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In vitro bioactive potential of *Azadirachta indica* and *Melia azedarach* extracts

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Abstract

Antimicrobial resistance (AMR) is recognized as a critical and growing global health threat affecting modern healthcare systems, while remarkable antimicrobial and antioxidant potential has been demonstrated by medicinal herbs. In this study, the antimicrobial and antioxidant activity of aqueous ethanol extracts of *Azadirachta indica* (A indica) and *Melia azedarach* (M azedarach), individually and in different formulation ratios, was investigated. In the described study, the leaves of *A. indica* (Azadirachta indica) and *M. azedarach* (likely *Azadirachta jussae* or a variant) were extracted using an aqueous ethanol medium in predetermined ratios. These extracts were then condensed, and the formulations were created in ratios of 25:75, 50:50, and 75:25, likely to investigate the effects of different concentrations of each plant extract on microbial growth and antioxidant activity.

The culture media for two bacteria, *Bacillus subtilis* and *Serratia marcescens*, were prepared, and the discs containing the plant extracts were placed on the cultured agar plates. The *zone of inhibition*—the area where bacteria were unable to grow around the discs—was observed as an indicator of antibacterial activity. The diameter of the inhibition zone was measured to assess the extent of antimicrobial effectiveness. Additionally, the antioxidant activity of the extracts was studied, which would involve determining how effectively the plant extracts could scavenge free radicals' potential.

Moderate antimicrobial activity against *Bacillus subtilis* was exhibited by *M. azedarach* when tested individually. However, when it was combined with *A. indica*, a significant increase in antimicrobial activity was observed. The maximum inhibitory effect against *Bacillus subtilis* was shown by *A. indica*, followed by the 25:75, 50:50, and 75:25 formulations, respectively.

In the case of *Serratia marcescens*, the maximum inhibitory effect was observed with the 25:75 formulation, followed by the 75:25 formulation. The remaining extracts exhibited almost similar levels of antimicrobial activity. Antioxidant activity was also exhibited by all the extracts and formulations tested.

Significant antimicrobial activity against *Serratia marcescens* was exhibited by *A. indica*, and their various formulations. Antimicrobial activity against *Bacillus subtilis* was shown by *A. indica* both individually and in combination with *M. azedarach*, whereas only moderate activity was observed with *M. azedarach* alone. Among

the tested formulations, the 25:75 combination demonstrated better antimicrobial activity compared to the other samples. Antioxidant potential was also exhibited by the extracts and formulations, like that reported earlier for related plant extracts.

Keywords Antimicrobial, Antioxidant, *Azadirachta indica*, *Melia azedarach*, *Serratia marcescens* and *Bacillus subtilis*

Introduction

Plants have been used as a source of food, fiber, and medicine since the start of human society. The use of plants and their products have had a long history, beginning with folk medicine and, over the years, being included into traditional and allopathic medicine systems (Dubey et al., 211). Medicinal herbs have been applied to produce a variety of pharmaceuticals that are currently used to alleviate various health disorders or diseases. Numerous research has been performed to discover new antimicrobial treatment alternatives that can effectively prevent the grow of bacteria or eradicate them without endangering humans, through complete screenings of medicinal herbs (Paritala et al., 2015 and Shafizadegan S et al., 2018). *Azadirachta indica* and *Melia azedarach* are versatile medicinal plants with a broad range of biological activities. They are naturalized in tropical and subtropical regions and can grow up to 30 m tall, belonging to the Meliaceae family. *Azadirachta indica* and *Melia azedarach* have been extensively used in Ayurveda, Unani, and homeopathic medicine, as medicinal properties are exhibited by their leaves, bark, stem, and root. Numerous biologically active compounds are present in *A. indica* and *M. azedarach*, including azadirone, promeliacin, limonoids, gedunin, vilasinin, C-secomeliacins, azadirachtin, nimbin, salanin, and other non-isoprenoids, as well as proteins, amino acids, polysaccharides, sulphurous compounds, and polyphenolics such as flavonoids, glycosides, dihydrochalcone, coumarins, tannins, and aliphatic compounds etc. (Biswas et al., 2002 and Vishnukanta et al., 2010). Various pharmacological actions of neem have also been reported, and it can be used as an abortifacient, analgesic, anthelminthic, antibacterial, anti-yeast, anti-ulcer, antifertility, antifilarial, antifungal, antihyperglycemic, anti-inflammatory, antiviral, antimalarial, diuretic, antinematodal, antipyretic, antispasmodic, insecticidal, antispermatogenic, antitumor, hypercholesteremic, hypoglycemic, and immunomodulator (Parotta, 2001; Ross, 2001). They have been well documented for their antitussive, antifungal, cholesterol-lowering, anticancer, anti-rheumatic, and other properties in their different extracts (Neycee M.A.et al., 2012, Jaybeen K, et al., 2011, Saviozzi M, et al. 2019 and Sujarwo W et al., 2016). *A indica* is referred to as the source of numerous therapeutic compounds and is therefore regarded as a “pharmaceutical wonder,” with over 300 chemically diverse and structurally complex phytochemicals having been identified from the plant (Chowdhury et al., 2022). Concerns have been raised regarding the safety and potential adverse effects associated with the use of extracts from different parts of the plant, including the seeds, which have not been extensively investigated for their toxicological profiles. The isolation of bioactive compounds from *A. indica* seeds has also been identified as an area requiring further exploration, while efforts have been undertaken to identify and characterize compounds from the leaves and other parts using techniques such as thin-layer chromatography (Gurav et al., 2023).

Therefore, considering previous reports that *A. indica* and *M. azedarach* possess significant medicinal importance, this study was conducted to evaluate the physicochemical and preliminary phytochemical properties, as well as the antimicrobial and antioxidant activities, of the leaf extracts of *A. indica* and *M. azedarach* using aqueous ethanol as a solvent.

Material and Methods

Herbal Extraction Using Aqueous Ethanol

Leaves were procured from the local market Gwalior M.P. India and identified by the botanist. Verification was performed at the Dr Devendra Kadam Assistant professor in Botany Department in Govt college Karera Shivpuri M.P. India. The healthy leaves of *A. indica* and *M. azedarach* were then washed with distilled water and shade-dried for 8 weeks

Fresh leaves of *A. indica* and *M. azedarach* were extracted with water and ethanol using the cold percolation method. The extracts were filtered, concentrated under reduced pressure at 47 °C, and further incubated at 46 °C for 3 days to remove ethanol, yielding crude leaf extracts. The extracts were diluted with distilled water (5:1) to prepare stock solutions (200 mg/mL). *A. indica* and *M. azedarach* extracts were then combined in ratios of 25:75, 50:50, and 75:25. These formulations, along with the individual extracts, were used for further studies.

Phytochemical screening

The total phenolic content of the aqueous ethanol extracts of *A. indica* and *M. azedarach* was determined using the Folin–Ciocalteu method. Standard solutions were prepared by taking 10, 20, 30, 40, and 50 µl aliquots from a 10 mg/ml stock solution, and the total phenolic content was expressed as gallic acid equivalents (GAE) according to the method described by Sharma and Singh (2012). The proanthocyanidin content was expressed as rutin equivalents following the protocol described by Kikuzaki and Nakatani (1993). The total flavonol content in the plant extracts was also expressed as rutin equivalents according to the method of Kumaran and Karunakaran (2007). The extraction procedure was carried out using semi-polar solvents, as their use aligns with trends reported in the literature. Semi-polar solvents, such as ethanol or acetone, have been reported to effectively extract both polar and non-polar phytochemicals. A wide range of bioactive compounds—ranging from hydrophilic compounds such as flavonoids and tannins to lipophilic compounds such as limonoids and steroids—can be dissolved in these solvents (Susilo et al., 2023). The extraction of phenolic compounds is considered critical due to their well-documented antioxidant properties. Free radicals, which contribute to oxidative stress and are associated with diseases such as cancer, cardiovascular disorders, and neurodegenerative conditions, are neutralized by these compounds. In medicinal applications, anti-inflammatory, anti-aging, and immunomodulatory effects have been attributed to phenolic compounds. Cellular protection against oxidative damage has therefore made these compounds a major focus in the development of plant-derived therapeutic products (Zeeshan et al., 2024). A high occurrence of phenolic phytochemicals in the methanol extract has been consistently reported in previous studies. The antioxidant activities of plant-derived phenolic compounds have been extensively documented by Li et al. (2024) and Ishabiyi et al. (2023). It has been confirmed that the type of solvent used during extraction significantly influences the phytochemical composition of plant extracts, thereby affecting their potential medicinal applications.

Table 1 Phytochemical content of *A indica* and *M azedarach*

Phytoconstiuents	*A indica*	*M azedarach*
Alkaloids	+	+
Saponins	+++	+
Tannins	++	-
Steroid	+++	+++
Terpenoid	+++	+++
Glycoside	++	-
Flavonoid	+	+
Phenol	+	+++
Oxalic acid	+	-

+++ More present, ++ Moderate present, + Least present and – Not present

In Vitro Antimicrobial Assay and Culture Preparation

Nutrient broth for bacteria was prepared and divided into falcon tubes, which were inoculated with *Bacillus subtilis* and *Serratia marcescens* using sterile inoculating loops. Potato Dextrose broth for fungi was prepared and inoculated with *Piriformospora indica*. The bacterial cultures were incubated at 37 °C for 24 hours. Nutrient

agar and Potato Dextrose Agar were prepared, and discs made from Whatman blotting paper were sterilized along with the media and glass plates at 120 °C for 20 minutes. The discs, impregnated with plant extracts, were placed on the spread plates of each microorganism. Bacterial plates were incubated at 37 °C for 24 hours, while fungal plates were incubated at 37 °C for 10 days. Microbial inhibition around the discs was measured as the zone of inhibition, and its diameter was recorded.

DPPH-Based Free Radical Scavenging Activity

The 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity was measured according to the method of Ilhami et al. (2005). Various concentrations of the plant extract in methanol (10, 20, 30, 40, and 50 µl) were mixed with 2 ml of 600 µM DPPH solution in methanol and incubated at 25°C for 30 minutes. The absorbance of the test mixture was read at 517 nm using a spectrophotometer, with a DPPH control containing only 1 ml of methanol in place of the extract. All experiments were performed in triplicate, and the results were average. Ascorbic acid was used as the standard. Percent inhibition was calculated using the following expression.

Inhibition (%) = (A control – A sample /A control) x 100

Where, A control and A sample stand for absorbance of the control and absorbance of tested extract solution, respectively.

Results and Discussion

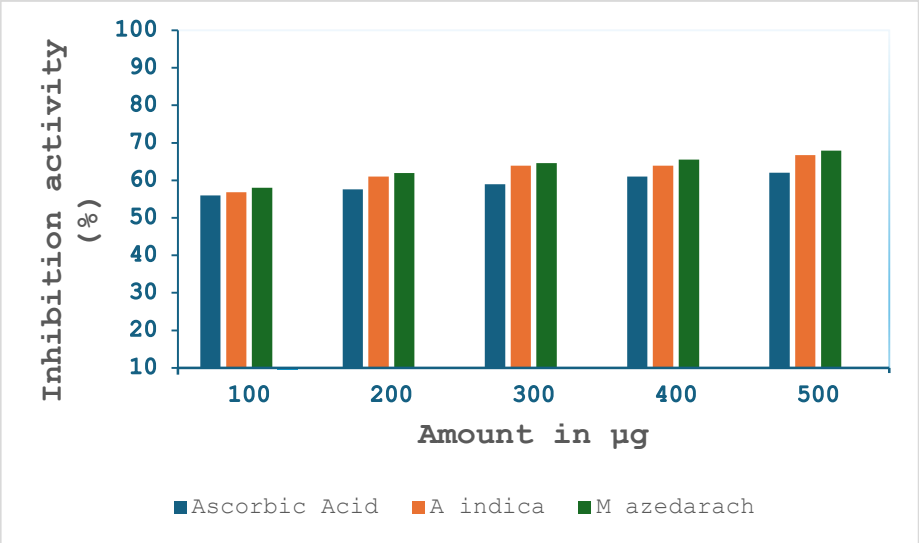
Phytochemical Profiling

The preliminary phytochemical analysis of the aqueous ethanol extract of *A. indica* and *M. azedarach* leaves revealed the presence of various secondary metabolites. Alkaloids, phenols, tannins, flavonoids, and saponins were the most prominent compounds, with the results of the phytochemical tests summarized in Table 1. Flavonoids, saponins, and phenolic compounds are major groups of compounds that function as primary antioxidants or free radical scavengers. Consequently, *A. indica* and *M. azedarach* may be utilized in the development of medicinal formulations, potentially contributing to the treatment of various ailments in the future. Therefore, due to the presence of these secondary metabolites, *A. indica* and *M. azedarach* may hold significant medicinal value.

Antioxidant and Free Radical Neutralizing Activity

Figure 1 presents the results of the free radical (DPPH) scavenging activity expressed as a percentage of inhibition. The results showed that the aqueous ethanol fraction (10–50 µl) of the 10 mg/ml *M. azedarach* solution was measured, with ascorbic acid used as the standard. The inhibition activity of the plant extract was found to be comparatively lower than that of ascorbic acid. The results revealed that the 50 µl (500 µg) plant extract exhibited the highest inhibition activity at 71.23%, followed by decreasing inhibition activity at lower concentrations. The highest radical scavenging activity was observed at 67.93±0.07 for *M. azedarach*, while the extracts of *Azadirachta* showed lower scavenging activity, with the aqueous ethanol extract displaying the highest value at 66.68±0.05. Overall, the aqueous ethanol extracts of both *Azadirachta* and *Melia* demonstrated the highest scavenging activity. Methanol and ethanol have been proven to be effective solvents for extracting phenolic compounds (Siddhuraju and Becher, 2003). In this study, the values obtained from the aqueous ethanol extract of *M. azedarach* were slightly higher than those from *A. indica*. Among the solvents used, aqueous ethanol was found to be the most effective for extracting phenolic components. Ethanol is preferred for the extraction of antioxidant compounds primarily due to its lower toxicity (Karadeniz et al., 2005).

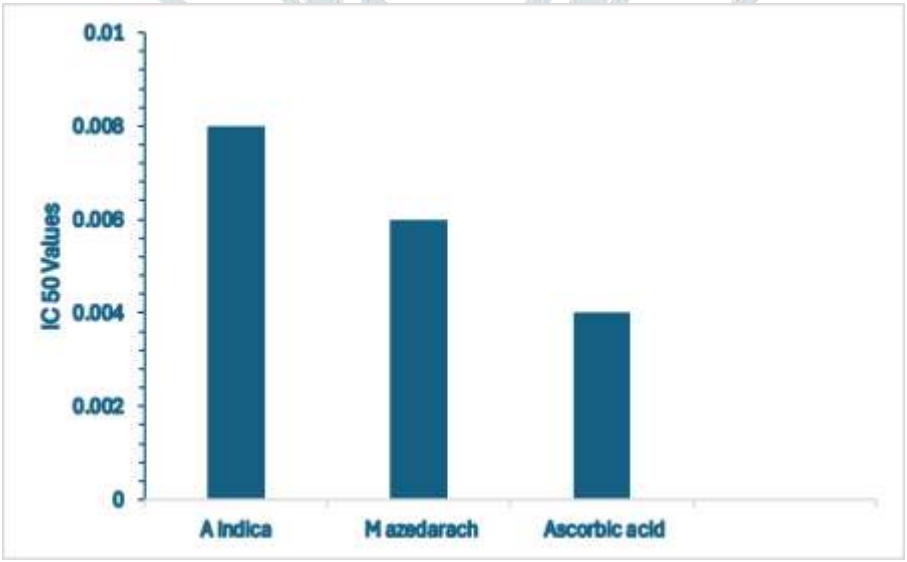
Figure 1. Antioxidant activity was evaluated using the DPPH radical scavenging assay.



IC50 (Half-Inhibitory Concentration)

The IC50 value, defined as the concentration of substrate that causes 50% inhibition of DPPH activity, was calculated by linear regression of plots of the percentage of antiradical activity against the concentration of the tested compounds. The results presented in figure 3 showed that the aqueous ethanol extract of *Azadirachta* exhibited an IC50 value of 0.008 µg/mg. In comparison, the extracts of *M azedarach* showed lower IC50 values, with the aqueous ethanol extract exhibiting the lowest value. Significant activity was demonstrated by the ethanolic extract of *M azedarach*, which showed a lower IC50 value compared to *Azadirachta*. The antioxidant activity of *Azadirachta* and *M azedarach* extracts was observed to increase with the polyphenol content of the extracts.

Figure. 2. IC50 value of A indica and M azedarach leaf extracted in aqueous ethanol in comparison to Ascorbic acid.



Antimicrobial activity

The antimicrobial activity of different formulations was evaluated by measuring the diameter of the zone of inhibition against *Serratia marcescens* and *Bacillus subtilis*, as summarized in Table 2.

Antimicrobial activity against *Serratia marcescens*

Among the tested formulations, the **25:75 formulation** exhibited the highest antimicrobial activity against *Serratia marcescens*, producing a zone of inhibition of **27 ± 4 mm**. This was followed closely by the **75:25 formulation** (26 ± 3 mm), while the **50:50 formulation** showed slightly lower activity (24 ± 3 mm). Notably, the antimicrobial effect of the 50:50 formulation was comparable to that of **1% gentamycin** (24 ± 5 mm), indicating that the formulated plant extract combinations possess antimicrobial efficacy like the standard antibiotic.

The enhanced activity observed in the 25:75 formulation suggests that a higher proportion of one extract may contribute more significantly to antibacterial action, possibly due to increased availability of bioactive phytoconstituents such as flavonoids, phenolics, or terpenoids known for membrane-disrupting and enzyme-inhibitory effects. The comparable or superior activity of the formulations relative to gentamycin highlights their potential as alternative or complementary antimicrobial agents.

Antimicrobial activity against *Bacillus subtilis*

In contrast, against *Bacillus subtilis*, the largest zone of inhibition was produced by **gentamycin** (25 ± 2 mm), demonstrating the superior effectiveness of the standard antibiotic toward this Gram-positive bacterium. Among the formulated extracts, both **50:50** and **75:25 formulations** exhibited similar antimicrobial activity, each producing inhibition zones of **23 ± 3 mm**, whereas the **25:75 formulation** showed slightly reduced activity (22 ± 4 mm).

Although all formulations demonstrated measurable antibacterial effects, their activity remained marginally lower than that of gentamycin, suggesting that *B. subtilis* may be less susceptible to the phytochemical combinations compared with *S. marcescens*.

Synergistic effects of combined formulations

The improved antimicrobial performance of the combined formulations compared with previously reported individual extracts indicates the possibility of **synergistic interactions** between phytoconstituents. Such synergy may enhance antibacterial efficacy through multiple mechanisms, including disruption of bacterial cell walls, inhibition of metabolic pathways, and increased permeability facilitating bioactive compound entry into microbial cells.

Overall interpretation

Overall, the findings demonstrate formulation-dependent antimicrobial activity. The **25:75 formulation** was identified as the most effective against *Serratia marcescens*, whereas **gentamycin** remained the most potent agent against *Bacillus subtilis*. The results support the hypothesis that optimized combinations of plant extracts can enhance antimicrobial efficacy and may serve as promising candidates for the development of plant-based antimicrobial formulations.

Table 2. Antibacterial effects of aqueous ethanol extract formulations derived from *A. indica* and *M. azedarach.*

Diameter of zone of inhibition (mm)		
Formulation amount in mg	*Serrata marcescens*	*Bacillus subtilis*
25:75	27±4	22±4
50:50	24±3	23±3
75:25	26±3	23±3
Gentamycin (1%) Vancomycin	24±5	25±2

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