Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
https://www.phytojournal.com
JPP 2024; 13(1): 127-132
Received: 21-11-2023
Accepted: 23-12-2023

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Phytochemical composition and antibacterial activity of *Azadirachta indica* (Neem) against *Enterococcus faecalis*: Implications on benefits of traditional medicines

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DOI: https://doi.org/10.22271/phyto.2024.v13.i1b.14821

Abstract
Azadirachta indica A. Juss (Meliaceae), a family of Mahogany trees, is native to the Indian subcontinent. *A. indica*, known as neem, has been used in traditional medicine for centuries due to its various medicinal properties, including antibacterial activity. This study analysed the phytochemical composition and antibacterial activities of *A. indica* against *Enterococcus faecalis (E. faecalis)*. This study was conducted between August 2023 and October 2023. Extraction of the active ingredients from *A. indica* leaves was done using the aqueous and ethanolic solvents. Various concentrations of both extracts of *A. indica* were tested for antibacterial activity against *E. faecalis* using the disc diffusion method and sensitivity was measured using the zones of inhibition. This study found the presence of phytochemicals including phenolics and tannins in both extracts. Further, alkaloids, flavonoids, and saponins were only found in the aqueous extract. Furthermore, steroids were found in the ethanolic extract only. Intriguingly, both aqueous and ethanolic extracts produced activity against *E. faecalis* with a minimum inhibitory concentration for both tbeing 20 mg/mL. The zones of inhibition increased with an increase in extract concentration, and the largest observed was 9.6 mm using the ethanolic extract. This study highlights the promising antimicrobial properties of both the aqueous and ethanolic extracts of *A. indica* as exhibited by significant inhibitory activity against *E. faecalis*. These findings support the traditional use of Neem in folk medicine as an antibacterial agent and highlight its potential for the development of novel therapeutic agents.

Keywords: Antibacterial activity, *Azadirachta indica*, Enterococcus faecalis, Neem, Phytochemicals, Traditional medicine, Zambia

Introduction
Infectious diseases are a common threat to both humans and animals, causing considerable suffering and mortality globally [1]. These diseases, often caused by harmful microorganisms like bacteria, viruses, parasites, and fungi are common in developing countries, especially in Africa [2]. Studies have shown that traditional medicines are effective in the management of many diseases, including infectious diseases [3, 4]. Subsequently, more than 80% of the population in sub-Saharan Africa depends on plants and traditional medical practices as the primary source of health care [5]. *Azadirachta indica (A. indica)* is among the traditional medicines that are effective against certain diseases [6, 7].
Enterococci are part of the normal flora of the gastrointestinal tract [8-10]. The use of broad-spectrum antibiotics in hospitalized patients causes the elimination of enterococc leading to a decreased thickness of the protective gastrointestinal mucus layer [11]. This may enable unnnecessary outgrowth of hospital-associated enterococcal clones that tend to be resistant to antibiotics [12]. Among *Enterococcus* species, two major species, *E. nterococcus faecalis (E. faecalis)* and *Enterococcus faecium (E. faecium)*, are pathogenic to humans. *E. faecalis* causes about 85-90% of enterococci infections while 5-10% are caused by *E. faecium* [11]. These two species also result in bacterial infections of surgical wounds, endocarditis, neonatal sepsis, and very infrequent meningitis [13].
As these infectious diseases continue to challenge healthcare systems worldwide, a growing concern is the rise of antimicrobial resistance (AMR) [14, 15].

AMR presents a significant challenge to global health [16, 17], contributing substantially to both illness and death worldwide [18]. This problem is worsened by the emergence of multi-drug resistant (MDR) bacteria [19]. According to recent studies, *E. faecalis* has been reported to exhibit a high level of resistance to ampicillin, especially due to beta-lactamase production [16, 20, 21].

A. indica (Meliaceae), also called Neem, is native to India, has medicinal effects, and is found in most tropical and subtropical countries [22, 23]. *A. indica* (neem) contains phytochemicals that are responsible for its medicinal effects and exert a physiological action on the human body [23]. Some of the phytcochemicals contained in Neem include alkaloids, anthraquinones, flavonoids, glycosides, steroids, saponins, and tannic acid [24, 25]. Extracts of Neem leaves have antibacterial, antifungal, antiviral, antidiabetic, insecticidal, diuretic, cardiac, antipyorrhoiec, antiscabic, and many other biological activities [7, 23, 26-28]. Neem extracts from leaves, roots, bark, and seeds have been shown to have antimicrobial activity against *Streptococcus* variants and *E. faecalis* [29-31]. A wide range of Gram-positive and Gram-negative microbes, including *Mycobacterium tuberculosis* and strains resistant to streptomycin, are susceptible to the oil from the leaves, seeds, and bark of Neem [32].

In Zambia, approximately 70% of the indigenous population use traditional medicines for their ailments, and plants are used as medicines in the treatment and prevention of several diseases [33]. Some studies have reported the various traditional medicines used among the Zambian people [7, 34-38]. This highlights the importance of traditional medicines in the treatment of diseases. It is against this background that this study analysed the phytochemical constituents and antibacterial activities of the *A. indica* extracts against *E. faecalis*, a diverse group of gram-positive, facultative anaerobic bacteria, presenting a promising avenue for the development of alternative therapeutic intervention.

Materials and Methods

Collection of plant material: This laboratory-based study was conducted from August 2023 to October 2023. *A. indica* leaves were collected from the National Institute of Public Administration (NIPA) Main Campus in Lusaka, Zambia. The plant was identified by the University of Zambia (UNZA) School of Natural Sciences Department of Biological Sciences.

Plant extraction preparation: We used a knife to remove *A. indica* leaves from the plant stem and washed them under running tap water to ensure dust removal and then dried under shade for 14 days. We used a mortar and pestle to pound the the dried leaves into a powdered which we later sieved to homogenous powder. Overall, we weighed 50 g of the *A. indica* coarse powder and then extracted the active ingredients with 750 mL of the solvent (water /ethanol). The mixture was filtered using Whitman's number one filter paper. We dried the filtrates in beakers over a water bath for 12 hours at 60˚C. The extracts were then covered and stored in a refrigerator at a temperaturw of 5˚C and their percentage yield were determined as follows:

Extract yield% = R/S x100

Note: S; weight of plant raw sample; R=weight of extracted plant residues

Phytochemical Analysis: Phytochemical analysis of the plant extracts was performed according to the procedure outlined in the University of Zambia's Pharmacognosy laboratory manual according to Hikaambo *et al.*, 2022 and Kabuka *et al.*, 2022 [7, 35]. We tested for phytochemicals including Alkaloids, Flavonoids, Phenolics, Tannins, Saponins, Steroids, and Terpenoids, based on a previous study methods [7].

Collection, culture, and inoculation of isolates: The *E. faecalis* (ATCC 29212) isolates used in the experiment were cultured in the Microbiology Laboratory located in the Department of Pathology and Microbiology at the University Teaching Hospitals (UTH) in Lusaka, Zambia. The *E. faecalis* isolates were isolated by standardised methods as explained in a previous study [16]. The petri dishers containing *E. faecalis* isolates were then incubated at 37ºC for 18 to 24 hours as reported in another study [39].

We used the direct state suspension and turbidity principles to prepare 0.5 McFarland standard. We ensured that the turbidity was balanced using clearn saline until the point when it coordinated the one for 0.5 McFarland turbidity standard. The process was completed by holding the suspension and the 0.5 McFarland turbidity gauges next to source of light opposite to a white foundation accompanied by differentiating dark lines [22, 40].

Mueller-Hinton agar was prepared on petri dishes and consideration was taken to ensure that excessive wetness and dryness of the agar did not occur. *E. faecalis* colonies were picked using a sterile cotton swab and inoculated onto the Mueller-Hinton agar petri dihes. This was followed by spreading the *E. faecalis* by swabbing the agar surfaces at a 90-degree edge, then at a 45-degree edge [7, 22]. Before applying the A. *indica* extracts and ciprofloxacin, the inoculated petri dishes were kept for 20 minutes to allow for assimilation of the inoculum [7, 22].

Antimicrobial Activity Determination: The disk diffusion method was used to evaluate the antimicrobial activity of *A. indica* against *E. coli*. We prepared concentrations of 1 mg/mL, 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL, and 50 mg/mL of the aqueous and ethanolic extracts of *A. indica* from a stock solution of 50 mg/mL which was diluted using sterile distilled water for the aqueous extract and Dimethyl sulfoxide (DMSO) for the ethanol extract, sterilised through Millipore filter and loaded their requisite amount over sterilised filter paper discs (8 millimetres in diameter). The loaded filter paper discs with the various concentrations of the plant extract were placed on top of the Mueller-Hilton agar plates and incubated at 37°C for 24 hours. The zones of inhibition were measured using a plastic ruler in mm and recorded against the concentrations of the effective plant extracts. We used ciprofloxacin 5 µg standard discs as positive control against *E. faecalis* (ATCC 29212) while DMSO and sterile water were used as negative controls. We determined the minimum inhibitory concentration (MIC) at the lowest concentration that inhibited the growth of *E. faecalis*, as outlined in a previous study [41].

Data Analysis: The collected data were entered into Microsoft Excel 2013 and cleaned. Analysis of Variance (ANOVA) was performed to compare the activity of the extracts and ciprofloxacin (the control drug). Additionally, to compare the results between the concentrations of two extracts, we performed Tukey's Honestly Significant Difference (HSD) Test for multiple comparisons. We

presented the results in Tables and a Figure. Finally, we conducted all statistical analyses at a 95% confidence level and findings a $p<0.05$ were considered significant.

Ethical consideration: Prior to carry out the study, we obtained ethical approval from the University of Zambia Health Sciences Research Ethics Committee (UNZAHSREC), protocol ID 2023301270009. All laboratory procedures were done based on the standard operating procedures.

Results

Percentage extraction value

Table 1 summarises the extracted *A. indica* using two solvents, ethanol, and water. The aqueous-based extraction method yielded a higher percentage of extract (26.42%) compared to the ethanol-based method (17.20%), *indica*ting that water was more effective in extracting the desired components from the plant material.

Table 1: Weight of the *A. indica* extract, initial weight, and calculated percentage yield of the plant.

Solvent to extract A. indica	Initial Weight of the plant in grams	Weight of extract recovered in grams	Percentage extraction yield
Ethanol	50	8.6	17.20%
Water	50	13.21	26.42%

Phytochemical Analysis

Table 2 summarises the phytochemical analysis of *A. indica* extracts in aqueous and ethanol solvents. In the aqueous extract, found phytochemicals including and alkaloids, flavonoids, phenolics, saponins, and tannins (Table 2). However, the ethanolic extract showed the absence of flavonoids, saponins, and alkaloids while retaining the presence of phenolics and tannins (Table 2).

Table 2: Phytochemical analysis of the *A. indica* extract

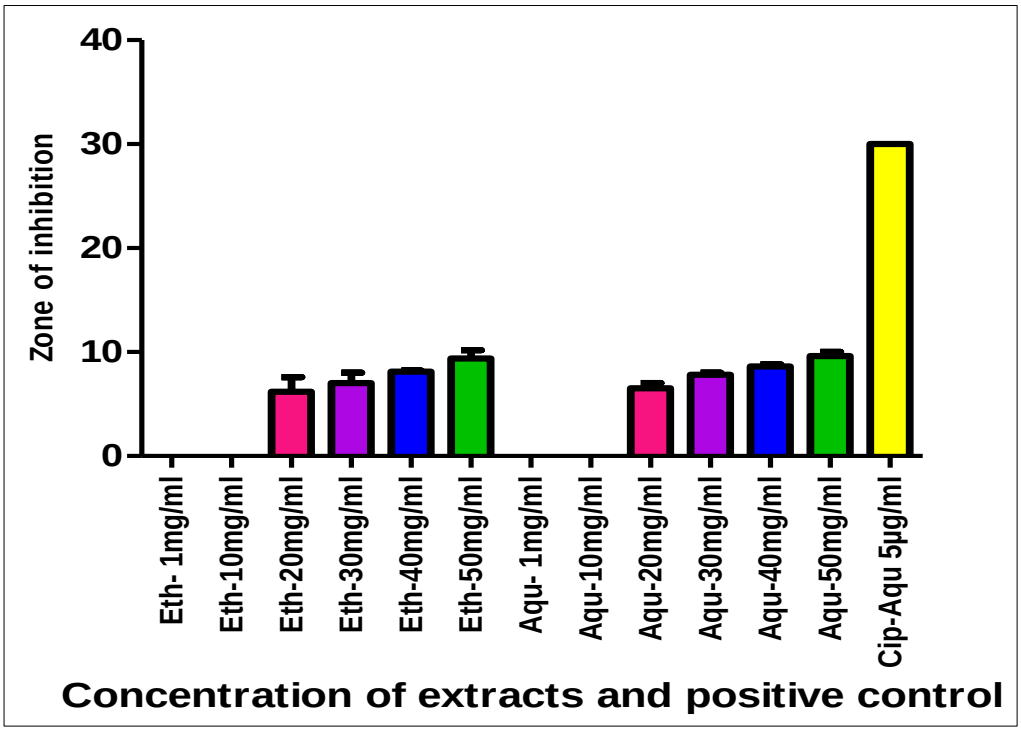
Phytochemicals	Aqueous extract	Ethanol extract
Alkaloids	Present	Absent
Flavonoids	Present	Absent
Phenolics	Present	Present
Saponins	Present	Absent
Steroids	Absent	Present
Tannins	Present	Present
Terpenoids	Absent	Absent

Antibacterial activity of aqueous and ethanolic extracts of *A. indica* against *E. faecalis*

The antibacterial activities of aqueous and ethanolic extracts of *A. indica* against *E. faecalis* are shown in Table 3. At a low concentration (1mg/mL), neither the aqueous nor the ethanolic extract showed significant inhibition of *E. faecalis* growth (Table 3). However, as the concentration was being increased, both extracts exhibited increased antibacterial activity with a maximum effect being observed at 50 mg/mL for both extracts. No statistical significance was observed between the two extracts at their maximum concentration (p-value = 0.896) (Table 3). However, the aqueous and ethanolic extracts at different concentrations showed statistically significant with p-value = 0.028 in both the extracts (Table 3).

Table 3: Antibacterial properties of aqueous and ethanolic extract of *A. indica* and ciprofloxacin

Concentration of extract	Mean zone of inhibition (mm) for the aqueous extract	Mean zone of inhibition (mm) for the ethanolic extract	P - Value	Mean zone of inhibition (mm) for ciprofloxacin
1mg/ml	0	0		30 ± 0
10mg/ml	0	0		
20mg/ml	6.5 ± 0.71	6.2 ± 1.98	0.028	
30mg/ml	7.8 ± 0.28	7.0 ± 1.41		
40mg/ml	8.6 ± 0.28	8.1 ± 0.14		
50mg/ml	9.6 ± 0.57	9.4 ± 1.13	0.896	



Eth: Ethanol extracts, Aqu: Aqueous extracts, Cip-Aqu: Ciprofloxacin aqueous solution

Fig 1: A graph of concentration vs. zone of inhibition of the extracts compared to ciprofloxacin.

Both the aqueous and ethanolic extracts of *A. indica* had less antibacterial activity against *E. faecalis* compared to ciprofloxacin.

Discussion

This study found that *A. indica* contained a variety of phytochemicals, including flavonoids, phenolics, tannins, saponins, alkaloids, terpenoids, and steroids. Furthermore, we found that *A. indica* exhibited a dose-dependent antibacterial activity against *E. faecalis* at a minimum inhibitory concentration of 20 mg/mL.

The current study found that the *A. indica* extract contained phytochemicals including flavonoids, phenolics, tannins, saponins, alkaloids, and steroids. Further, the current study revealed that phenolics and tannins were present in both the ethanolic and aqueous extracts. Furthermore, our study found that alkaloids, flavonoids, and saponins were only present in the aqueous extract and not in the ethanolic extract. Similarly, a study that was conducted in India reported that saponins were absent in the ethanolic extract of *A. indica* while phenolics and tannins were present in the ethanolic extract [42]. Contrary to the findings of the current study where terpenoids were absent in both the aqueous and ethanolic extract, a study in Ethiopia by Dereje and colleagues reported the presence of terpenoids in both the aqueous and ethanolic extracts of *A. indica* [43]. In our study, steroids were only present in the *A. indica* ethanolic extract. Our findings are similar to the results that were reported in other studies which also found the presence of steroids and tannins in the *A. indica* ethanolic extract [44, 45]. The variation in phytochemical content observed can be explained by variations in the polarities of the solvents used. Additionally, considering the influence of geographical and environmental conditions on the plant, research conducted in different regions worldwide might yield different findings regarding the presence or absence of the phytochemicals identified in this study. The antibacterial activities of *A. indica* are reported to be due to the presence of phytochemicals which possess antibacterial effects against various bacteria [18, 46-48].

The present study found that *A. indica* exhibited antibacterial activity against *E. faecalis*. These findings are in line with those reported in the Kingdom of Saudi Arabia by Mustafa *et al.* [49]. Additionally, the study conducted by Mohammed *et al.* also identified the antibacterial activity of neem against *E. faecalis* [50]. Furthermore, the activity of Neem leaf methanolic extract on uropathogens was documented in Saudi Arabia [18], Pakistan [23], and India [51]. The study done by Dhanya et al, in India, showed that ethanolic extract of *A. indica* at 50% concentration had no activity on the *E. faecalis* which is different from our study where at 50 mg/mL had an activity with the zone of inhibition of 9.4±1.13mm [52]. This variation could be due to differences in the environmental conditions, climate change, storage, and processing of *A. indica* which can affect the phytochemical constituents of plants [53].

The findings in this research show that the ethanolic and aqueous extracts of *A. indica* leaf exhibit concentration-dependent antibacterial activity against tested organisms. Some studies have been done to investigate the antimicrobial activity of neem leaf extract and their results are almost similar to our results. One of the studies by Tamizh Paavai *et al.* conducted in India supports the idea that the inhibitory action of neem against the tested organism increases with higher concentrations of neem extract [54]. This aligns with the results observed in our study, where higher concentrations of neem extracts (50 mg/mL) exhibited larger zones of inhibition compared to lower concentrations. The findings from both studies collectively underscore the concentration-dependent antimicrobial effect of neem [54].

The MIC for both the aqueous and ethanolic extracts of *A. indica* was determined to be 20 mg/mL in our study. This finding contrasts with the observations made by Muhammad *et al.* in their research, where the MIC for *A. indica* ethanolic extract against *E. coli* and *K. pneumoniae* was reported to be 50 mg/mL and 75 mg/mL, respectively [55]. This variation is due to the different organisms that were used in these studies. Further, our study found that A. *indica* extracts did not have antibacterial activity against E. faecalis at concentrations lower than 20 mg/mL. Similarly, a study conducted by Angel *et al.* and colleagues reported that the methanolic leaf extract did not show any antibacterial activity against *E. faecalis* at lower concentrations [56]. These variations underscore the significant influence of the solvent's polarity on the antimicrobial activity of *A. indica*.

We are aware of the limitation of our study. The achieved yield of the extracts was less than 30% of the 50 g extracts. This could have influenced the presence and amounts of active ingrendiets obtained. Consequently the quantity of the active constituents was not measured. Nevertheless, our study *indica*te the importance of traditional medicines as a source of antimicrobial agents. Additionally, further research should be conducted to support the use of Neem as a medicinal product.

Conclusion

This study highlights the promising antimicrobial properties of both the aqueous and ethanoic extracts of *A. indica* (neem) as they exhibited significant inhibitory activity against *E. faecalis*, with a concentration-dependent effect observed. These findings support the traditional use of Neem in folk medicine as an antibacterial agent and highlight its potential for the development of novel therapeutic agents. The presence of various phytochemicals, including flavonoids, phenolics, tannins, saponins alkaloids, and steroids, underscores the complex composition of *A. indica* extracts and their potential as sources of novel antimicrobial agents.

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