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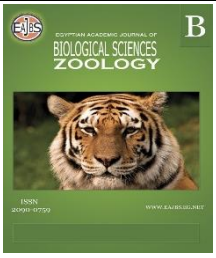

In vitro Antibacterial and Cytotoxic Activity of The Leaf Extract of *Anisomeles malabarica* against HepG2 Cancer Cell Lines

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ABSTRACT

Plant-based therapies have made an impact on human health. In the present study, aqueous *A. malabarica* leaf extract was subjected to determine the qualitative phytochemical screening, antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, cytotoxic activity, and Acridine Orange/Ethidium Bromide dual staining assay for apoptotic effect against the HepG2 cell lines. The aqueous extract contained secondary metabolites such as quinones, carbohydrates, flavonoids, phenols, terpenoids, and amino acids, which were revealed in preliminary phytochemical screening. Among the bacterial species tested, *P. aeruginosa* (23.1mm) had the highest zone of inhibition, whereas *K. pneumoniae* (13.2mm) had the lowest, indicating that the leaf extract had significant antibacterial activity. After 24 hours of observation, the highest concentration of aqueous leaf extract suppressed 65.52% of cancer cell proliferation. The aqueous extract that had a concentration-dependent apoptotic impact against HepG2 cells was ascertained using the Acridine Orange/Ethidium Bromide assay. However, more research is needed before it can be used as a therapeutic tool. The findings of this study will be useful in the development of new antibacterial and anticancer therapeutic medicines derived from *A. malabarica* in the future.

INTRODUCTION

Plants are widely employed as therapeutic agents nowadays due to their accessibility, adaptability, affordability, therapeutic efficacy, and lack of adverse effects (Joseph *et al*., 2010). The presence of phytochemicals in plant extracts plays an important role in therapeutics. Because of the occurrence of numerous bioactive secondary metabolites such as alkaloids, terpenoids, glycosides, steroids, flavonoids, and phenolic chemicals, modern pharmaceuticals rely on plant-based medication (Ouerghemmi *et al*., 2017). Plant extracts contain a wide range of biochemical actions, including anti-allergic, anti-inflammatory, antioxidant, anti-microbial, anti-fungal, antiviral, and anti-cancer properties (Pisoschi and Pop; Friedman, 2007).

Medicinal plants are used by about 80% of the world's population in developing countries to heal a variety of diseases (Rai and Lalramnghinglova, 2010). Bacterial infections are regarded as a major public health concern since germs have been genetically

modified to resist certain treatments resulting in a variety of worldwide resistant bacterial species (Aarestrup, 2005). Antimicrobial resistance must be combated by novel therapeutics (Ali *et al*., 2018). Bacterial infection is responsible for around 15 % of cancer cases worldwide, making it a severe public health concern (Mager, 2006).

Hepatocellular carcinoma (HCC) is the fifth most frequent kind of liver cancer worldwide, with Asia and Africa accounting for the majority of cases **(**Bruix *et al*., 2004)**.** Therefore, more effective methods for cancer control and apoptosis induction are needed, contributing to cancer treatment. It is also worth noting that dietary supplements and bioactive substances generated from medicinal plants and herbs have potent pharmacological effects and can help prevent cancer Solowey *et al*., 2014)**.**

Anisomeles malabarica, which is commonly known as Malabar Catmint, belongs to the Lamiaceae family. It is known for inhabitation in dry regions or plains and is found throughout India. It is a traditional medicinal plant used to cure cancer, liver disorder, fever, cold, cough (Ranganathan and Vijayalakshmi, 2012) and wounds (Ignacimuthu *et al*., 2006). *A. malabarica* has been reported to have significant pharmacological therapeutic potential with anti-inflammatory, (Sudha and Srinivasan, 2014) antiepileptic activity, (Choudhary *et al*., 2011) cytotoxic activity in cancer cells (Preethy *et al*., 2013).

Therefore, the present study reports the phytochemical constituents and therapeutic validation of *A. malabarica* leaf extract, particularly with antibacterial and anti-cancer effects against the HepG2 cancer cell lines.

MATERIALS AND METHODS

Plant Material Collection:

The leaves of the plant "*Anisomeles malabarica*" were collected from the Western Ghats, Tamil Nadu, India. To remove moisture, healthy leaves were cleansed with tap water and double distilled water before being sliced into small pieces and dried in the shade for 20 days. Dried leaves were ground into a fine powder using a blender and stored in an airtight container in the refrigerator until needed.

Preparation of Leaf Extract:

The aqueous extract was made by dissolving 10 g of *A. malabarica* leaves powder in a 250 ml Erlenmeyer flask containing 100 ml of distilled water at a ratio of 1:10. For 1 hour, the mixture was maintained in a boiling water bath at 60°C. The extract was then filtered again using Whatmann No.1 filter paper before keeping it at 4°C for subsequent analysis.

Phytochemical Analysis:

Following the standard methods described by Roghini and Vijayalakshmi (2018), phytochemical analysis was performed to detect the presence of carbohydrates, proteins, phenols, flavonoids, terpenoids, steroids, anthraquinones and quinones in the plant extract.

Antibacterial Activity:

Agar-well Diffusion Assay:

Microbial cultures were prepared by following the method described by Boyanova *et al*. (2005). MHA was poured onto the sterile Petri dishes which were used for the growth of microbial cultures. Using a sterilized swab, freshly prepared inoculum containing pathogens was swabbed on the MHA plates. Five wells were made on the MHA plates using a cork borer and the wells were loaded with different concentrations of leaf extract (20-100 µl/ml). To compare the efficacy of leaf extract, Streptomycin was used as a control. After 24 hours of incubation period at 37°C, the zone of inhibition was measured in mm (diameter). The experiment was repeated thrice.

Minimum Inhibitory Concentration of Leaf Extract:

The broth microdilution method was used to estimate the minimum inhibitory concentrations (MIC) of the *A. malabarica* leaf extract. Using Mueller-Hinton, two-fold serial dilutions of known quantities of leaf extract (100, 50, 25, 12.5, 6.25, 3.12, 1.5, 0.78, 0.39, and 0.19 µl/ml) and kept under appropriate controls. An overnight fresh culture of 5L inoculum (≈5×105 CFU/ mL) was added to each of the wells and the plates were incubated at 37°C for 24 hours. This experiment was carried out three times.

Anticancer Activity:

Cell culture and Maintenance:

HepG2 (human hepatoma) cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were grown in Dulbecco's Modified Eagles Medium (DMEM-Himedia), which included 10% heat-inactivated Fetal Bovine Serum (FBS) and a 1% antibiotic cocktail that included Penicillin (100U/ml), Streptomycin (100 µg/ml), and Amphotericin B (2.5 µg/ml). In a cell culture incubator (Galaxy® 170 Eppendorf, Germany), cell-containing TC flasks (25cm^2) were incubated at 37°C in a humidified environment with 5% CO_2.

Cytotoxicity Analysis:

HepG2 cells (2500 cells/well) were seeded on 96 well plates and allowed to acclimate to the culture conditions of 37°C and 5% CO_2 for 24 hours in the incubator. Aqueous leaf extract was further diluted in DMEM media and added to the wells containing cultured cells at final concentrations (6.25, 12.5, 25, 50, and 100 µg/ml). The plates were then incubated for another 24 hours after being treated with the test samples. The wells were filled with 100 mL of 0.5 mg/mL MTT solution in PBS. The plates were then incubated for another 2 hours to allow for the formation of formazan crystals. The supernatant was collected, and 100 mL of 100% DMSO was applied to each well. A microplate reader was used to measure the absorbance at 570 nm. All of the experiments were carried out in triplicate. The viability of the cells was calculated using the formula below:

$$\text{Percentage of cell viability} = \frac{\text{Average absorbance of treated}}{\text{Average absorbance of control}} \times 100$$

IC_{50} value:

The IC_{50} value is the half-maximal inhibitory concentration of the sample. The IC_{50} values were calculated using the equation for slope (y = mx + C) obtained by plotting the average absorbance of the different concentrations of the test sample (6.25-100 µg/mL) in Microsoft Excel.

Acridine Orange-Ethidium Bromide Dual Staining:

HepG2 cells were cultured in Dulbeccos modified Eagles media and grown to 60-70% confluency and treated with sample and incubated for 24 hours. The cells were rinsed in cold PBS after being treated with various concentrations and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10 minutes. The labelled cells were rinsed twice in 1X PBS before being viewed using a fluorescence microscope with a blue filter (Olympus CKX41 with Optika Pro5 camera).

Statistical Analysis: All the tests were conducted in triplicates. The data of all the parameters were statistically analyzed and the values were expressed as mean ± S.D. Significant differences between the means of parameters were determined ($p<0.05$).

RESULTS AND DISCUSSION

Phytochemical Analysis:

Phytochemical screening was carried out in an aqueous extract of *A. malabarica*

and the results are given in Table 1. Secondary metabolites of the plant are directly responsible for different activities such as antimicrobial, antioxidant, antifungal, and anticancer (Anyasor *et al*., 2010). In the study, preliminary analysis revealed the presence of various phytochemical constituents. Quinones, carbohydrates, phenols, flavonoids, terpenoids and steroids were found to be present in the aqueous extract. An earlier study reported that the phytochemical analysis of the methanolic extract of *Anisomeles malabarica* L. has indicated the presence of steroid, flavonoid and terpenoid types of compounds (Roy and Barik, 2010). Phenols and amino acids were found in higher concentrations in the extract. The phenolic groups in the extract may inhibit the growth of bacteria through the formation of protein-phenolic groups between hydroxyl groups and the cell membrane of protein, which disrupts the cell membrane and causes the death of bacteria (Mangunwardoyo *et al*., 2012). Anthraquinones were absent in the leaves of *A. malabarica*.

Table 1: Preliminary qualitative phytochemical analysis of *A. malabarica* aqueous leaf extract

S. No	Phytochemical Constituents	Aqueous *A. malabarica* leaf extract
1	Carbohydrates	+
2	Flavonoids	+
3	Phenols	+
4	Terpenoids	+
5	Steroids	+
6	Anthraquinones	-
7	Aminoacids	+
8	Steroids	+

'-'indicates absence, '+' indicates presence

Antibacterial Assay:

In the present study, the antibacterial efficacy of *A. malabarica* aqueous leaf extract was evaluated against Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) by well diffusion method. Antibacterial activity was expressed in terms of a clear zone of inhibition after 24 hours. Aqueous extract yielded excellent antibacterial activity against the tested microorganisms as shown in Figure 1. A study made by Kavitha, *et al*. (2012) showed that ethanolic and methanolic extract of *A. malabarica* showed less activity compared to that of aqueous extract.

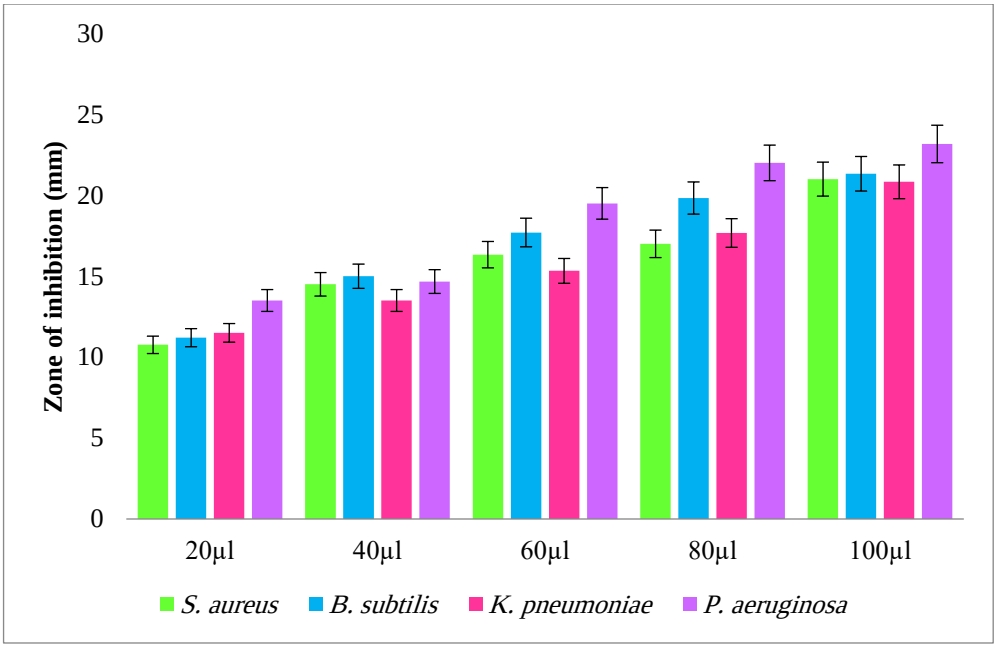

Fig. 1: Antibacterial efficacy of *A. malabarica* aqueous leaf extract**.**

Among the tested microorganisms, *S. aureus* showed the lowest antibacterial activity of 10.76 mm at the least concentration of 20 µl. The highest zone of inhibition was found in *P. aeruginosa* (23.17 mm) and the lowest inhibition was exhibited by *K. pneumoniae* (20.83 mm) at a concentration of 100 µl. While comparing the antibacterial efficacy of gram-negative and gram-positive microorganisms, leaf extract showed excellent bacterial inhibition against *P. aeruginosa* in all concentrations. An earlier report by Packialakshmi and Nilofer Nisha (2014) stated that the antibacterial activity of *A. malabarica* exhibited higher inhibition against gram-negative organisms which agrees in line with the results of the present study. Similarly, from the results, it was evident that leaf extract showed very good antibacterial activity against *P. aeruginosa* compared to the other tested microorganisms. The result clearly shows that alkaloids, tannins and terpenoids which were abundantly found in the aqueous extract were found to be responsible for the antibacterial activity. The antibacterial activity of the extract might be attributed to the presence of the aforesaid secondary metabolites in the extract.

Minimum Inhibitory Concentration (MIC):

The MIC values of the *A. malabarica* leaf extract are given in Table 2. For the leaf extract, 6.25 µl was known to be MIC for *S. aureus* and 3.12 µl for *B. subtilis* while *K. pneumoniae* and *P. aeruginosa* recorded MIC at 6.25 µl and 1.78 µl. An earlier study reported by Britto *et al*. (2012) revealed that *A. malabarica* methanolic leaf extract had MIC values in the range of 1.5-12.5 µl/ml against all the tested pathogens which coincide with the results of the present study.

Table 2: Minimum Inhibitory Concentration (MIC) of *A. malabarica* leaf extract

Bacterial strain	***A. malabarica* leaf extract MIC (µl/ml)**
Staphylococcus aureus	6.25
Bacillus subtilis	3.12
Pseudomonas aeruginosa	1.78
Klebsiella pneumoniae	6.25

Values are expressed as Mean± SD for triplicates

Anticancer Activity:

Various anticancer drugs have been developed throughout time, but nowadays, resistance and side effects of cancer treatments demand new, highly efficient anticancer substances with low toxic effects (Abolhasani *et al*., 2018). The *in vitro* cytotoxic effects of *A. malabarica* leaf extract were screened against the HepG2 cell lines. The results of the study show that cell inhibition increased with an increase in the concentration of the aqueous leaf extract as shown in Figure 2. Cancer cell inhibition was observed in all the concentrations but the highest percentage of 65.52% inhibition was found at the concentration of 100 µg/ml and the lowest of 11.77% was recorded at 6.25 µg/ml.

A study made by Murati *et al.* (2019) showed that the moderate anti-proliferative effect of *P. cornuta* extracts in HepG2 cells may demand a higher dose to produce effective inhibition such as the significant inhibitory effect of *P. spinosa*. flower extracts in HepG2 cells were 150–200 µg/ml. IC_{50} obtained for the *A. malabarica* extract was 60.99% (Figure 3). Control cells appeared normal in shape throughout the study. Results showed that *A. malabarica* aqueous leaf extract showed considerable anticancer activity against the HepG2 cell line.

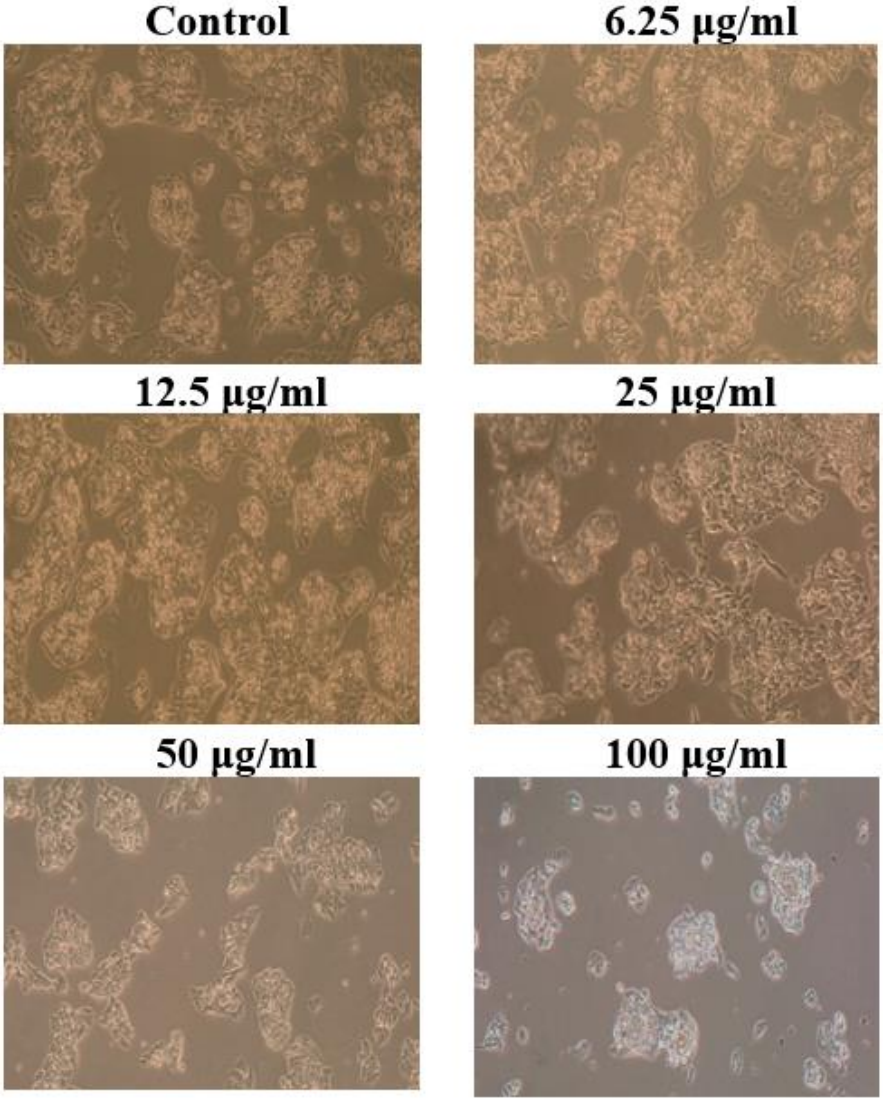

Fig. 2: Photomicrograph of HepG2 cells on different concentrations of *A. malabarica* aqueous leaf extract.

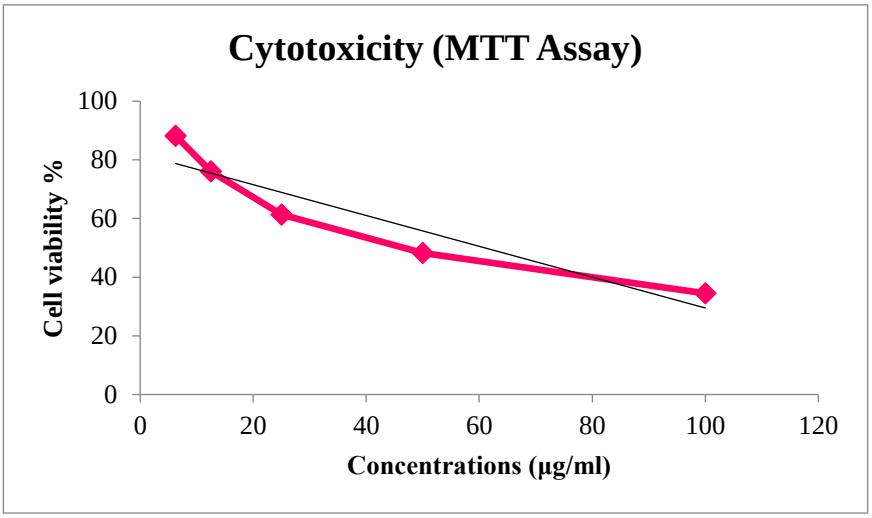

Fig. 3: Percentage of cell growth inhibition of *A. malabarica* leaf extract on HepG2 cell line by MTT assay.

Determination of Apoptosis by Acridine Orange (AO) and Ethidium Bromide (EB) Double Staining:

Apoptosis is a formed cell death process characterized by loss of plasma membrane phospholipid asymmetry, enzymatic cleavage of the DNA into oligonucleosomal fragments, and segmentation of the tumour cells into membrane-bound apoptotic bodies. The present study also examined the induction of apoptosis in human liver cancer cells upon treatment with the aqueous extract of *A. malabarica*. Aqueous leaf extract of *A. malabarica* emits light orange and red stained nuclei with chromatin condensation while in control cells normal green nuclei were seen as shown in Figure 4. Acridine orange-ethidium bromide assay by fluorescent microscopy revealed that the leaf extract persuaded early and late apoptotic cells in HepG2 liver carcinoma cells.

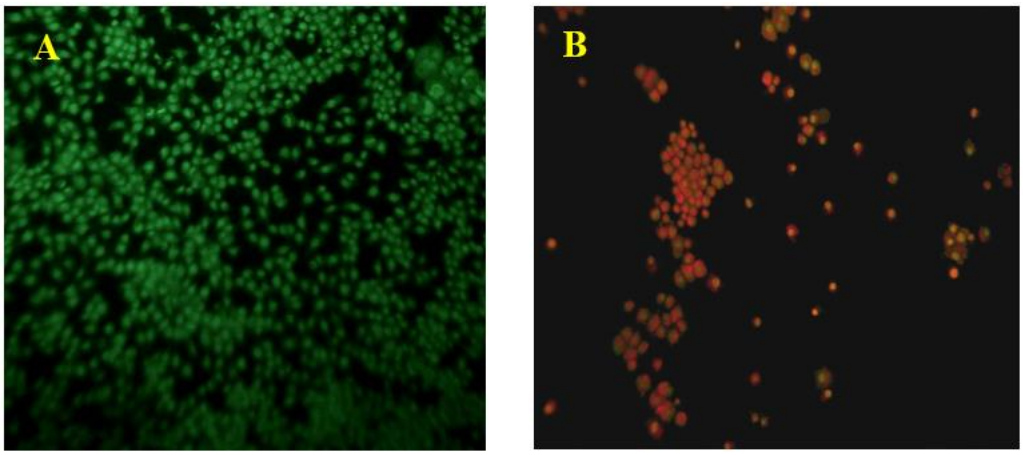

Fig. 4: AO and Ethidium Bromide staining of Hep G2 cells (A) Control HepG2 cells, (B) *A. malabarica* leaf extract IC50 concentration.

CONCLUSION:

A. malabarica possesses a varied spectrum of secondary metabolites, as specified in the study, indicating that it is a valuable therapeutic plant. Leaf extract of *A. malabarica* demonstrated good antibacterial activity against all pathogens tested, with particularly very high activity against *P. aeruginosa*, and could be used as a powerful

antimicrobial agent in the future to prevent many diseases. *A. malabarica* aqueous leaf extract exhibited a significant cytotoxic effect against HepG2 cancer cell lines. To uncover and investigate the bioactive components discovered in *A. malabarica* aqueous leaf extract, more research is needed, and hence this current study was expanded to look at its other biological properties.

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Conflicts of interest:

The authors declare no conflict of interest.

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