Cytotoxic and antiproliferative potential of methanolic extract of *Mallotus philippensis* in MCF-7 cell line

Bimitha Benny, A Sandesh Krishna, Sujith Samraj, Preethy John, Uma Radhakrishnan

**ABSTRACT**

*Mallotus philippensis* is a shrub used for anti-filarial anti-bacterial anti-inflammatory anthelmintic and immune-regulatory purposes for centuries in the current study methanolic extract of *M. philippensis* leaves was examined *in vitro* for cytotoxicity and apoptotic potential in (MCF-7), breast cancer cell lines Various biochemical tests used to investigate the phychochemical contents of the plant extract. Qualitative phytochemical analysis of *M. philippensis* leaves revealed the presence of phenolic compounds steroids flavonoids alkaloids diterpenes and tannins (MTT), dye reduction test was used to assess the cytotoxicity of the methanol extract of *M. philippensis* (MCF-7), cells were grown in (RPMI), medium supplemented with (10%), serum and (1%), antibiotic and antymycotic solution *M. philippensis* leaf extract reduced cell viability in a dose-dependent manner, with a half maximal inhibitory concentration (IC50), of (190g/mL), Acridine orange Ethidium bromide and fluorobupe benzimidazole-carcobacaine iodide 5, 5', 6, 6'-tetrachloro-1, 1', 3,3'-tetra ethyl (JC-1), staining were used to examine the influence of the extract on the development of apoptotic characteristics in (MCF-7), cells. The (AO/EB), staining revealed apoptotic cells with orange-red fluorescence in extract-treated cells which increased in a dose-dependent manner whereas control cells showed green fluorescence. Control cells stained with (JC-1), dye fluoresced red-orange whereas cells treated with extract showed red-green fluorescence whose intensity changed in a dose-dependent manner The methanolic extract of *M. philippensis* leaves showed *in vitro* cytotoxic potential as well as induced apoptotic cell death in human breast cancer (MCF-7), cell lines and may be studied further to generate a potent anti-cancer drug.

**Keywords:** *Mallotus philippensis*, (MCF-7), Cell line, Breast Cancer, (MTT), Assay, Apoptosis.

**INTRODUCTION**

Cancer is a condition in which certain cells grow out of control and invade the rest of the body The suppression of programmed cell death also known as apoptosis is critical for cancer formation and suppression of apoptotic mechanisms can intensify resistance to treatment. Breast cancer is one of the most prevalent types of cancer and a complex disease that primarily affects women Chemotherapy, hormone radiation therapies and surgical treatment are currently available options for treatment of breast cancer They have shown benefit in patients but have frequently resulted in adverse and harmful health risks [1]. Cancer cell lines are frequently employed as a preclinical model for anticancer drug development (MCF-7), a commonly used cell line is estrogen receptor (ER), positive, progesterone receptor (PR), positive and human epidermal growth factor receptor (HER), negative. About (60%), of anticancer drugs are developed from medicinal herbs, and there are still a variety of species that have anticancer properties which have not been scientifically validated [10]. As a result, the use of natural therapies is an alternative to the adverse effects of synthetic drugs [6].

*Mallotus philippensis* (Euphorbiaceae family), is a forest a dye-producing plant native to the Indian subcontinent that is commonly referred to as “Kamala” Whole plant parts are rich in bioactive compounds which provide the plant's therapeutic properties. Different parts of this plant are claimed to have medicinal values such as antibacterial antifungal antileukemic and hepatoprotective based on folk tale practices [5]. Nevertheless, this plant has not been evaluated for its *in vitro* cytotoxic and apoptotic potential against breast cancer cell lines Hence we planned to evaluate cytotoxic potency of *Mallotus philippensis* against (MCF-7), breast cancer cell lines.

**MATERIALS AND METHODS**

**Plant Extraction**

The leaves of *M. philippensis* were collected from poked, Wayanad district, Kerala, India. The plant materials were authenticated by the (MSSRF), Kalpetta. The leaves of *M. philippensis* were shade-dried, powdered using blender and the powder was extracted with methanol using a Soxhlet extraction apparatus The methanol extract was subsequently concentrated using a rotating vacuum evaporator at
low pressure and temperature 40°C the solvent was completely evaporated and frozen in an airtight container until further usage.

Cell line and culture conditions

Adherent human breast adenocarcinoma cell line (MCF-7), procured from National Centre for Cell Science Pune Maharashtra India was utilized for in vitro antiproliferative studies. These adherent cells were cultured in RPMI, 1640 supplemented with (10%), fetal bovine serum and (1%), antibiotic antymycotic solution (penicillin-streptomycin and amphotericin B), in an incubator at 37°C with (5%), (CO₂). The cells were trypsin zed using (0.25%), trypsin/1 mM (EDTA), solution.

Phytochemical analysis

The qualitative phytochemical analysis was performed [6].

Sample preparation

A stock solution of methanolic extract of M. philippensis (MMP), prepared in (10%), tween at concentration (130mg), further diluted using (RPMI), 1640 medium for required concentrations.

In vitro cytotoxic evaluation of (MMP), in (MCF-7), cell lines

In vitro cytotoxic potential of the extract of (MMP), was assessed in (MCF-7), using (MTT), reduction assay as per Rises et al., (2004) [7]. The extract was diluted to (320), (160), (80), (40), (20), and (10µg/ML.), and used for the study (96), well plates were seeded with (1x10⁵cells/mL), and was allowed to proliferate for 24 hours. Then the extract at the desired concentrations was added to the cells again incubated for 24 hrs. After 24 hrs the media was removed (MTT), was added to each well at (10µL), incubated for 4 hours with serum free media. The reaction was stopped by adding (100µL), of (DMSO), and the absorbance was read at (570nm), in a Varioskan (ELISA), Plate reader.

The following formulae were used to compute the percent cell viability and percent cell inhibition: Per cent cell viability = (Average absorbance of treated cells/Average absorbance of untreated cells) × 100

Per cent cell inhibition = 100 - per cent cell viability the net absorbance from the control wells was taken as 100 per cent viable. The IC₅₀ values of extracts were calculated by plotting the concentration against per cent cell inhibition using (AAT), Bio quest.

Selection of concentrations

Based on the (MTT), assay, three concentrations of the extract, i.e., double (IC₅₀), (IC₃₀), and half (IC₀), were selected for the study. Thus, the concentrations that were used for the study were (380), (190), (95µg/mL), for (MCF-7), cells respectively.

Acridine orange / Ethidium bromide (AO/EB) staining

A concentration of (3x10⁵), cells were seeded into a six well cell culture plate and treated with of extract for 24 h. The (AO/EB), staining procedure was followed to detect the live apoptotic and necrotic cells. Twenty-five (µL), of the treated or untreated cells were stained with five µL of acridine orange (10µg/ML), and ethidium bromide (10 µg/mL), and examined under Trinocular Research fluorescence microscope (DM 2000 LED), Leica with blue excitation (488nm), and emission (550nm), filters at (20X), magnification [8].

JC-1 Staining

(MCF-7), cells were plated at a seeding density of (3x10⁵), cells per well in six well plates. After 24 h of treatment with extracts at concentrations (380), (190), (95µg/mL), and the cells were incubated with five M. flor probe, 5. 5', 6. 6'-tetrachloride- 1', 1', 3,3'-tetra ethyl benzimidazole-carboxylic acid (JC-1), for 30 min at room temperature in the dark. The cells were examined using fluorescent microscope with filters having blue excitation/emission of (540/570), nm and red excitation/emission of (590/610 nm), filters (DM 2000 LED, Leica) [9].

Statistical Analysis

All the results expressed as mean + SE with n equal to number.

RESULTS

Phytochemical Analysis

The phytochemical analysis of (MMP), revealed the presence of alkaloids flavonoids tannins diterpenes steroids and phenolic compounds (Table 1).

Table 1: Depicted various phytochemicals present in the MMP

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>MMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroids</td>
<td>Present</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Present</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>Present</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Present</td>
</tr>
<tr>
<td>Tannins</td>
<td>Present</td>
</tr>
<tr>
<td>Diterpenes</td>
<td>Present</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Absent</td>
</tr>
<tr>
<td>Saponins</td>
<td>Absent</td>
</tr>
</tbody>
</table>

In vitro cytotoxic evaluation of (MMP), and Calculation of (IC₅₀)

There was a dose dependent decrease in the viability of cells exposed to different concentrations of extract with the viability being least at (320µg/mL), (Figure 1), (Table 2), shown percent inhibition of (MCF-7), cells after treatment with (MMP). The graph showing the analysis of (IC₅₀), is depicted in Figure. 2, The (IC₅₀), of methanolic extract of M. philippensis was (190µg/mL), as obtained from (MTT), assay.

Table 2: Percent inhibition of cells exposed to (MMP), presented as (Mean±SE), with n=3 replicates

<table>
<thead>
<tr>
<th>Concentrations(µg/mL)</th>
<th>Percent cell inhibition (Mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>320</td>
<td>74.42±0.05</td>
</tr>
<tr>
<td>160</td>
<td>39.08±0.24</td>
</tr>
<tr>
<td>80</td>
<td>35.69±0.19</td>
</tr>
<tr>
<td>40</td>
<td>35.28±0.18</td>
</tr>
<tr>
<td>20</td>
<td>37.33±0.10</td>
</tr>
<tr>
<td>10</td>
<td>33.43±0.04</td>
</tr>
<tr>
<td>IC₅₀(µg/mL)</td>
<td>190</td>
</tr>
</tbody>
</table>

Figure 1. Per cent viability of (MCF-7), cells treated with (MMP)
After treatment with (MMP), live, necrotic, early and late apoptotic cells were detected (Figure 3), represent the images of cells of treatment after (AOEB), staining. Control cells showed greenish fluorescence, with a circular nucleus uniformly placed in the centre. Treatments of cells with half (IC50), doses of the (MMP), resulted, in early apoptotic cells with localized crescent-shaped or granular green stained nucleus. With (IC50), and twice (IC50), concentrations, orange to red fluorescent cells in late apoptotic stage were observed. Treated cells also displayed visible morphological changes, such as membrane blebs, nuclei fragmentation, chromatin condensation and apoptotic bodies.

**JC1 Staining**

(JC1), aggregates with reddish/orange fluorescence were observed in control cells, indicating a higher mitochondrial membrane potential. After 24 h treatment of cells with (MMP), a dose-dependent shift from red-to-green fluorescence was seen, indicating a concentration dependent lowering of mitochondrial membrane potential (figure 4). As lowering of mitochondrial potential in treated cells confirming involvement of intrinsic pathway of apoptosis.

**Acridine Orange/ Ethidium Bromide staining**

After treatment with (MMP), live, necrotic, early and late apoptotic cells were detected (Figure 3), represent the images of cells of treatment after (AOEB), staining. Control cells showed greenish fluorescence, with a circular nucleus uniformly placed in the centre. Treatments of cells with half (IC50), doses of the (MMP), resulted, in early apoptotic cells with localized crescent-shaped or granular green stained nucleus. With (IC50), and twice (IC50), concentrations, orange to red fluorescent cells in late apoptotic stage were observed. Treated cells also displayed visible morphological changes, such as membrane blebs, nuclei fragmentation, chromatin condensation and apoptotic bodies.

**DISCUSSION**

Breast cancer is the most common type of cancer found in women and is highly invasive. The occurrence of molecular markers for oestrogen or progesterone receptors as well as human epidermal growth factor classifies breast cancer into several subtypes Thus the current work focused on the development of novel agents that might be used in the treatment of the (MCF-7), breast cancer cell line, which possess both oestrogen and progesterone receptor and it lack human epidermal growth factor receptor.

Phyto-constituents were evaluated using a variety of biochemical tests. Qualitative phytochemical analysis revealed the presence of alkaloids tannins flavonoids steroids phenolics and diterpenes in (MMP), Natural phenolic compounds have been shown to have cytotoxic properties in human cancer cell lines [10]. Polyphenols and flavonoids have previously been shown to have anticancer properties through inducing apoptosis [11]. Preliminary in vitro cytotoxic activity of (MMP), was determined in (MCF-7), cell line using the (MTT), test. Only live cells can use the (NADH), enzyme to convert yellow-coloured (MTT), into a purple-coloured formazan product. When a cell dies, it loses its ability to convert (MTT), and generate colour. The intensity of colour and the vitality of cells have a linear correlation (MMP), showed growth inhibition of (MCF-7), cells in dose dependent manner with (IC50), value (190µg/mL). The major drawback of (MTT), assay is its inability to distinguish between apoptosis and necrosis as the cause of cell growth inhibition. Apoptosis is an important preventive mechanism against carcinogenesis as it eliminates genetically defective cells. Induction of apoptosis is therefore a highly desired mechanism for cancer management [11]. Drugs kill cancer cells by causing apoptosis, a process whose sensitivity is determined by the drug proportionate to the level of apoptosis [12]. The (AO/EB), staining was done to assess morphological and apoptotic mechanism of cell which gives a clear contrast between live early and late apoptotic cells. The nuclei of normal cells are stained by (AO), penetration which become green via attaching to (DNA), (EB), on the other hand dyes the nuclei of late apoptotic and necrotic cells red and the result shows that (MMP), is inducing apoptosis in a dose dependent manner. The present study shows a dose dependent induction of apoptosis.

The intrinsic pathway of apoptosis is accomplished by increasing the mitochondrial membrane permeability there by releasing the apoptotic factors by a decrease in the mitochondrial trans membrane potential. (JC-1), is a dye that accumulates in the membrane at high trans membrane potential producing red fluorescence and changes from red to green at low transmembrane potential [13]. Cells with integral mitochondrial membrane has high transmembrane potential. In current study (MMP), decreased the transmembrane potential in a dose dependent manner showing possibility for mitochondrial-dependent intrinsic apoptotic pathway.

**CONCLUSION**

The current study showed cytotoxicity of *M. philippensis* in vitro against (MCF-7), cells (AO/EB), and (JC-1), staining showed the potential of *M. philippensis* for inducing apoptosis through the intrinsic pathway. Phytochemical analysis revealed the presence of phenolic flavonoids and terpenoids which contributed for cytotoxicity of (MMP) and hence *M. philippensis* can be a lead molecule for development of an anticancer drug; additional phytochemical studies are required to discover and define the anticancer activities of specific compounds present in the extract.

**Acknowledgements**

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**Figure 2.** Calculation of (IC50)

**Figure 3.** Morphological changes of (MCF-7), cells after treatment with various concentrations of (MMP), by (AO/EB), staining. 200X A-control cells, B-cells treated with half (IC50), C-cells treated with (IC50), and D-cells treated with twice (IC50).

**Figure 4.** Morphological changes of (MCF-7), cells after treatment with various concentrations of (MMP), by (JC-1), staining. 200X A-control cells, B-cells treated with half (IC50), C-cells treated with (IC50), and D-cells treated with twice (IC50).
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Conflict of Interest
None declared.

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REFERENCES


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