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Bimitha Benny
MSc Scholar, Department of Veterinary Biochemistry and Molecular Biology, College of Veterinary and Animal Sciences, Mannuthy-680651, Kerala, India

A Sandesh Krishna
MSc Scholar, Department of Veterinary Biochemistry and Molecular Biology, College of Veterinary and Animal Sciences, Mannuthy-680651, Kerala, India

Sujith Samraj
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy-680651, Kerala, India

Preethy John
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode-673576, Kerala, India

Uma Radhakrishnan
Head, Department of Veterinary Biochemistry and Molecular Biology, College of Veterinary and animal Sciences, Mannuthy- 680651, Kerala, India

Correspondence:
Dr. Sujith Samraj
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy-680651, Kerala, India
Email: sujith@kvasu.ac.in

Cytotoxic and antiproliferative potential of methanolic extract of *Mallotus phillippensis* 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. phillippensis* leaves was examined in vitro for cytotoxicity and apoptotic potential in MCF-7 breast cancer cell lines. Various biochemical tests used to investigate the phytochemical contents of the plant extract. Qualitative phytochemical analysis of *M. phillippensis* leaves revealed the presence of phenolic compounds, steroids, flavonoids, alkaloids, diterpenes and tannins. The MTT dye reduction test was used to assess the cytotoxicity of the methanol extract of *M. phillippensis*. MCF-7 cells were grown in RPMI medium supplemented with 10% serum and 1% antibiotic and antimycotic solution. *M. phillippensis* leaf extract reduced cell viability in a dose-dependent manner, with a half maximal inhibitory concentration (IC_{50}) of 190 g/mL. Acridine orange/ Ethidium bromide and fluoroprobe, benzimidazol-carbocyanine 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. phillippensis* leaves showed invitro 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 phillippensis*, 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 [2,3]. As a result, the use of natural therapies is an alternative to the adverse effects of synthetic drugs [4].

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 folktale practices [5]. Nevertheless, this plant has not been evaluated for its *invitro* cytotoxic and apoptotic potential against breast cancer cell lines. Hence, we planned to evaluate cytotoxic potency of *Mallotus phillippensis* against MCF-7 breast cancer cell lines.

MATERIALS AND METHODS

Plant Extraction

The leaves of *M. phillippensis* were collected from poked, Wayanad district, Kerala, India. The plant materials were authenticated by the MSSRF, Kalpetta. The leaves of *M. phillippensis* 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

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 antimycotic solution (penicillin-streptomycin and amphotericin B), in an incubator at 37°C with 5% CO2. The cells were trypsinized 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. phillippensis* (MMP) prepared in 10% tween at concentration 130 mg, 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 Riss *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 $1x10^5$ cells/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 100 µL of DMSO and the absorbance was read at 570 nm in a Varioscan 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 IC_{50} values of extracts were calculated by plotting the concentration against per cent cell inhibition using AAT Bioquest.

Selection of concentrations

Based on the MTT assay, three concentrations of the extract, i.e., double IC_{50}, IC_{50} and half IC_{50} were selected for the study. Thus, the concentrations that were used for the study were 380, 190, 95 µg/mL for MCF-7 cells respectively.

Acridine orange / Ethidium bromide (AO/EB) staining

A concentration of 3 x 10^5 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 (10 µg/mL) and ethidium bromide (10 µg/mL) and examined under Trinocular Research fluorescence microscope, DM 2000 LED, Leica with blue excitation (488 nm) and emission (550 nm) filters at 20X magnification [8].

JC-1 Staining

MCF-7 cells were plated at a seeding density of 3 x 10^5 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 *µM* fluoroprobe, 5. 5', 6, 6'- tetrachloro- 1, 1', 3,3'-tetra ethyl 61benzimidazole-carbocyanine iodide (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 of replicates and IC_{50} values- determined using online curve fitting (www.aatbioquest.com).

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

Phytochemicals	MMP
Steroids	Present
Alkaloids	Present
Phenolic compounds	Present
Flavonoids	Present
Tannins	Present
Diterpenes	Present
Glycosides	Absent
Saponins	Absent

***In vitro* cytotoxic evaluation of MMP and Calculation of IC_{50}**

There was a dose dependent decrease in the viability of cells exposed to different concentrations of extract with the viability being least at 320 µg/mL (fig.1). Table.2. shown percent inhibition of MCF-7 cells after treatment with MMP. The graph showing the analysis of IC_{50} is depicted in Fig 2. The IC_{50} of methanolic extract of *M. phillippensis* was 190 µg/mL as obtained from MTT assay.

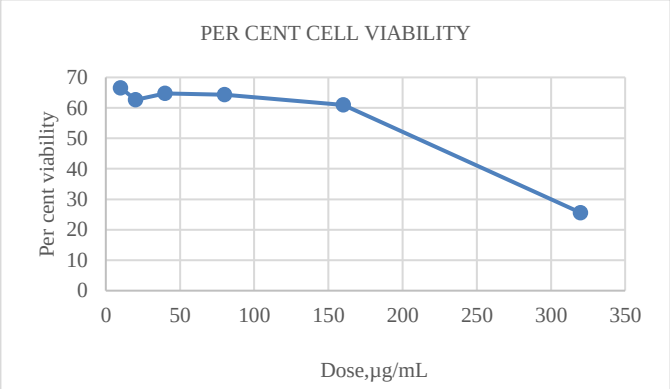

Figure 1: Per cent viability of MCF-7 cells treated with MMP

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

Concentrations(µg/mL)	Percent cell inhibition (Mean±SEM)
320	74.42+0.05
160	39.08+0.24
80	35.69+0.19
40	35.28+0.18
20	37.33+0.10
10	33.43+0.04
IC_{50}(µg/mL)	190

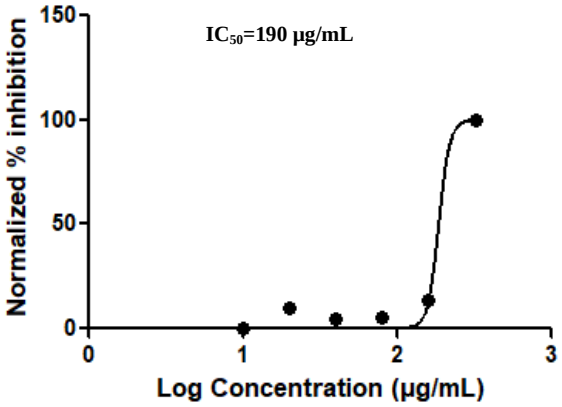

Figure 2: Calculation of IC_{50}

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 IC_{50} doses of the MMP resulted, in early apoptotic cells with localized crescent-shaped or granular green stained nucleus. With IC_{50} and twice IC_{50} 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.

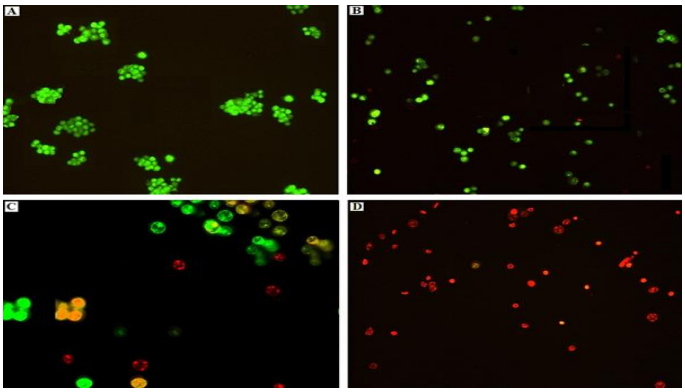

Figure 3: Morphological changes of MCF-7 cells after treatment with various concentrations of MMP by AO/EB staining.200X A-control cells, B-cells treated with half IC_{50}, C-cells treated with IC_{50} and D-cells treated with twice IC_{50}

JC-1 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.

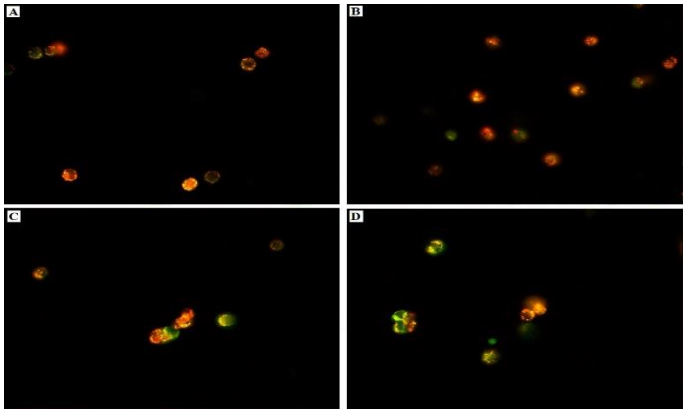

Figure 4: Morphological changes of MCF-7 cells after treatment with various concentrations of MMP by JC-1 staining.200X A-control cells, B-cells treated with half IC_{50}, C-cells treated with IC_{50} and D-cells treated with twice IC_{50}

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 IC_{50} value 190 µg/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 [12]. Induction of apoptosis is therefore a highly desired mechanism for cancer management [13]. Drugs kill cancer cells by causing apoptosis, a process whose sensitivity is determined by the drug proportionate to the level of apoptosis [14]. 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 lower trans membrane potential [15]. 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. phillippensis* for inducing apoptosis through the intrinsic pathway. Phytochemical analysis revealed the presence of phenolic, flavnoids and terpenoids, which contributed for cytotoxicity of MMP and hence *M. phillippensis* 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.

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Conflict of Interest

None declared.

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