

ORIGINAL RESEARCH ARTICLE

Assessment of antibacterial, anti-inflammatory, and anti-cancer activities of *Melia azedarach* L. leaf extract

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Abstract

The purpose of the present study was to evaluate the antibacterial, anti-inflammatory, and cytotoxic properties of *Melia azedarach* leaf. The phytoconstituents found in the leaves were extracted with four different solvent systems based on their polarity index. Antimicrobial activity of selected plant extracts was evaluated using disc diffusion and micro-broth dilution methods against four different pathogenic bacterial strains. The ethanolic extract of *M. azedarach* leaf showed a significant inhibitory effect on the growth of *Escherichia coli* (15.07 mm) and *Staphylococcus aureus* (18.23 mm) ($P < 0.005$), followed by *Bacillus subtilis* and *Salmonella typhi*. Further, the mechanism of action was confirmed by live/dead cells analysis, which revealed the death of *E. coli* and *S. aureus* upon treatment with ethanolic extract. *In vivo* anti-inflammatory test conducted using carrageenan-induced rat paw edema model revealed that the 300 mg/kg ethanolic extract exhibited a significant anti-inflammatory activity of 51.78% compared with that of standard drug diclofenac ($P < 0.001$). Further, the cytotoxicity of ethanolic extract against human hepatocarcinoma cell lines (HepG2) was evaluated by MTT assay, and the findings showed a moderate level of toxicity against the HepG2 cell line with an IC_{50} value of $540.00 \pm 0.6 \mu\text{g/mL}$ compared to doxorubicin. HepG2 cells treated with doxorubicin (2 $\mu\text{g/mL}$) and ethanolic extract showed a 2.33- and 1.35-fold increase in p53 gene expression, respectively. Apoptosis activity was measured in terms of DNA laddering, which indicated the late stage of apoptosis. The results showed that the extract-treated cell lines induced DNA fragmentation, which was found to be a potent anti-cancer mechanism in HepG2 cells. This study corroborated that the leaf extract of *M. azedarach* is a good source of active phytochemicals, with promising biological activities.

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1. Introduction

Herbal drugs are widely regarded as a safer option for consumption as compared to synthetic drugs. Most herbs with medicinal properties were recognized and applied based on traditional knowledge, but validated scientific evidence pertaining to their efficacy is lacking. At present, we are not equipped with a proper monitoring system and adequate knowledge to determine the safe dosage level of these natural products. These inadequacies serve as the prime reason why the natural products have yet to be integrated in the health-care system.¹⁻³

The beneficial properties of plants are accounted for by the phytochemicals they possess. Antibacterial, anti-inflammatory, and anticancer activities are among the important biological and medicinal qualities ascribed to these phytochemicals. Cancer remains the second leading cause of death worldwide, with about 18 million new cases and 9 million cancer-related deaths reported every year as per the World Health Organization (WHO).⁴ Inflammation is a complex cascade caused by many external factors and eventually changes the functional and phenotypic traits of the cells, thereby precipitating the development of cancer. According to WHO, 80% of people living in developing nations, including India, depend on herbal medicine to meet their health-care needs.⁵ India is one of the countries blessed with numerous medicinal plant resources, and the therapeutic potency of such plants has been demonstrated to check their bioefficacy by several researchers.

Melia azedarach (Meliaceae) is a deciduous tree species capable of growing up to 50 feet in height. Touted with medicinal values, *M. azedarach* has a wide geographical distribution, commonly found in India, China, Indonesia, and throughout Southeast Asia. Its leaves are alternate, bi-pinnately compound; inflorescence is panicles; and fruits are berries. *M. azedarach* has the potential to grow and form a dense-thick canopy, restricting the growth of native vegetation.⁶ Fruits and leaf litter of this plant have been proved to potentially increase mineralizable nitrogen and increase soil pH in acidic soils.⁷ Extracts of the plant have been used for various medicinal purposes, including the treatment of viral infections like herpes. Dilute foliar extracts combined with triclopy-based herbicides have been utilized to control pests such as insects and other worms.⁸ Of the several compounds isolated from this plant, meliacine appears to be the predominant active constituent of *M. azedarach*. Meliacine – a peptide isolated from the plant's leaves – exhibits potent activity against herpes simplex type-1 and has also been used as an abortifacient, an antiseptic, a diuretic, an insect repellent.⁹

In the present study, screening of phytochemical, *in vivo* anti-inflammatory activity using the carrageenan-induced

rat paw edema method, bactericidal properties, and anticancer activity of the *M. azedarach* leaf extracts was carried out. Cytotoxicity of the extract was further confirmed by determining apoptosis level through the measurement of p53 expression in human hepatocarcinoma (HepG2) cell lines treated with ethanolic leaf extract of *M. azedarach*.

2. Materials and methods

2.1. Collection of plant materials and extract preparation

Fresh and healthy leaves of *M. azedarach* were collected from a local community in Mysore, Karnataka (11° 30' and 18° 30' N latitude and 74° 15' and 78° 30' E longitude) based on its ethnobotanical importance. The plant specimen was authenticated by a taxonomist, and the herbarium specimen (VsNo-MCbot02) was submitted to the Department of Studies in Botany, University of Mysore, Mysuru. Leaf materials were thoroughly washed with running tap water, shade-dried at room temperature, and made into a fine powder, and 100 g of powdered sample were extracted in 250 mL of petroleum ether, chloroform, ethyl acetate, and ethanol using soxhlet extractor for 48 h. The extracts obtained were subjected to flash evaporation and stored at 4°C in an airtight glass bottle until further studies.¹⁰

2.2. Phytochemical screening

Phytochemical analysis of different leaf extracts of *M. azedarach* was carried out to determine the presence or absence of secondary metabolites by following Harborne's standard method (1998).¹¹ The extracts were subjected to qualitative preliminary phytochemical screening to detect the presence of phytochemicals such as carbohydrates, proteins, steroids, flavonoids, tannins, alkaloids, and cardiac glycosides.

2.3. Antibacterial activity

The evaluation of antibacterial activity of all four solvent extracts was conducted using disc diffusion method (CLSI, 2012a).¹² After being cultured for 24 h, *Staphylococcus aureus* (MTCC441), *Bacillus subtilis* (MTCC220), *Salmonella typhi* (MTCC325), and *Escherichia coli* (MTCC452) were uniformly swabbed on Petri plates containing nutrient agar medium using a sterile glass spreader (1.5×10^8 CFU/mL). Each disc (6 mm) was loaded with 50 µL (5 mg/disc) of extract and placed equidistantly. Streptomycin (10 µg/disc) and the respective solvents served as positive and negative controls. The plates were incubated for 24 h at 37°C. The zone of inhibition was measured using HI Media Zone measuring scale, and the assay was performed in triplicates.

2.4. Minimum inhibitory concentration (MIC)

The MIC of the leaf extracts was tested using the micro-broth dilution method¹³ coupled with an enzyme-linked immunosorbent assay (ELISA) multi-plate reader. About 100 µL of nutrient broth and 10 µL of inoculum suspension of each test bacterium (1.5×10^8 CFU/mL) were added to each well. The extracts were prepared at 50 mg/mL concentration, which served as a stock solution. The solvent extract (100 µL) was added to the first well of each row, and two-fold serial dilution was carried out along the rows to create a series of extracts with different extract concentrations ranging from 5 to 0.002 mg/mL. The plates then were incubated at 37°C for 24 h. After incubation, each well was supplemented with 10 µL of 2,3,5-triphenyl tetrazolium chloride dye at 2 mg/mL and incubated for 1 h, and absorbance was measured at 620 nm using an ELISA multi-plate reader. The concentration at which color had stopped changing was considered the MIC value. The assay was performed in triplicates.

2.5. Bacterial cell morphology assay

The assay was performed to distinguish the morphology of the live and dead bacterial cells after treatment with extract at MIC value using the method described by Li *et al.*¹⁴ About 100 µL of bacterial cell suspension (1.5×10^8 CFU/mL) was treated with 50 µL of selected leaf extracts (100 µg) and incubated at $37 \pm 2^\circ\text{C}$ for 24 h. The untreated cells and streptomycin-treated cells served as controls. After incubation, the mixture was centrifuged at 5000 rpm for 5 min at 4°C, and the pellet was washed thrice with phosphate-buffered saline. Each obtained sample was mixed with a fluorescent dye solution of ethidium bromide (EB) and acridine orange (AO) at a 1:1 ratio and incubated for 30 min. Later, 5 µL of these samples were mounted on microslide for observing the dead and live cells under a Carl-Zeiss Fluorescence Microscope (Lawrence and Mayo, Germany) at 40× magnification. The absorbance was taken for the green fluorescence dye at 480/500 nm and the red fluorescence dye was read at 490/635 nm. The former stains the bacteria with both intact as well as damaged membranes, and the latter stains only the cells with damaged membranes.

2.6. Anti-inflammatory activity

2.6.1. Experimental animals

Healthy adult Wistar rats (120–150 g), regardless of their gender, were housed in polypropylene cages, maintained at room temperature of $22 \pm 2^\circ\text{C}$ under the conditions of 12-hour light-dark cycles and 40–60% humidity. The rats were given standard rodent diet and water *ad libitum*. The experiment was conducted based on the ethical guidance for the care and use of laboratory animals in research as

approved by the animal ethics committee of the University of Mysore, Manasagangotri, Mysore (animal sanction order UOM/IAEC/05/2017).

2.6.2. Acute toxicity study

The ethanol extract was subjected to determine the toxicity level of the animals. An acute toxicity study was performed as per the OECD-423 guidelines.¹⁵ The animals were randomly selected for the toxicity study. The rats were divided into five groups with six animals each and were fasted overnight but allowed to drink water. After that, the extract was administered orally at a dose of 5 mg/kg. Instead of administering extract, a similar quantity of 1% carboxyl methylcellulose solution was administered to the control group. The rats were kept for observation for 6 days. If no mortality was noted, the process was repeated using higher doses such as 50, 300, and 2000 mg/kg.

2.6.3. Carrageenan-induced rat paw edema

Paw edema was induced in the right hind paw of each rat, and the drugs were administered orally as suggested by Singh *et al.*¹⁶ In group A, the rats served as controls treated with 100 µL of 0.9% drug suspension in saline. The rats in group B were administered with standard anti-inflammatory drug diclofenac sodium (Novartis India Ltd.) at 40 mg/kg (p.o.), and the rats in groups C, D, and E were orally administered with 100, 200, and 300 mg/kg of ethanolic leaf extract of *M. azedarach*, respectively. After an hour of administration of drugs, 0.1 ml of 1% w/v carrageenan (Sigma Aldrich Chemical Co.) solution in normal saline was injected into the subplantar tissue of the right hind paw of each rat. The paw volume was measured using a plethysmometer, before and after injection of carrageenan at different time intervals of 1 h, 2 h, and 3 h. The percentage of edema induced was assessed in the drug- and extract-treated rats and was compared with the control and the inhibitory effects were studied. The comparative strength of drugs was determined by assessing the percentage of inflammation inhibition they achieved, and the percentage inhibition was calculated using the formula in the following:

$$\text{Percent of inhibition (\%)} = \frac{(C - T)}{C} \times 100 \quad (I)$$

Where T represents the difference in increased paw volume after the administration of test drugs, and C represents the difference in increased volume in the control group.

2.7. In vitro cytotoxicity assay

2.7.1. Cell culture

HepG2 cell line was procured from National Centre for Cell Sciences, Pune, India, and maintained in Dulbecco's

Modified Eagle Medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 µg/mL), streptomycin (100 µg/mL), and amphotericin B (5 µg/mL) under humidified air of 5% CO₂ at 37°C until confluent.

2.7.2. Cytotoxicity assay

HepG2 cells maintained in DMEM were treated with ethanol extract of different concentrations (62.5, 125, 250, 500, and 1000 µg/mL) to evaluate the cytotoxic effects of the ethanolic extract on the HepG2. The MTT assay was carried out to analyze the cytotoxicity of the HepG2 cells after 24 h of incubation as described by Mosmann.¹⁷ A microplate reader with 96 wells was used to detect the optical density at 570 nm, and the percentage of toxicity was calculated using the formula in the following:

$$\begin{aligned} &\text{Percent of growth inhibition(\%)} \\ &= 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100 \quad (\text{II}) \end{aligned}$$

The mRNA levels of *p53* were determined using a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). HepG2 cells were cultured on 60 mm Petri dish and cultivated in DMEM supplemented with FBS and amphotericin for 24 h. The dish was filled with the ethanolic extract of desired concentration (at IC₅₀ value) and incubated for 24 h. Total cellular RNA was extracted from untreated (control) and treated cells using TRI Reagent (Sigma Aldrich, Germany) in adherence with the manufacturer's protocol. cDNA was synthesized from the extracted RNA using a reverse transcriptase kit (Thermo Scientific, USA) according to the manufacturer's instructions. The reaction mixture (20 µL) was then subjected to PCR for amplification of *p53* cDNAs using precisely designed primers obtained from Eurofins India, and the housekeeping gene *GAPDH* was co-amplified with each reaction as an internal control reference. The optimum thermal cycling conditions for PCR are as follows: 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 10 min.

During the first strand synthesis, oligo dT primers were used. For second strand synthesis, the product generated has a size of 371 bp. The sequences of primers used in this experiment are as follows:

- Forward: 5'-CTGAGGTTGGCTCTGACTG TACCACCATCC-3'
- Reverse: 5'-CTCATTCAGCTCTCGGAACATC TCGAAGCG-3'

2.7.3. DNA fragmentation studies

DNA fragmentation study was carried out using the method proposed by Vandghanooni *et al.*¹⁸ cHepG2 cells (3 × 10⁶/mL) were seeded onto 60 mm Petri dishes and cultured for 24 h at 37°C in an atmosphere of 5% CO₂. Afterward, the cells were washed with media and treated with 2 µg/mL doxorubicin (standard drug) and ethanolic extract at IC₅₀ value; the cells were incubated for 24 h at 37°C and 5% CO₂. At the end of the incubation period, the G Bioscience apoptotic DNA ladder kit (Thermo Scientific, USA) was used to isolate the cancer cell's chromosomal DNA as per the instructions of the kit. For three hours, the DNA samples were run at 5 V/cm on a 2% agarose gel electrophoresis. The gel was imaged and visualized using the UVP gel documentation system (Thermo Fisher Scientific, USA).

2.8. Statistical analysis

The data of triplicate values are expressed as mean ± standard deviation. The statistical differences among the test groups were analyzed using a one-way analysis of variance coupled with a *post hoc* Duncan's multiple range test. The statistical analyses were performed using SPSS Version 18.0 (SPSS Inc., Chicago, IL, USA). Differences with *P* < 0.05 are regarded as statistically significant. The IC₅₀ value was determined using a logarithmic equation, *i.e.*, *Y* = *M*(*x*) + *C*. Here, *Y* = 50, and *M* and *C* values were derived from the viability graph.

3. Results

3.1. Preliminary phytochemical analysis

The preliminary phytochemical screening of leaf extracts of *M. azedarach* showed the presence of different classes of phytochemicals. Among the different solvent extracts tested, ethanolic extract showed a maximum number of phytoconstituents followed by ethyl acetate extract, while the least number of phytochemicals were noted in petroleum ether and chloroform extract. The tested extracts showed the presence of carbohydrates, proteins, steroids, flavonoids, tannins, alkaloids, and cardiac glycosides (Table 1).

3.2. Antibacterial activity and MIC

Four different solvent extracts of *M. azedarach* leaf were subjected to antibacterial activity testing against *E. coli*, *B. subtilis*, *S. typhi*, and *S. aureus*. The overall results of the tested extracts and their antibacterial activity and MIC are depicted in Table 2. Our results showed that the four solvent ethanolic extract effectively inhibited the growth of tested bacterial strains and the largest zone of inhibition in *E. coli* (15.07 ± 1.54 mm) and *S. aureus* (18.23 ± 0.57 mm),

as shown in Figure 1A and E. Similarly, ethyl acetate extract also demonstrated inhibitory effect on pathogenic growth, but no such activity was observed in chloroform and petroleum ether extracts. The MIC assay showed maximum growth inhibition in ethanol and ethyl acetate extracts with a range of 0.625 mg/mL to 1.25 mg/mL, while streptomycin showed an MIC value of 0.0781 mg/mL. The present study indicates that ethanolic extract exhibits

significant bactericidal activity because of the presence of a higher number of phytochemicals and this can be attributed to several bioefficacy mechanisms.

3.3. Bacterial cell morphological studies

The maximum growth inhibition observed in *E. coli* and *S. aureus* was used as a reference to study the morphological changes that occurred during the process of inhibition by live/dead cells backlight assay. The ethidium bromide and acridine orange dyes were used to stain the bacteria: ethidium bromide can only enter the damaged bacterial cell through the ruptured cell membrane, while acridine orange adheres to both live and dead cells. The bacterial strains treated with ethanolic extracts along with control were observed under fluorescence microscopy. The staining results revealed the orange staining of extract- and streptomycin-treated cells, indicative of cell death (Figure 1B and 1F), and the reddish staining of the cells (Figure 1D and 1H), suggesting rupture of the cell membrane. Meanwhile, untreated cells appeared in green fluorescence, indicating normal growth without any abnormalities or damage in the cell membrane (Figure 1C and 1G). The fluorescence intensity of the dead and live cells in all the treatments is depicted in Figure 1I. Taken together, this study indicates the antibacterial efficacy of the ethanolic leaf extract of *M. azedarach*.

3.4. Anti-inflammatory activity

In vivo anti-inflammatory activity of *M. azedarach* ethanolic leaf extract was tested by carrageenan-induced paw edema method. Initially, the toxicity level of the ethanolic leaf extract was tested on the rats up to a dose of 2000 mg/kg of extract, and the extract did not cause any mortality even at higher dosage. Hence, the extract was considered safe for administration. The ethanolic leaf extract at dosage of 100, 200, and 300 mg/kg exhibited significant anti-inflammatory activity in all the tested rats ($P < 0.001$). There is a significant decrease in the edema

Table 1. Preliminary phytochemical analysis of various solvent leaf extracts of *M. azedarach*

Phytochemicals	Tests	Extracts			
		PE	CL	EA	ET
Carbohydrates	Molisch's test	-	-	+	+
	Fehling's test	+	+	+	+
	Barford's test	+	-	-	-
Proteins	Biuret test	-	-	-	-
	Million's test	-	+	-	+
	Ninhydrin test	-	+	+	+
Steroids	Salkowski's test	-	+	+	+
	Liebermann-Burchard test	-	-	-	+
	Test for triterpenoids	+	-	+	+
Flavonoids	Shinoda test	-	+	+	+
	Alkaline reagent test	-	-	-	+
	Zinc HCl test	-	+	-	-
Tannins	Ferric chloride test	+	+	-	+
Alkaloids	Mayer's test	-	-	-	-
	Dragendorff's test	-	-	-	-
	Hager's test	+	-	+	+
Cardiac glycosides	Killer-Killian's test	-	-	-	-
	Raymond's test	-	-	-	-
	Baljet test	+	-	+	+

Note: '+' Indicates presence; '-' Indicates absence.

Abbreviations: PE: Petroleum ether; CL: Chloroform; EA: Ethyl acetate; ET: Ethylene; *M. azedarach*: *Melia azedarach*.

Table 2. Antimicrobial activity of various solvent leaf extracts of *M. azedarach*

Pathogens	Zone of inhibition (mm)					Minimum inhibition concentration (mg/L)				
	Extracts					Extracts				
	PE	CL	EA	ET	Standard	PE	CL	EA	ET	Standard
<i>E. coli</i>	-	-	8.10±0.49 ^c	15.07±1.54 ^b	23.41±0.18 ^a	-	-	1.25	0.625	0.0781
<i>S. aureus</i>	-	-	10.44±0.15 ^c	18.23±0.57 ^b	22.32±1.09 ^a	-	-	1.25	0.625	0.0781
<i>B. subtilis</i>	-	-	-	14.23±0.85 ^b	18.23±0.74 ^a	-	-	-	0.625	0.0781
<i>S. typhi</i>	-	-	8.23±0.44 ^c	11.74±0.23 ^b	19.53±0.66 ^a	-	-	1.25	0.625	0.0781

Notes: Values are represented as means±SE for three independent replicates. ^{a,b,c}Represented as significantly different value according to Dunken Multiple test, $P < 0.05$.

Abbreviations: PE: Petroleum ether; CL: Chloroform; EA: Ethyl acetate; ET: Ethanol; *M. azedarach*: *Melia azedarach*; *E. coli*: *Escherichia coli*; *S. aureus*: *Staphylococcus aureus*; *B. subtilis*: *Bacillus subtilis*; *S. typhi*: *Salmonella typhi*.

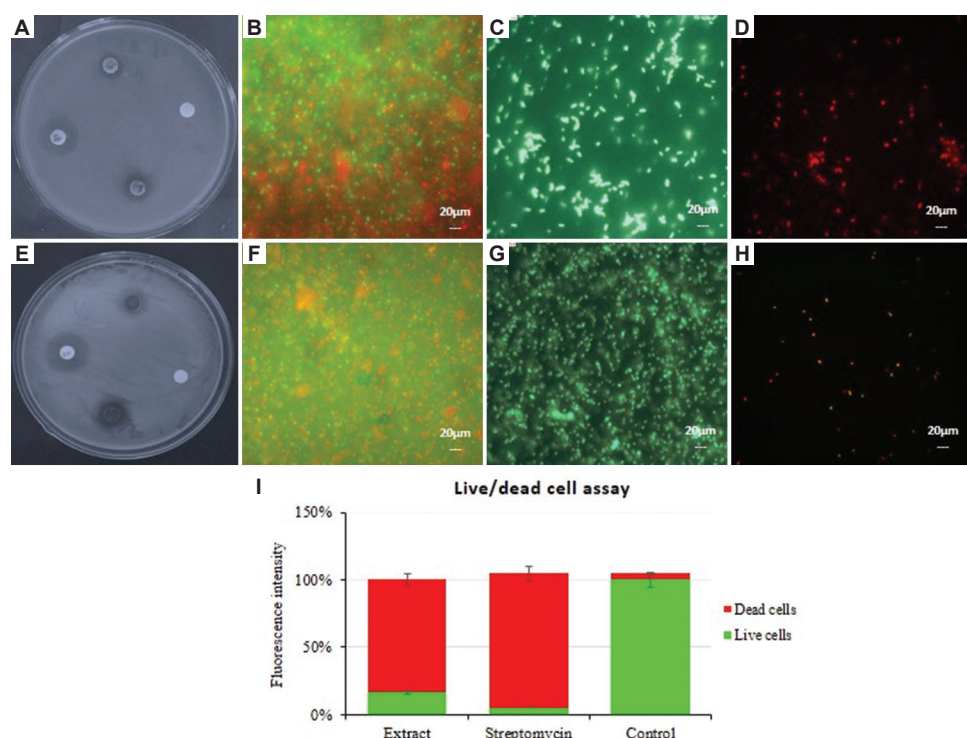


Figure 1. Antibacterial and live/dead cell backlight assay. (A and E) Zone of inhibition in *Escherichia coli* and *Staphylococcus aureus*. (B and F) Dead cells after exposure to extract at 50 mg/L concentration. (C and G) Live cells (negative controls). (D and H) Dead cells after being treated with streptomycin at 10 mg/L concentration. (I) Fluorescence intensity and the percentage ratios of live/dead cell distribution of *E.coli* and *Staph. aureus*. The cells were viewed under the magnification of $\times 40$ using fluorescence microscope.

volume of rats administered with extract after 3 h, when compared to the control group (Table 3). The extract at 300 mg/kg exhibited significant anti-inflammatory activity of 51.78 % in carrageenan-induced rat paw edema (Figure 2). In addition, the results showed that the extract inhibited inflammation in treated rats in a dose-dependent fashion.

3.5. Cytotoxic effect of *M. azedarach* leaf extract on HepG2 cell line

The MTT test was used to assess the cytotoxic effects of ethanolic extract on the HepG2 cells. Half maximum inhibitory concentration (IC_{50}) was computed from the dose-response curve of 62.5, 125, 250, 500, and 1000 $\mu\text{g/mL}$ concentration. The cytotoxicity of the ethanolic extract is depicted in Figure 3, and it was found that there was a significant difference ($P < 0.05$) between different concentrations of the tested sample. The IC_{50} value of ethanolic extract on the HepG2 cells after a 24-h exposure was determined as $540.00 \pm 0.6 \mu\text{g/mL}$. Further, using phase contrast inverted microscope, the bacterial cell morphology after being treated with ethanolic extract and control-treated cells was observed (Figure 4).

3.6. Analysis of apoptosis-related gene expression

The upregulation of *p53* gene expression on the HepG2 cells after being exposed to ethanolic extract of *M. azedarach* at a concentration of 540 $\mu\text{g/mL}$ for 24 h was detected by means of qRT-PCR (Figure 5A and B). Precisely, the cells treated with doxorubicin (2 $\mu\text{g/mL}$) and ethanolic extract showed an upregulation of *p53* gene expression by 2.33 and 1.35 fold, respectively, whose levels were much higher in comparison to the untreated control.

3.7. DNA fragmentation detection assay

Apoptosis was further evaluated by measuring the DNA laddering as a result of DNA fragmentation, which is indicative of the late stage of apoptosis. In this study, HepG2 cells treated with doxorubicin (2 $\mu\text{g/mL}$) and ethanolic extract exhibited DNA laddering, as shown in Figure 5C.

4. Discussion

In the current study, we successfully unveiled the presence of various secondary metabolites in *M. azedarach* leaf extracts by means of phytochemical screening. Ethanolic extract boasts a huge amounts of phytochemicals such

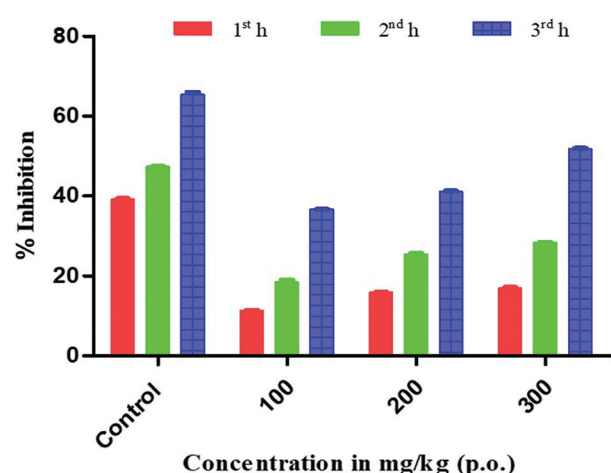
Table 3. Anti-inflammatory activity of leaf ethanolic extract of *M. azedarach* at different hours in carrageenan-induced paw edema model

Groups	Dose of extract, p.o. (mg/kg)	Change in paw thickness (mm) mean±SD		
		1 st h	2 nd h	3 rd h
Group A, (saline; negave control)	-	0.766±0.07 ^e	0.818±0.08 ^e	0.99±0.05 ^a
Group B (diclofenac sodium, positive control)	40	0.461±0.05 ^d	0.431±0.01 ^d	0.372±0.02 ^b
Group C	100	0.679±0.03 ^c	0.667±0.02 ^c	0.627±0.04 ^e
Group D	200	0.645±0.02 ^b	0.610±0.04 ^b	0.584±0.01 ^d
Group E	300	0.636±0.05 ^a	0.586±0.01 ^a	0.477±0.01 ^c

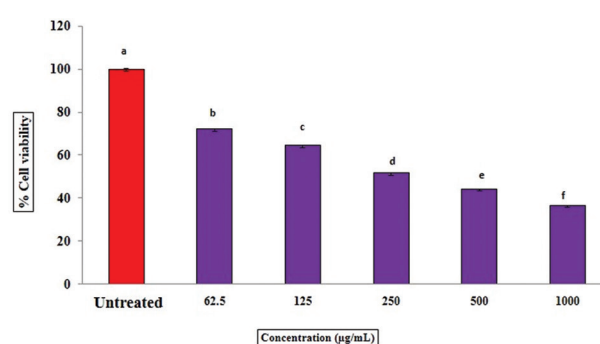
Notes: Values are represented as means±SE for three independent replicates. ^{a,b,c,d,e}: Represented as significantly different according to Dunken Multiple test $P<0.05$.

Alphabets with the same letter show that there is no significant difference. Alphabets with different letter show that there is significant difference.

Abbreviation: *M. azedarach*: *Melia azedarach*.

**Figure 2.** Anti-inflammatory effect of leaf ethanolic extracts of *Melia azedarach* and percent inhibition of the carrageenan-induced paw edema in different time intervals.

as carbohydrates, proteins, steroids, flavonoids, tannins, alkaloids, and cardiac glycosides, followed by ethyl acetate extract. These phytochemicals are known to play a crucial role in health-care domain. Through the disc diffusion assay, we found that the growth of the tested bacterial strains such as *E. coli*, *B. subtilis*, *S. typhi*, and *S. aureus* was effectively suppressed by ethanolic leaf extract of *M. azedarach*, as compared to its ethyl acetate extract, whose inhibitory effects on growth were exerted in a dose-dependent manner. No activity was observed in petroleum ether and chloroform extracts. Significant bactericidal activity was observed in ethanolic leaf extract, which contains a variety of phytochemicals compared to the remaining solvent extracts, as confirmed in the preliminary phytochemical screening. Similar results have been reported by Gaggia *et al.*,¹⁹ who reported that the aqueous leaf extract of chinaberry exhibited antimicrobial activity against certain phytopathogenic bacteria.

**Figure 3.** Cytotoxic properties of ethanolic leaf extracts of *Melia azedarach* and percentage of cell viability against HepG2 cells at different concentrations.

Furthermore, we also found that ethanolic extract was highly toxic and deadly for *E. coli* and *S. aureus*, as confirmed using a fluorescent viability probe (Live/dead® Backlight assay). Our results are in agreement with the previous reports of De CC Pinto *et al.*,²⁰ who found that *Annona muricata* extract could effectively damage the cell membrane of Gram-positive and Gram-negative bacteria. In general, bacterial cells that have lost the plasma membrane integrity and are unable to maintain an electrochemical potential are considered dead.²¹ Although the bacterial potential of *M. azedarach* has been reported previously, this study showed that tested extract showcases a broad spectrum of antibacterial activities against both Gram-negative and Gram-positive bacteria in a dosage-dependent manner.

Carrageenan-induced acute inflammation is one of the widely adopted *in vivo* tests for screening the anti-inflammatory activity of natural products. Induced inflammation can be observed in two phases: the early stage that features intense inflammation and the later phase that results in a gradual increase in the swelling of the rat paw edema.²² The initial inflammatory response

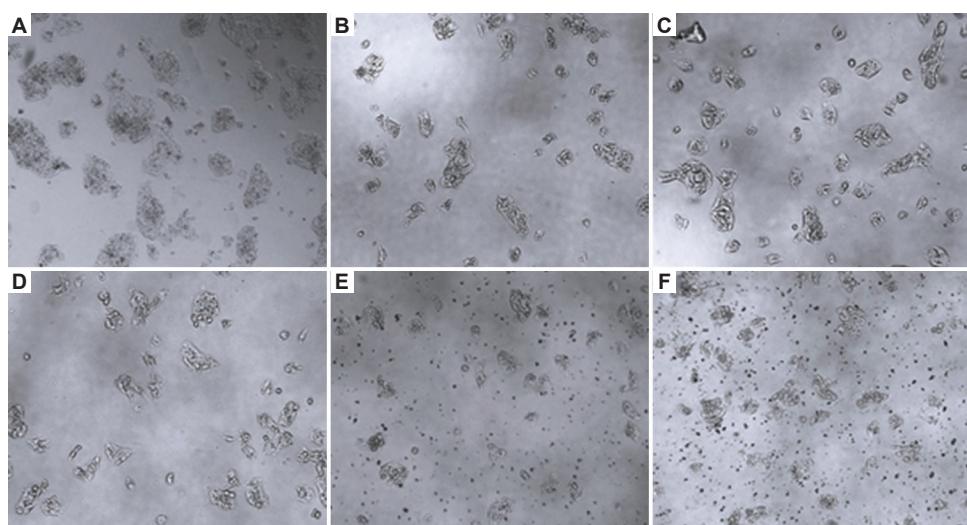


Figure 4. Morphological changes in HepG2 cells as observed under phase contrast inverted microscope at 24 h: (A) Untreated cells, (B) cells treated with *Melia azedarach* ethanolic extracts at 62.25 µg/mL, (C) at 125 µg/mL, (D) at 250 µg/mL, (E) at 500 µg/mL, and (F) at 1000 µg/mL. The cells were viewed under the magnification of $\times 10$.

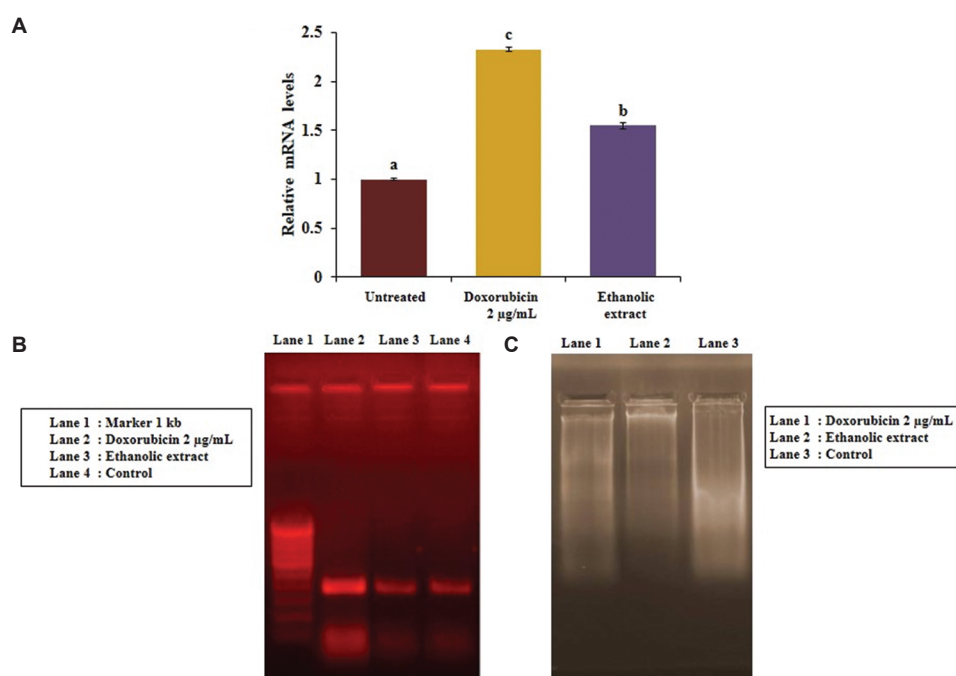


Figure 5. Effect of *Melia azedarach* ethanolic extract at 500 µg/mL concentration on the mRNA levels of *p53* in HepG2 cells. Total RNA was isolated from treated and control samples, and the gene expression was analyzed using reverse transcription polymerase chain reaction (A and B). DNA laddering was visualized in HepG2 cells upon treatment with ethanolic extract at 500 µg/mL concentration for 24 h (C).

begins within an hour of the carrageenan injection, influenced partly by the injection trauma and also by the presence of histamine and serotonin components. In the present study, there is no significant inhibition of paw edema: the ethanolic leaf extract of *M. azedarach* at 100, 200, and 300 mg/kg concentrations led to only 11.32%, 15.84%, and 17.10% of paw edema inhibition, respectively,

in the first few hours upon administration. This indicates no inhibition of histamine and serotonin. In the late hours, after 3 h, the inhibition of paw edema was increased up to 51.78 %. This increase can be caused by the responsiveness of carrageenan-induced paw edema to cyclooxygenase inhibitors. Carrageenan-induced rat paw edema model can be employed to assess the impact of non-steroidal anti-

inflammatory agents that chiefly target the cyclooxygenase responsible for the excessive prostaglandin synthesis.²³ In the present study, the results align well with earlier reports on anti-inflammatory effects of *M. azedarach* leaf extract.²⁴

The ethanolic leaf extract also showed strong cytotoxic impacts on the HepG2 cells. Doxorubicin is frequently used to serve as the standard drug for comparison with potential anti-cancer drug candidates because of its well-known pro-apoptotic effect. In the present study, the viability of HepG2 cells noticeably decreased with an increase in extract concentration. This particular finding does not concur with a study by Zeng *et al.*,²⁵ who discovered that a compound isolated from *M. azedarach* leaves effectively reduced the viability of the similar cell line. This result discrepancy is due to potential variations in phytochemical content influenced by extraction procedures, leaf maturity, solvent choice, and dosage used or duration of exposure to cells. Another study conducted by Zhang *et al.*²⁶ proved that *M. azedarach* extracts are rich in bioactive chemicals, primarily limonoids, phenolics, and oxygenated triterpenoids, which are considered potential compounds with high therapeutic values against malignant cells.

Apoptosis is regarded as the pharmacodynamics endpoint of anticancer drug therapy as it prevents cancer from developing resistance to chemotherapy.²⁷ Furthermore, apoptosis prevents the inflammatory response caused by necrosis through self-dismantling of individual cell components. Thus, apoptosis of cancer cells will not cause harmful effects to the adjoining normal cells. In this study, the qRT-PCR method was used to investigate molecular pathways by inducing apoptosis with ethanolic leaf extract of *M. azedarach*. Following treatment with ethanolic leaf extract, *p53* expression was increased. Based on our results, we postulate that ethanolic leaf extract upregulates the expression of *p53*, which causes *p21* protein accumulation, thereby leading to cell cycle arrest through apoptosis induction in HepG2 cells. Our results are consistent with several studies that showed how *p53* and *Bcl-2* function to induce apoptosis.²⁸ The tumor suppressor gene *p53* also plays a vital role in apoptosis. In fact, the majority of medications used to treat cancer patients nowadays elicit antitumor effects by reducing tumor growth in a *p53*-dependent manner. Joray *et al.*²⁹ reported that *p53* tumor suppressor is necessary for the efficient execution of the apoptosis following treatment with a cytotoxic limonoid derived from *M. azedarach*. In this study, the ethanolic leaf extract was cytotoxic to HepG2 cells and also upregulated *p53* expression in these cells. These findings are in support of *M. azedarach* extract as a potential anticancer agent and provide insights into

the development of chemotherapeutic drugs based on herbs.

The HepG2 cells treated with ethanolic leaf extract showed evidence of DNA fragmentation, a sign of confirmed apoptosis. However, no fragmentation was observed in the control cells. A possible sign that DNA replication is being inhibited because of inter-nucleosomal cleavage caused by apoptosis is DNA molecule breakdown. Previous research has demonstrated that *Avicennia marina* extract is an apoptotic agent against MDA-MB 231 cells, resulting in DNA fragmentation.³⁰

5. Conclusion

M. azedarach leaf extracts possess a broad spectrum of phytochemicals with a promising bactericidal effect against human pathogenic bacteria and with moderate anti-inflammatory and anti-cancer properties against HepG2 cancer cell lines. Further, the leaf extract also induced apoptosis in MCF-7 cells, probably mediated through *p53* expression. This study lends credence to the concept that ethanolic leaf extract can be employed in some forms of anti-cancer therapy. Our findings indicate that *M. azedarach* leaves are a potential reservoir of natural bioactive compounds, providing an invaluable source of novel biomolecules with anti-inflammatory and anti-cancer properties.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: All authors

Investigation: All authors

Writing—original draft: All authors

Writing—review & editing: All authors

Ethics approval and consent to participate

The experiment was conducted based on the ethical guidance for the care and use of laboratory animals in research as approved by the animal ethics committee of the University of Mysore, Manasagangotri, Mysore (animal sanction order UOM/IAEC/05/2017).

Consent for publication

Not applicable.

Availability of data

The data of this study are available from the corresponding author upon reasonable request.

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