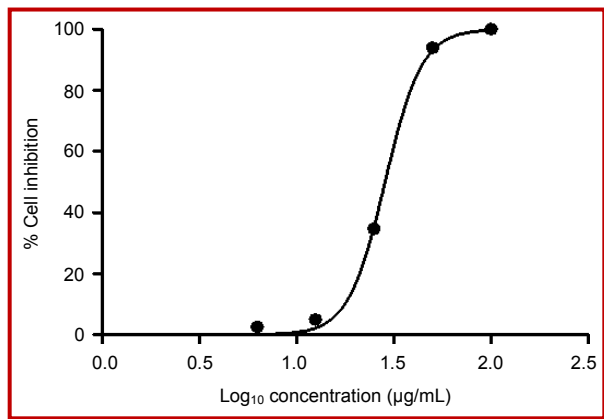


Figure 1: Cytotoxic effect of *O. stamineus* ethyl acetate fraction 3 on MCF-7 cells

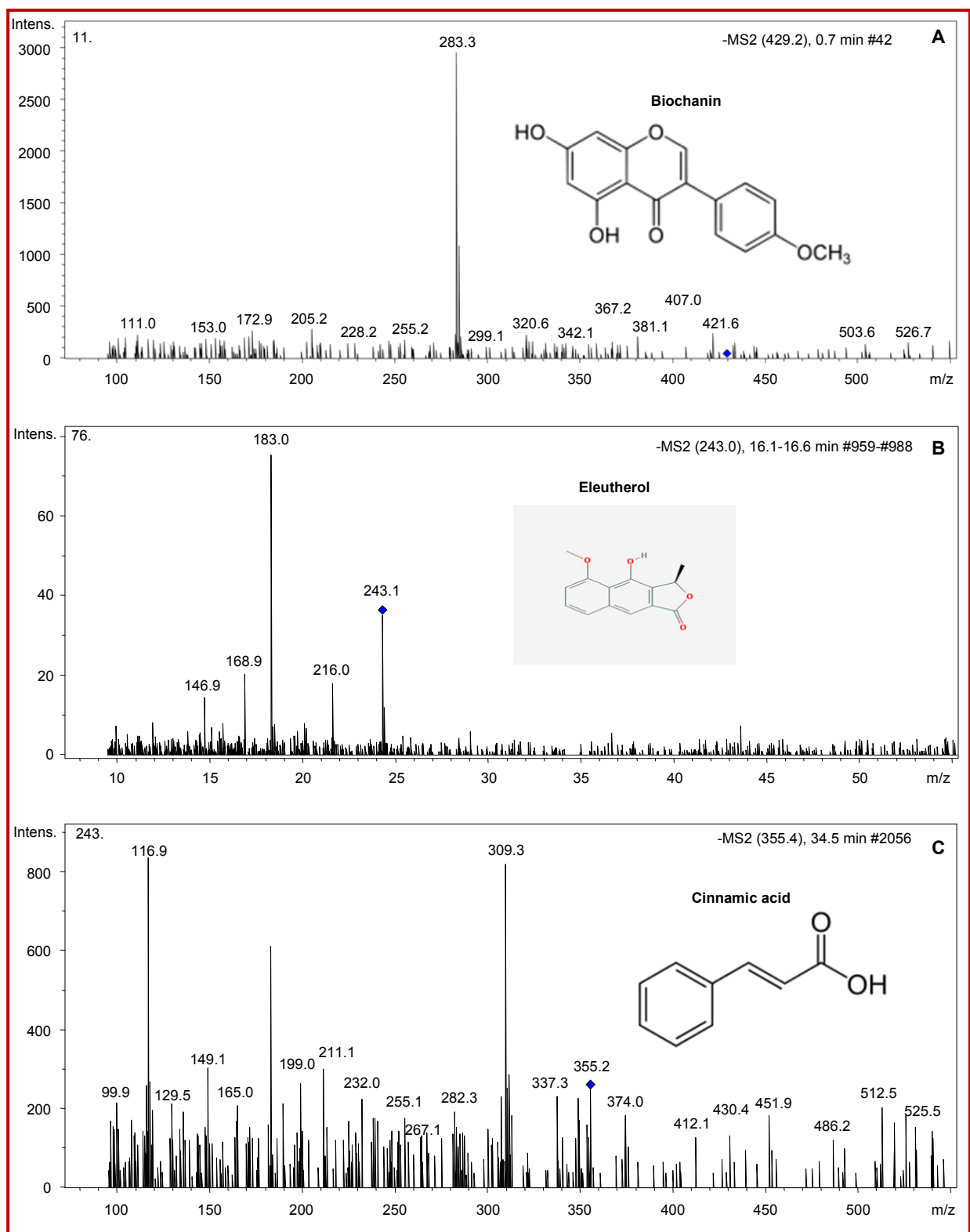


Figure 2: LC-MS/MS spectrum of ethyl acetate fractions of *O. stamineus* leaves. A) Biochanin, B) Eleutherol and C) Cinnamic acid

Table I				
ESI-MS and ESI-MSMS product ions of fractions from ethyl acetate extract of <i>O. stamineus</i> leaves				
Identified compound	Rt [min]	Mass	Parent ion [M-H] ⁻	MSMS [Product ion]
Biochanin A-O- rhamnoside	0.7	430	429	283, 367, 77, 74
Eleutherol	16.1-16.6	244	243	183, 243
Cinnamic acid-o-hexoside	34.5	356	355	310, 309

are listed in Table I. Most of the phytomolecules identified were eluted at 0.7-34.5 min and then later at 35-45 min. It was also observed that all the identified flavone glucosides were eluted with 21- 32% acetonitrile in acidified water (14-24 min), similarly majority of identified flavones/flavonols were eluted with increase in acetonitrile content nearing to 100% (35-45 min).

It has been observed that a dose-dependent decrease in nucleolin and bcl₂ expression with concomitant increase

in bax and cytochrome c expression in MCF-7 cells (Figure 3). Data was analyzed using image J software and the intensity values were schematically represented by densitometry analysis.

Fluorescence microscopy analysis of nuclear fragmentation

Fluorescence microscopy images of MCF-7 cancer cells in the absence and presence of compounds are shown in Figure 4. From the images, untreated MCF-7 cancer cells (control) did not show any significant adverse

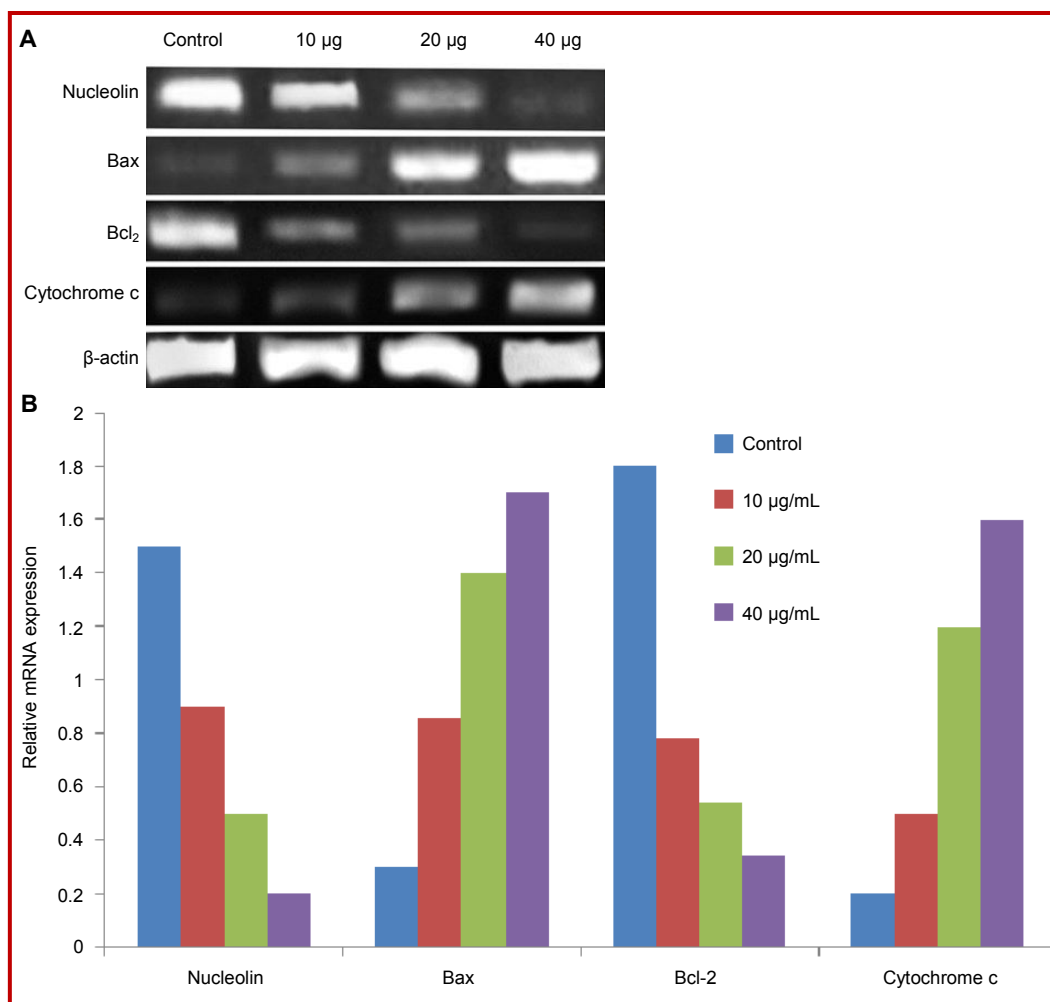


Figure 3: Expression level of nucleolin, bcl₂, cytochrome c and bax in MCF-7 cell extract treated with different concentration of ethyl acetate extract of *O. stamineus* (A); Graphical representation of expression level of nucleolin, bcl₂, cytochrome c and bax was determined by RT-PCR in MCF-7 cell extract (B)

effect as compared to cells treated with fractions. It has also been observed a concentration dependent change in apoptotic effect which is significant.

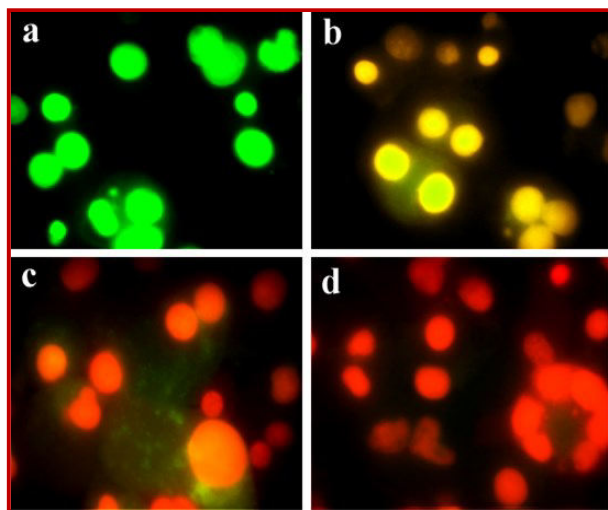


Figure 4: Fluorescent microscopic analysis of MCF-7 cells treated with ethyl acetate extract of *O. stamineus* leaves; a) Control; b) 10 µg/mL; c) 20 µg/mL; d) 40 µg/mL

Discussion

The cytotoxic effect of fraction 3 on MCF cells was promising. It could be due to the presence of biochanin, eleutherol and cinnamic acid in the extract. These compounds were reported as antitumor and antioxidant agents. Decreased level of bcl₂ and nucleolin with concomitant decrease in bax and cytochrome c could induce apoptosis in MCF cells. However, the exact mechanism of down-regulation of bcl₂ and nucleolin and up-regulation of cytochrome c and Bax by the identified compounds remains to be explored.

The biochanin A, the flavanoid compound 1 which is isolated from ethyl acetate fractions of *O. stamineus* was identified by LC-MS with the published data in literature (Medjakovic and Jungbauer, 2007).

The occurrence of biochanin A in large quantities in the leaves of *O. stamineus* isoflavonoids have been screened for their pharmacological effects such as anti-inflammatory, antitumor, hypotensive, locomotor and bioassay studies were reported. Studies showed that cell cycle analyses of biochanin A treatment showed an obvious delayed mitosis and this phenotype was associated with the elevation of p21. Elevation of p21 after PLK-1 inhibition might be a general phenomenon in normal and cancer cells (Kreis et al., 2009). In normal cells, stabilization of p21 induces PLK-1 depletion, which causes the elevation of p53. In cancer cells (p53-defective), PLK-1 depletion reduced Mdm2 expression, a negative regulator of p21 protein stability (Lei and Erikson, 2008). Thus, anti-cancer effects of biochanin A was associated with down-regulation of PLK-1 and up-regulation of p21 mechanism.

Researchers suggest that biochanin A selectively inhibits SK-BR-3 breast cancer cells as compared to MCF-10A normal breast epithelial and NIH-3T3 normal fibroblast cells. It also indicates that biochanin A has a biphasic effect on SK-BR-3 cell viability with increased cell viability at subapoptotic concentrations (2–20 µM) and a dose-dependent inhibition of cell viability at higher concentrations (Sehdev et al., 2009).

Eleutherol is a naphthofuran, one of its derivatives eleutherine showed interesting inhibitory activity against human topoisomerase II, and compounds with this action can be considered as part of first-line chemotherapy for a wide variety of tumors (Kusuma et al., 2010).

Cinnamic acids are abundant in various natural resources. Cinnamic acid and its natural analogues are known for the treatment of cancer for over centuries. 2-Methyl cinnamide isolated from a fermentation beer of *Streptomyces griseoluteus*, showed significant anti-invasive or antimetastatic effects (Del et al., 2011). Natural products bearing the cinnamoyl moiety have attracted much attention due to their broad spectrum of biological activities and low toxicity. Additionally, trans-cinnamic acid derivatives, both isolated from plant sources and synthesized, are well known for their antioxidant (Chung and Shin, 2007), antitumor (Bezerra et al., 2006), antimicrobial (Naz et al., 2006) and antimycobacterial properties (Carvalho et al., 2008). Cinnamic acid derivatives, especially those combining the cinnamoyl moiety with hydroxyl groups, present strong free radical scavenging properties.

Bcl₂ protein is an anti-apoptotic protein, its overexpression has been implicated in different type of cancer and associated with resistance to chemotherapy (Myer et al., 1997). Overexpression of bcl₂ protein is the result of enhanced stability of bcl₂-mRNA, which is controlled by AU rich element present in the 3' UTR region. Nucleolin is highly conserved among eukaryotes and is ubiquitously expressed. It has been reported that nucleolin protein binds with AU rich elements of bcl₂-mRNA and thereby increases the stability of the mRNA (Ishimaru et al., 2010). We observed a dose-dependent decreased level of the expression of nucleolin and bcl₂, on contrary, increased the level of bax and cytochrome c in MCF-7 cell. Bax, a bcl₂ family protein, interacts with bcl₂ protein and induces apoptosis. Bax is reported to increase the opening of the mitochondrial voltage-dependent anion channel, which leads to the loss in membrane potential and the release of cytochrome c. The increase in bax and cytochrome c is in accordance with the previous findings (Gómez et al., 2013).

AS1411, an aptamer which is a short G-rich oligonucleotide, forms G-quadruplex structure which is resistant to serum enzymes. This compound was reported to decrease the level of nucleolin and bcl₂ in MCF-7 cells (Sridharan et al., 2008). Baicalein, a flavanoid, induces

apoptosis in cancer cells.

Most of the studies reveal that the bcl₂ protein prevents apoptotic mechanism, whereas bax protein induce the cell death in cancer cells. Down-regulation of bcl₂ leads to the release of cytochrome c from mitochondria to the cytosol, resulting in cell death. Eriocalyxin B inhibits the proliferation and induces apoptosis through down-regulation of bcl₂ (Rasul et al., 2013). Studies examined that the cellular levels of cytochrome c, caspase 3, PARP, p53, bax, bcl₂ and β-actin expression after baicalein treatment. This compound was reported to increase the expression of bax and cytochrome c (Krishnamoorthy et al., 2016).

Conclusion

The ethyl acetate fraction of *O. stamineus* leaves revealed that the presence of biochanin, eleutherol and cinnamic acid. A marked decrease of nucleolin and bcl-2 expression level was observed in MCF cells treated with the fraction. It is clear that ethyl acetate fraction showed up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes, which in turn induces apoptosis. This is the first report eleutherol isolated from the leaf of *O. stamineus*.

Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

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Author Info

Sivakumar Ramalingam (Principal contact)

e-mail: rsiva@src.sastra.edu