Research article

Augmentation of in vitro cytotoxic potentiality of crude methanolic leaf extract of *Olea dioica* as a pivotal anticancer source

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ABSTRACT

Introduction and Aim: Tumor is the major cause of death world-wide, with an approximate 10 million fatalities in 2020, or almost one in every six deaths. The concerns with the current commercially available anticancer medications’ low selectivity and persistent side effects have driven the research of safer and more effective chemotherapeutic drugs. The current work is focused on determining the cytotoxic potential of crude methanolic extract of *Olea dioica* leaves against various cancer cell lines viz., A549 (human lung cancer), HCT116 (human colorectal cancer), HeLa (human cervical cancer), MCF-7, and MDA-MB-231 (human breast cancer).

Materials and Methods: The cells were treated with crude methanolic extract at five different concentrations viz., 25, 50, 100, 250 and 500 µg/ml to assess the cell viability.

Results: The results from the assay performed for five cell lines have shown that methanolic extract of *O. dioica* has an exponential cytotoxic potential on HeLa cell line followed by MCF-7 (IC₅₀ value 81.05), HCT116 (106.86) and MDA-MB-231 with IC₅₀ value 81.05, 106.86, 141.34 and 499.24 µg/ml respectively, whereas the A549-human lung cancer cell has shown no cytotoxic activity.

Conclusion: The methanol extract of the *O. dioica* leaf could be recognized as a pivotal source of anticancer compounds, but furthermore research is needed to investigate the chemical components of *O. dioica* extracts that are mainly accountable for anticancer action and to expound more precise molecular processes of cell death.

Keywords: Anticancer; HeLa cell line; breast cancer; MTT assay; *Olea dioica*; methanol.

INTRODUCTION

Tumor is distinguished by undifferentiated/abnormal cell growth and has become one of the most devastating disease globally, causing around 10 million fatalities in 2020, or almost one in every six deaths (1). Breast cancer, followed by lung cancer, is the most common cancer diagnosed worldwide, with an expected 2.3 million new cases 11.7%, followed by lung 11.4%, colorectal 10.0%, prostate 7.3%, and cervical 3.1% tumors (2).

Some of the chemotherapeutic medications currently in use, including paclitaxel for breast cancer, vinca alkaloids, flavopiridol have potential on leukemia, colorectal cancer respectively and cisplatin for lung cancer, were originally produced from plants (3-5). There are still no curative treatments or preventative measures available for the management of malignant illnesses, despite extensive research carried out over the previous decades. Standard cancer treatment often involves the use of cytotoxic medications, radiation, chemotherapy, and surgery(6). But these treatments are expensive, which causes a financial burden on individuals and very painful procedures. Also, conventional pharmaceutical drugs will have side effects on diseased people. Hence, the present strategies have concentrated on using edible medicinal plants as sources of supplies that could successfully manage cancer. As a result, to better understand how cancer develops, to detect it early, and to provide effective cancer treatments, future curative techniques should be developed (7).

The findings of numerous investigations show that medicinally significant plants have provided bioactive compounds for the management of cancers (8). Alternative medications, such as herbal treatments, are frequently used by cancer patients receiving palliative care. The oldest and well-established of these treatments, traditional medicine, have been practiced from ancient times. A distinct herbal extracts and mixtures had been developed to treat various disorders, including cancer (9). As a result, the present study is being conducted to determine the potential anticancer activity of leaf methanolic crude extract of *O. dioica* in cultures of the human cancer cell lines A549-Human Lung Cancer, HCT116-Human Colorectal Cancer, HeLa-Human Cervical Cancer, MCF-7, and MDA-MB-231 Breast cancer.

The genus *Olea* has about 34 species that are discovered for different activities based on ancient established applications. These plants’ bioactive metabolites have shown efficient anticancer activity(10). Among them, *O. dioica* is a moderate

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tree native to South Indian evergreen and semi-evergreen forests, primarily the Western Ghats. This plant bark is tough and looks brownish in color. The leaves are simple, arranged in opposite, and elongated with serrated edges; the leaf apex is pointed; the inflorescence consists of axillary divaricate spikelets; the flowers are polygamoecious and creamy white in colour; and the fruit is a fleshy drupe with one seed that becomes black when ripe. Flowers blossom from February through April (8). The high antioxidant effectiveness of O. dioica leaf extract was previously reported in a research study (11), as also with its antimicrobial (12), anticholinesterase (13), and analgesic activities (14). A cytotoxic study was reported on the bark and leaves of O. dioica for colorectal and breast cancers using ethanol solvent extract (8,15). However, none of the studies reported on methanolic crude extract with different cancer cell lines.

The MTT test is a spectrometric assay that is widely used to assess the cytotoxic effects of numerous chemicals, medicines, environmental contaminants, and plant extracts. This in-vitro assay measures cell survival after toxicant exposure and acts as a marker of antiproliferative activation, cell activation, and cytotoxicity (16). The current work examines the in-vitro cytotoxic potential of several cell lines using the MTT assay of methanolic crude extract of O. dioica leaves.

MATERIALS AND METHODS

Chemicals

Methanol, DMEM-F12-(AL127G, Himedia), Fetal Bovine Serum (RM10432, Himedia), DMEM High Glucose-(AL007A, Himedia) D-PBS (Phosphate Buffer Saline) (TL1006, Himedia) DMSO (PRH1309, Sigma), MTT reagent (5 mg/ml) (4060 Himedia), Quercetin (Q4951, Sigma), and trypsin-EDTA.

Preparation of crude extract

The freshly collected O. dioica leaves were washed twice with dH2O, shade dried, and powdered into fine particles. The powdered 100 g sample was subjected to the maceration method of extraction using 500 ml of methanol as the extraction solvent for each cycle keeping on a rotary shaker for 72 hours at room temperature. Cycles were repeated until the colorless mixture appeared (17). The rotary evaporator at 40°C after being filtered with Whatman Grade-1 filter paper was used to concentrate extracts. The concentrated extract was kept at 4°C for further examination (18).

Assessment of cell proliferation by MTT assay

Cell lines

A549-Human Lung cancer cell line, HCT116 (Human colorectal cancer), Hela (Human Cervical cancer) MCF-7 and MDA-MB-231 (Human Breast cancer) cell line was produced from NCCS (National Centre for Cell Science), Pune.

Cell culture

The cytotoxicity of Olea dioica plant crude extract was tested on five cell lines. For the development, DMEM (Dulbecco’s modified eagle medium) was supplemented with 10 percent v/v FBS, 2 mM L-glutamine, 40 μg/ml of gentamicin, 100 units/ml penicillin, and 1040 μg/ml of streptomycin. An approximate of 1 x 10^4 cells per well in 100 μl aliquots of medium on 96-well cell culture plates are seeded (17). The cells were allowed to adhere for 24 hours. The incubator was regulated at 37°C and 5% CO2(19). After washing twice with PBS (phosphate buffer saline), cells were treated for a few minutes with 0.25 % trypsin/1 mM EDTA (ethylene di-amine tetra acetic acid). Finally, new serum-containing media was added to the detached cell to inactivate trypsin (19,20).

Drug preparation for treatment

The crude methanolic extract of O. dioica leaf, was analyzed for cell antiproliferative against Human lung cancer, breast cancer, colorectal cancer, and human cervical cancer cell lines. These cells were treated with a series of concentrations (25, 50, 100, 250 and 500 μg/ml) of crude samples were prepared in DMEM (100 μl) containing dimethyl sulfoxide (DMSO), maximum; 0.01 %) cultured and incubated at 37°C for 48 h, and the cellular morphology was observed. Following incubation, the cells were observed under LYNX inverted phase contrast microscope (24). Following the first screening findings, the MTT test was used to determine apparent IC50 values (17).

Cytotoxic MTT assay

Following the 24 h test period, toxic endpoints were estimated using the spectrometric technique methyl-thiazolyl tetrazolium (MTT) assay (17). The methanolic crude extract of O. dioica leaves was subjected to an MTT assay using five different cell lines; A549-(human lung cancer), HCT116-(human colorectal cancer), HeLa-human cervical cancer, MCF-7, and MDA-MB-231 (human breast cancer) to examine the cell viability of each cell to break the tetrazolium salt MTT. Cell suspensions of each cell were seeded into 96-well plates at required cells of 2 x 10^4 without a test agent. Allow the cells to grow for about 24 h. The cancer cells used in the assay were treated with appropriate concentrations of methanolic crude extract test agents (25, 50, 100, 250 and 500 μg/ml) and quercetin was used as a standard at about 10 μM concentration. The plate was incubated for 24 hours at 37 °C in a 5% CO2 atmosphere(17). After incubation, the plates were washed twice with phosphate buffer saline (PBS) to isolate spent media. To a total volume concentration of 0.5 mg/ml, 10 μl of

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MTT reagent was added. Incubated the plate for 3 hours, wrapped in aluminium foil to prevent light exposure(21). The incubation time varies depending on the cell line (while performing comparisons, keep incubation period consistent within one experiment). After the incubation, the cells were washed with PBS twice to remove MTT reagent, and then 100 µl/well DMSO was added as a solvent to solubilize the insoluble crystalline formazan products. Gentle stirring in a gyratory shaker or pipetting up and down will enhance the dissolution of MTT formazan crystals. The absorbance was recorded using an ELISA reader at 570 nm wavelength. Each experiment was performed In triplicate to calculate the standard error of the mean (7). The concentration necessary to inhibit cell viability by 50 % was designated as the half-maximal inhibitory concentration (IC₅₀). The linear regression equation, \( y = mx + c \), was used to calculate the IC₅₀ value. The viability graph was used to obtain \( y = 50, m, \) and \( c \) values (22). To compute the standard error of the mean. The following method was used to calculate the percent growth inhibition of control (untreated) cells compared to test (treated) cells (17).

\[
\% \text{ Growth inhibition} = \frac{\text{Control} - \text{actual absorbance}}{\text{Control}} \times 100
\]

Statistical analysis

The result obtained is shown as mean SD (standard deviation). The MTT test was conducted in triplicate. The statistical data was shown as standard error of the mean. The statistical software GraphPad prism version 9.0 for windows was used. The Tukey’s test was used along with one-way ANOVA (Analysis of variance)

RESULTS

In vitro cytotoxic activity of methanolic extract of O. dioica by MTT assay

The methanolic crude extract of O. dioica leaves was tested for antiproliferative against five human cancer cells at various concentrations to calculate the IC₅₀ value (50% growth inhibition) using MTT assay. The results of O. dioica methanol leaf extract tests against different cells at various doses (25, 50, 100, 250 and 500 µg/ml) are recorded.

Cytotoxic activity against MCF-7 and MDA-MB-231 cell lines

The MTT result revealed the viability of cancer cells decreases with increasing plant extract concentration. The cell viability of MCF-7 and MDA-MB-231 (human breast cancer) cells was unaffected by O. dioica methanol leaf extract concentration of 25 g/ml after 24 h. The maximum growth inhibition of MCF-7 was observed at 250 µg/ml with 31.8 % and 500 µg/ml with 26 %. The IC₅₀ value of this extract on MCF-7 was 106.86 µg/ml. The MDA-MB-231 showed 64.1 and 45.8 % were highest, at 250 and 500 µg/ml concentration, respectively with an IC₅₀ value 499.24 µg/ml (Table 1). The inhibition of viable cell counts of MCF-7 and MDA-MB-231 cancer cell lines from O. dioica leaf extract as represented in Figs 1 and 2.

Fig. 1: The MTT assay of O. dioica methanol leaf extract against MCF-7 cell lines
Cytotoxic activity against colorectal cancer (HCT-116) cell line

The cytotoxic effect of methanol extract against human colorectal cancer (HCT116) cells was performed at different concentrations. The survival rate of cells is reduced with increase in the concentration of leaf extract. The results showed that at increased concentrations of 250 and 500 µg/ml, inhibited the growth of HTC116 cells in a dose-independent manner, with 33.6 and 26.2% viability at 24 h. The IC₅₀ value of the extract was 141.3 µg/ml. Fig. 3 clearly depicts that the cell viability of HCT-116 cell lines was inhibited as the concentration of leaf extract increased.

Cytotoxic activity against cervical cancer (HeLa) cell line

Using the MTT assay, a potential antiproliferative effect on HeLa cell line was achieved in a concentration range 25 to 500 µg/ml. At 250 µg/mL concentration, the survival of HeLa cells decreased from 100% to 31.7 percent. The potential antiproliferative activity of this extract against HeLa cells was found at a 500 µg/ml concentration with 22.4% cell growth inhibition over a period of 24 h. The IC₅₀ value on HeLa cells was 81.05 µg/ml by MTT assay, as detailed in Table 1. The O. dioica methanol leaf extract showed a significant cytotoxic potential for all the cell lines when administered at a concentration greater than 250 µg/ml as shown in Fig. 4.
Table 1: Cytotoxic activity of *O. dioica* methanolic leaf extract against HeLa, MCF-7, HCT116, MDA MB 231 and A549 cancer cell lines at different concentrations by MTT assay

<table>
<thead>
<tr>
<th>Cell viability %</th>
<th>Untreated</th>
<th>Standard</th>
<th>25 µg/ml</th>
<th>50 µg/ml</th>
<th>100 µg/ml</th>
<th>250 µg/ml</th>
<th>500 µg/ml</th>
<th>IC_{50} µg/ml</th>
</tr>
</thead>
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<tr>
<td>HeLa</td>
<td>100</td>
<td>38.39506173</td>
<td>72.83950617</td>
<td>52.34567901</td>
<td>46.79012346</td>
<td>31.72839506</td>
<td>22.46913580</td>
<td>81.05</td>
</tr>
<tr>
<td>MCF-7</td>
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<td>24.33333333</td>
<td>82.13333333</td>
<td>58.93333333</td>
<td>48.8</td>
<td>31.86666667</td>
<td>26.06666667</td>
<td>106.86</td>
</tr>
<tr>
<td>HCT116</td>
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<td>49.71772676</td>
<td>89.34888973</td>
<td>72.45013173</td>
<td>56.19119307</td>
<td>33.68460670</td>
<td>26.23259315</td>
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</tr>
<tr>
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<td>97.0593719</td>
<td>88.95976447</td>
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<tr>
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<td>56.91158157</td>
<td>51.93026152</td>
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</tbody>
</table>

Fig. 4: The MTT assay of *O. dioica* methanol leaf extract against HeLa cervical cancer cell lines

Fig. 5: The MTT assay of *O. dioica* methanol leaf extract against A549 lung cancer cell lines

Fig. 6: Cell viability % of *O. dioica* methanolic leaf extract against HeLa, MCF-7, HCT116,
MDAMB-231 and A549 cancer cell lines at different concentrations by MTT assay

Cytotoxic activity against A549 lung cancer cell lines

The cytotoxic effect of methanol extract against A549-human lung cancer cells was studied at different concentrations. The survival rate of cells did not decrease even with the increased concentration of leaf extract. The result showed that there was no significant inhibition of the growth of A549 cancer cells. Though the concentrations increased in a dose-independent manner at the concentrations of 250 µg/mL and 500 µg/mL, the survival of the cells was 56.9 and 51.9% of viability at 24 h (Fig. 5). The IC₅₀ value of the extract was not observed.

Although *O. dioica* methanol leaf extract was treated against the cell lines HeLa and HCT-116, they have shown the greatest cytotoxic effect among all cell lines in comparison with the standard drug quercetin (Fig. 6). The morphological differentiation of each cell line is shown in Fig. 1-5, which confirms that after treatment with leaf extract, the size of the cells reduced and they became detached from the surface, suggesting that the methanol extract of *O. dioica* induced potent cytotoxicity in all the cell lines dose-dependently. The anti-cancer characteristics are found in all five treated cell lines, but seemingly more prominently in HeLa cervical cancer and HCT-116 colorectal cancer cells.

**DISCUSSION**

From ancient years, medicinal herbs have been used in the prevention and treatment of tumors, and various therapeutically potent plants with anticancer activity have been described in the literature. Herbal screening medications may tend to the identification of novel mutagenic agents that can be used as an analogue to expensive antiproliferative chemotherapy treatments. Some medicinal plants have received interest as potential for cancer treatment due to their low noxious and inexpensive cost (23).

The *Olea* plants had been used to cure various diseases, including cancer, since ancient times. Several additional species present in genus *Olea* have already been found to be cytotoxic against many human cancerous cells. There have been relatively few investigations into the species *O. dioica*, which possesses highly therapeutic characteristics attributed to secondary metabolites such as alkaloids, carotenoids, tannins, flavonoids, phospholipids, and phenolics with diverse biological activities (12).

This study aims to evaluate the cytotoxic potential of *O. dioica* methanol leaf extract on human cancer cell lines; A549-(human lung cancer), HCT116-(human colorectal cancer), HeLa-(Human cervical cancer), MCF-7, and MDA-MB-231 cell lines. To compare the anti-cancer effects of leaf extract on cell viability to a routinely used anti-cancer drug, we chose quercetin, an anti-cancer agent that has been recorded in various *in vitro* and *in vivo* studies that performed on different cell lines and animal models. *O. dioica* crude methanol leaf extract was treated to evaluate the cellular viability of five cell lines in concentrations of 25, 50, 100, 250 and 500 µg/ml after 24 h. However, concentrations of 250 µg/ml and 500 µg/ml showed a highly significant cytotoxicity effect on HeLa and HCT-116 cells.

The cell viability is dependent on the concentrations treated, with the cytotoxic effect increasing with the rise in concentration levels and resulting in increased toxicity. As a result, the findings show that cytotoxicity increases in direct proportion to the concentration treated. *O. dioica* methanol leaf extract at a concentration of 500 µg/ml and an incubation period of 24h provide the best cytotoxic effect on HeLa and HCT-116 cells. In lower concentrations, the cytotoxic effect of the sample was very low (Fig. 6).

The MTT assay is a sensitive, quantitative, and reliable colorimetric assay, where the cell viability was measured to determine the cytotoxic ability of a test sample. The test relies on the ability of living cells’ mitochondrial dehydrogenase enzyme to metabolize the yellow water-soluble substrate methyl-thiazolyl tetrazolium (MTT) into a blue or purple formazan product which is insoluble in water. In a variety of cell lines, the total volume of formazan generated is directly proportional to the cell number (24).

Previously, the cytotoxic capability of *O. dioica* plant ethanol extracts on DLA (Dalton lymphoma ascites carrying mice) and Ehrlich’s ascites carcinoma (EAC) in Swiss albino mice was tested by the MTT assay, and they have shown outstanding efficacy (15). The methanolic leaf extract was tested for cell viability on HT-29 and MDA-MB-231 cells, yielding IC₅₀ values of 131.87 and 140.25 µg, respectively (8). In contrast, our study used HTC116 and the same MDA-MB-231 cells. The results were observed at 141.34 µg/ml respectively (Table 1). Similarly, the methanolic leaf extract was tested for cell viability on MCF-7 human breast cancer cells and HeLa-human cervical cancer. The IC₅₀ values were 106.86 and 81.05 µg/ml. Four cancer cells showed substantial activity when compared to the IC₅₀ values of the five cell lines, except for the A549-human lung cancer cell, which did not indicate the influence of cell viability. The cytotoxic effect among all five cells, the HeLa-human cervical cancer cell, has shown outstanding efficacy with an IC₅₀ value of 81.05 µg/ml.

Breast tumor is the most ubiquitous type of cancer in women and the most diagnosed, it begins when cells in the breast (such as those lining the ducts and lobules) begin to develop abnormally. The various

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therapies for this malignancy are determined by the stage of the disease. It might include chemotherapy, radiation, hormone treatment, and surgery (mammography). Colorectal cancer (CRC) is among one of the most ubiquitous types of cancer in the world. It is the second most common cancer in women (9.2 %) and the third most prevalent cancer in males (10 %). The popular of CRC instances are seen in Western nations (55 %), but this trend is changing (25). Cervical cancer is the fourth most frequent female cancer in the world. It is caused by a chronic infection with the human papilloma virus (HPV). Chemotherapy, radiation, hormone therapy, and surgery are the most common therapies. Treatments for the impoverished are difficult to get due to their high cost. Choosing natural sources such as medicinal plants for therapy is thus the best strategy in all aspects. The study chose O. dioica as the plant source because there has been no countable research on this plant. The current investigation was conducted to validate the cytotoxic potential against a subset of cancer cells.

The morphological differentiation of each cell line is shown in Fig. 1-5, which confirms that after treatment with the leaf extract, the cell size reduced to smaller and detached from the surface, indicating that the methanol extract of O. dioica induced potent cytotoxicity in all cell lines dose dependently. All five treated cell lines exhibit anti-cancer properties, but HeLa-cervical cancer and HCT-116 colorectal cancer cells appear to be more prominent. Hence, the findings of this study demonstrated that the plant extract could be developed as an anti-cancer drug.

CONCLUSION

Herbal medication screening is a key to the identification of novel mutagenic agent that can be used as an analogue to expensive antiproliferative chemotherapy treatments. Few medicinal plants have received interest as potential cancer treatments due to their low noxiousness and inexpensive. The MTT assay is a sensitive one among cancer assays, quantitative, and reliable colorimetric assay that measures cell viability. The quantity of formazan produced complex is directly proportional to the cell number in a range of cell lines. The methanolic leaf extract of O. dioica was tested for cell viability on A549-(human lung cancer), HCT116-(human colorectal cancer), HeLa-(human cervical cancer), MCF-7, and MDA-MB-231 human breast cancer cell lines. Compared to the IC50 values of the five cell lines, four cancer cells have shown significant activity, except for the A549-human lung cancer cell. In conclusion, the utilization of crude methanolic extract allowed us to determine the antiproliferative significant phytochemicals from medicinal plants from the Western Ghats of Karnataka. Future studies will concentrate on identifying the chemical components of O. dioica extracts relevant for anticancer effects as well as clarifying more detailed molecular pathways of cell apoptosis.

CONFLICT OF INTEREST

Authors declare no conflicts of interest.

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