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Phytochemical Analyses and In Vitro Antibacterial Effect of *Azadirachta indica* Leaf Ethanol and Aqueous Crude Extracts on *Klebsiella pneumoniae* Strains from Farmed *Clarias gariepinus*

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Abstract

Azadirachta indica is endowed with several phytocompounds that are responsible for its role in health management. This study evaluated phytochemical analyses and the antibacterial effects of Azadirachta indica leaf ethanol and aqueous extracts on Klebsiella pneumoniae strains from farmed Clarias gariepinus. The crude extracts were produced based on standard methods. In-vitro antibacterial effects and minimum inhibitory concentration (MIC) of ethanol and aqueous crude extracts of Azadirachta indica leaf against Klebsiella penumoniae were examined using the agar disc diffusion method and broth dilution assay. The phytochemicals present in A. indica aqueous and ethanol extracts were: flavonoids, phenols, tannins, and others. The minimum inhibitory concentrations (MIC) for the ethanol and aqueous extracts against the K. pneumoniae strains were found to be 5 mg/ml and 10 mg/ml, respectively. The crude ethanol and aqueous extracts demonstrated in-vitro antibacterial effects against Klebsiella pneumoniae strains from farmed Clarias gariepinus.

Keywords: Aqueous, *Azadirachta indica*, Crude, Ethanol, *Klebsiella pneumoniae*, Leaf.

Introduction

A therapeutic plant employed in African traditional medicine, described as having antimicrobial, antioxidant, anti-inflammatory, and antibacterial activities is *Azadirachta indica (A. indica), commonly known as Nepu*, belonging to the family Meliaceae, usually present in Asia and Africa (Patil *et al*., 2022). Virtually all parts of the Neem tree were reported to have an extensive variety of pharmacological characteristics (Moin *et al*., 2022). *Azadirachta indica* (AI) has multifarious components, including limonoids, nimbin, nimbolide, nimbidin, and other forms of constituents that are important in disease control (Asghar *et al*., 2022). The principal polyphenol flavonoids extracted from *Azadirachta indica* fresh leaf were ß-sitosterol and quercetin, which were reported to have antibacterial properties (Asghar *et al*., 2022).

Azadirachta indica is endowed with several types of compounds that are responsible for its role in the management of health. Azadirachtin and triterpenes have the highest therapeutic use (Islas *et al*., 2020). Triterpenes consist of tannins, which are epoxy-azadiradione, azadirachtins, and nimbins. The major compounds in the extracts are limonoids, glycoproteins, catechins, nimbins, flavonoids, terpenes, phenols, tannins, and saponins (Islas *et al*., 2020). A variety of ingredients in different portions of the Neem tree include epoxy-azadiradione, nimbin, quercetin, azadirachtin, liminoids, and nimbosterol (Islas *et al*., 2020). The compounds found in the leaf are 7-desacetyl-7-benzoylgedunin, n-hexacosanol, amino acid, nimbanene, nimbolide, nimbandiol, ascorbic acid, Nimbin, 6-desacetylnimbinene, nimbiol, and 17-hydroxyazadiradione (Hossain *et al*., 2011). Quercetin, flavonoids, and polyphenols isolated from AI fresh leaves possessed anti-bacterial activities. The seeds contain important elements such as gedunin and azadirachtin (Hossain *et al*., 2011). In addition, the leaf aqueous extract recently analyzed showed higher quantities of glycosides, tannins, and saponins (Hossain *et al*., 2011). Meanwhile, leaf methanolic extracts contain an abundance of flavonoids, tannins, and alkaloids (Dash *et al*., 2017). Remilekun *et al.* (2022) stated that infective diseases caused by anaerobic bacteria are accountable for significant financial losses in aquaculture all over the world. One of these anaerobic bacteria is *Klebsiella pneumoniae*, reported by Bera *et al.* (2022) to be responsible for the mass mortalities of the Cyprinid and Silurid families of fish. Infection of fish with *Klebsiella pneumoniae* was previously reported in *C. gariepinus* fries (Oladele *et al.*, 2010), *C. gariepinus* adults Adeshina *et al.* (2020), *Clarias batrachus, Oreochromis niloticus*, *Anabas testudineus, Cirrhinus mrigala*, *Labeo catla*, and *Labeo rohita* others include, Indian major carp (Das *et al*., 2018; Das, 2021), *Nemipterus japonicus* (Diana, 2012), Tilapia Zilli (Ogbonna, 2008), *Nishikigoi Cyprinus* Carpio Carp, and Maldive's clownfish which is called Amphiprion nigripes (Gopi *et al*., 2016). Transmission of *K. pneumoniae* occur through contaminated handling of the fish, polluted water sources such as lakes, streams, and rivers, and unsuitable preservation of the culture system. However, this bacterium is not involved in fish natural microflora, yet it can cause severe infections in fish (Oliveira *et al.*, 2014). *Klebsiella pneumoniae* isolates obtained from these fishes showed antibiotic resistance genes, predisposing the whole food chain to resistant gene transfer (Anifowose *et al.* (2025). The main challenge with antibacterial drug usage in aquaculture is its capacity to influence the increase in antibacterial resistance genes, which may be finally transmitted to new bacteria of clinical and economic importance, particularly through the food chain (Miranda *et al*., 2013). The change in global climate might degenerate the challenges of antimicrobial resistance (AR), as it could alter the dynamics and occurrence of new and prevailing bacteria and subsequently the need for the prevalence of AR and antibacterial agents (Miranda *et al*., 2013). Treatment choices for aquatic species are currently limited, and this has put more pressure on the available therapeutic options, which predisposes AR to the classes of antibiotics in use (Caruso, 2016). Cabello *et al.* (2013) reported that various researchers have concluded that AR bacteria are a worldwide issue due to widespread or wrong usage of antibiotics in the bid to avoid probable infections and solve important production challenges. Hence, there is a need for extensive phytochemical analyses and in-vitro antibacterial effects of *Azadirachta indica* leaf ethanol and aqueous extracts on *Klebsiella pneumoniae*, with the view to developing new drug candidates for the treatment.

Materials and Methods

Plant Sample Preparation

The leaf of *A. indica* A. Juss was sampled from the Department of Veterinary Medicine car park, University of Ibadan, and confirmed at the Herbarium in the Department of Botany Plant Laboratory (UIH-23205). The collected leaves were air-dried for 21 days and pulverized into a fine powder with a blender. The leaves were macerated in distilled water or 100% ethanol (25 g of *A. indica* dried leaves per liter) with intermittent shaking for three days at room temperature, respectively (Figure 1). Afterward, the mixture was filtered by Whatman® filter paper to acquire

clear filtrates. The filtrate was condensed with a rotary evaporator, and the remaining moisture was removed over a water bath at 30 °C. Aqueous and ethanol extracts (2.40 g and 5.50 g) were obtained, respectively. The extracts were stored at 4 °C (Hikaambo *et al*., 2022). The extraction was performed in triplicates.

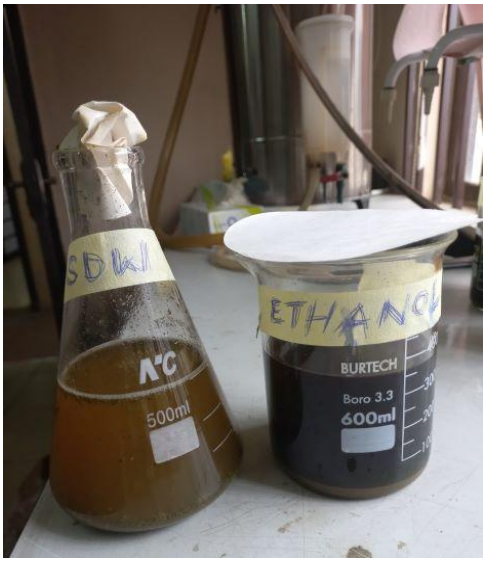

Fig. 1: Aqueous and ethanol extract of *Azadirachta indica* leaf before evaporation

Phytochemical Analyses of *Azadirachta indica* Leaf Aqueous and Ethanol Extracts

Qualitative Phytochemical Screening of *Azadirachta indica* Leaf Extracts

The procedure used for conducting the phytochemical analyses of *Azadirachta indica* leaf crude extracts (ethanol and aqueous) was based on previous studies described by Ogidi *et al*. (2021) and Hikaambo *et al*. (2022).

Saponin Test

A stock solution of ethanol and aqueous extracts of *A. indica* (100 mg/ml) was prepared in distilled water and vortexed to form a stable, consistent froth. Thereafter, three drops of olive oil were added to the frothing and vortexed, and emulsion formation was detected (Hikaambo *et al*., 2022).

Terpenoids Test

Acetic anhydride of 1ml and concentrated H_2SO_4 of 2ml were mixed with 0.5 g of *A. indica* extracts (ethanol and aqueous). Formation of reddish violet colour was observed (Hikaambo *et al*., 2022).

Tannins Test

Azadirachta indica extracts of 0.5g (ethanol and aqueous) of 0.5g were mixed with water 10ml in a test tube and boiled. The presence of blue-black or brownish-green colouration after the addition of 0.1 % ferric chloride few drops indicated a positive result (Hikaambo *et al*., 2022).

Cardiac Glycosides (Keller-Killian) Test

Glacial acetic acid solution of 2 ml, which contained ferric chloride solution, was mixed with 5 ml of water and 0.5 g of *A. indica* extracts (ethanol and aqueous). Meanwhile, 1 ml of concentrated H_2SO_4 was used as an underlay. The existence of the deoxy sugar appearance of cardenolides was confirmed by a brown ring at the interface. There was a formation of a greenish ring above the brown ring that spread out on the whole layer, resulting in the appearance of the violet ring formed under the brown ring in the acetic acid layer (Hikaambo *et al*., 2022).

Flavonoids Test

Azadirachta indica extracts (ethanol and aqueous) of 0.5g was added to 5ml of diluted ammonia. The addition of 1ml concentrated Sulphuric acid was followed and the appearance of flavonoids was indicated by the formation of a yellow colour (Hikaambo *et al*., 2022).

Alkaloid Test

Diluted HCl of 1ml was added to 0.5g of *A. indica* extracts (ethanol and aqueous) followed by filtration. Mayer`s reagent which was potassium mercuric iodide was mixed with filtrate. The existence of alkaloids was confirmed by a yellow-colored precipitate (Ogidi *et al*., 2021).

Steroids Test

A. indica extracts (ethanol and aqueous) of 0.5g was mixed with 2ml of Acetic anhydride and filtered. 2ml of Sulphuric acid was mixed with filtrate. The existence of steroids was established by colour change from violet to blue or green (Ogidi *et al*., 2021).

Reducing Sugar Test

Dihydrogen monoxide of 1ml was added to 0.5g *A. indica* extracts (ethanol and aqueous). 5-8 drops of Fehling's solution were added and the solution was boiled. The presence of brick red precipitate indicated a positive result (Hikaambo *et al*., 2022).

Phenolic Test by Ferric Chloride

The distilled water of 1ml was added to 0.5 g of *A. indica* extracts (ethanol and aqueous) followed by filtration. 10% ferric chloride solution few drops were mixed with 2ml of filtrate. The existence of a phenolic hydroxyl group was established with a violet or green-blue colour (Hikaambo *et al*., 2022).

Quantitative Phytochemical Screening of *Azadirachta indica* Leaf Extracts

Quantitative analysis was carried out on the phytochemicals that were positive in the qualitative tests.

Estimation of Alkaloids

The spectrophotometric analytical method was used for the assessment of alkaloids (Sathishkumar and Baskar, 2014). Distilled water of 8.5 mL was added to 1.5 mL of *A. indica* leaf crude extracts (ethanol and aqueous) to form a volume of 10 mL in a 25 mL standard flask. 0.01 M sodium-meta periodate (SPI) 1 mL and 0.1 M acetic acid 0.5 mL were added to the previous solution and heated by boiling water bath for 10 minutes. 0.01M 3-methyl-2-benzotriazolinone hydrazine (2 ml) hydrochloride (MBTH) was added into flasks and boiled in the water bath for 2 minutes. Afterwards, there was cooling of the flask. Then, a spectrophotometric measurement of the blue color detected at 630 nm was done (Hikaambo *et al*., 2022).

Estimation of Total Phenolic Content (Folin-ciocalteau Method)

The reagent of Folin (0.5 ml) and distilled water (3.9 ml) was mixed with 0.5 g of *A. indica* leaf crude extracts (ethanol and aqueous). The incubation of the tube for 3 minutes was carried out at room temperature. Thereafter, 2 mL of 20% sodium carbonate was mixed and kept for 1 minute in the water bath. Finally, spectrophotometric measurement of the blue color detected at 650 nm was done (Ogidi *et al*., 2021).

Estimation of Total Flavonoid Content (Aluminum chloride Method)

Distilled water of 4.9 mL was added to 0.1 mL of *A. indica* leaf crude extracts (ethanol and aqueous). 0.3 ml of 5% NaNO3 was added, and 5 minutes later, 3 ml of 10% $AICl_3$ was added. Thereafter, the addition of 1 M NaOH in 2 ml of water for 6 minutes was done. The absorbance measurement was 510 nm (Ogidi *et al*., 2021).

Estimation of Tannins (Modified Prussian Blue Method)

Potassium ferric cyanide (0.008 M) in 1 ml, 6.9 ml of distilled water, and 0.2 M ferric chloride in 1 ml of 0.1 M HCI were added to 0.1 ml of the *A. indica* leaf crude extracts (ethanol and aqueous) and thoroughly mixed. The spectrophotometric measurement of the blue color detected at 700 nm was done (Ogidi *et al*., 2021).

Estimation of Steroids (Liberman-Burchard Method)

Azadirachta Indica leaf crude extracts (ethanol and aqueous) of 0.1 were added to 4.9 ml of chloroform to form a volume of 5 ml in a test tube. Liberman-Burchard reagent (2 ml), concentrated sulfuric acid (0.5 ml), and acetic anhydride (10 ml) were added and thoroughly mixed. Black paper was used to cover them for 15 minutes in the darkness. The spectrophotometric measurement of the green color detected at 640 nm was done (Ogidi *et al*., 2021).

Bacterial Isolation and Characterization

Klebsiella pneumoniae isolates were earlier characterized using species-specific primers and submitted to GenBank with the ascension numbers OQ341515.1, OQ345815.1, and OQ345817.1. (Anifowose *et al.,* 2025).

Antimicrobial Susceptibility Testing

The in-vitro antibacterial effect of *Azadirachta indica* leaf crude extracts on pure isolates of *Klebsiella pneumoniae* was carried out to determine the zone of inhibition. The procedures were carried out through the agar-disc diffusion method based on Mehta *et al.* (2022) and repeated twice. *Azadirachta indica* ethanol and aqueous crude extracts were used for the susceptibility test. A total of twelve *Klebsiella pneumoniae* isolates were analyzed for susceptibility testing.

Preparation of an Antibiotic Disk

The disc was constructed by the CLSI Mo2 disk diffusion guide. Three 6-mm sterilized Whatman filter paper disks were placed on a 100-mm plate. The discs were impregnated with the different concentrations of each ethanol and aqueous extract: 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 5 mg/ml, 7.5 mg/ml, 10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, 50 mg/ml, 60 mg/ml, and 100 mg/ml. Ciprofloxacin (CIP) and normal saline were used as a positive and negative control. Each isolate was inoculated on a sterile Mueller-Hinton agar plate and appropriately labeled with an indelible marker *(*Mehta *et al.*, 2022).

Preparation of Inoculums

By adjusting the suspension of the *Klebsiella pneumoniae* colony in sterile normal saline to the density of a McFarland 0.5 turbidity standard, the direct colony suspension method was employed. *Klebsiella pneumoniae* was grown overnight on Tryptic Soy Agar (HiMEDIA®), and pure cultures were harvested using a sterile loop. The colonies were suspended in sterile normal saline that had been blended to a consistent turbidity. Saline or additional bacteria were added to get the density of the *Klebsiella pneumoniae* suspension to McFarland 0.5. After that, Muller-Hinton agar (HiMEDIA®) was streaked with the suspended *Klebsiella pneumoniae* colonies. Quality control was performed using the *Pseudomonas aeruginosa* strain (Anifowose *et al*., 2025).

Inoculation of Agar Plates

The extract-impregnated disc was picked using sterile pointed forceps and applied to the surface of Mueller-Hinton agar plates. This allowed the discs to effectively touch the medium. Afterward, the plates were incubated for 18 hours at 37 °C (Anifowose *et al*., 2024).

Measurement of the Zone of Inhibition - Inhibition zone diameters were measured in millimeters with a Vernier caliper.

Minimum Inhibitory Concentration - Determination of minimum inhibitory concentration values of *A. indica* crude extracts (ethanol and aqueous) was carried out by broth dilution assay using tube dilution technique according to Muhammad *et al.*, 2019. Ciprofloxacin and blank normal saline were used as a positive and negative control

respectively.

Statistical Analysis

The zone of inhibition of *Azadirachta indica* aqueous and ethanol extracts tested against *Klebsiella pneumoniae* isolates was measured in millimeters. One way ANOVA and Duncan multiple range test (DMRT) were employed to calculate the level of significance observed in antibacterial activity of *A. indica* aqueous and ethanol leaf extracts; the level of statistical significance was determined at $p < 0.05$.

Result and Discussion

Qualitative and Quantitative Phytochemical Analyses of *Azadirachta indica* Aqueous and Ethanol Extracts

The qualitative phytochemical evaluation of *A. indica* aqueous and ethanol leaf extracts showed presence of flavonoids, phenols, tannins, saponin, alkaloids, terpenoids, steroids, glycosides, and reducing sugars. Meanwhile, quantitatively, flavonoids and terpenoids were significantly $p < 0.05$ higher in ethanol compared to aqueous extract. Conversely, phenols and saponin were significantly $p< 0.05$ higher in aqueous compared to ethanol extract (Table 1).

Table 1: Qualitative and Quantitative Phytochemical Analyses of *Azadirachta indica* leaf Aqueous and Ethanol Crude Extracts

Phytochemical	Qualitative Analysis		Quantitative Analysis	
	Aqueous Extract	Ethanol Extract	Aqueous Extract (µg/ml)	Ethanol Extract (µg/ml)
Flavonoids (Aluminium chloride test)	+	++	3.94 ± 0.42^{e}	8.4 ± 0.02^{f}
Phenols (Folin-Ciocalteau Test)	+	+	10.96 ± 0.31^{a}	7.12 ± 0.01^{b}
Tannins (Prussian blue method)	++	++	2.50 ± 0.23^{c}	3.70 ± 0.11^{c}
Saponin (Froth's Test)	+++	+	12.3 ± 0.34^{b}	3.2 ± 0.05^{c}
Alkaloids (Hager's Test)	++	++	4.20 ± 0.42^{a}	3.90 ± 0.31^{a}
Terpenoids (Salkowaski's Test)	+	+	2.93 ± 0.02^{d}	4.50 ± 0.74^{e}
Steroids (Burchard method)	+	+	4.81 ± 0.11^{c}	4.48 ± 0.52^{c}
Glycoside	++	+++	ND	ND
Reducing sugar	+	++	ND	ND

ND; not determined, -; absent, +; low concentration, ++; moderate concentration, +++; high concentration

Antimicrobial Activity of *Azadirachta indica* (Neem) Leaf Extract Against *Klebsiella pneumoniae* Strains

Fig. 2: Zone of inhibition of Antimicrobials, A. *indica* Aqueous and Ethanol extract against *Klebsiella pneumoniae* isolate**;** E – *Azadirachta indica* ethanol extract**,** AE – *Azadirachta indica* aqueous extract

Antibacterial Screening Showing the Mean Zone of Inhibition -Aqueous and ethanol extracts at 1 – 4 mg/ml

showed no zone of inhibition against *K. pneumoniae* strains. Ethanol extract at 7.5 – 100 mg/ml showed significant higher zone of inhibition against *K. pneumoniae* strains compared to aqueous extract (Figure 2). Meanwhile, all concentrations of ciprofloxacin showed significant higher zone of inhibition against *K. pneumoniae* strains compared to both aqueous and ethanol extracts (Table 2).

Table 2: Inhibition zone (M±S.E.M) of *Azadirachta indica* aqueous and ethanol extracts against *Klebsiella pneumoniae* (1.5×10^7cfu/ml) strains

Conc. of extract	*A. Indica (aqueous extract)*	*A. indica (ethanol extract)*	Ciprofloxacin	Normal Saline
1mg/ml	$(0.0\pm0.0)^{a}$	$(0.0\pm0.0)^{a}$	$(5.0\pm0.20)^{b}$	$(0.0\pm0.0)^{a}$
2mg/ml	$(0.0\pm0.0)^{a}$	$(0.0\pm0.0)^{a}$	$(7.0\pm0.12)^{b}$	$(0.0\pm0.0)^{a}$
3mg/ml	$(0.0\pm0.0)^{a}$	$(0.0\pm0.0)^{a}$	$(10.0\pm0.30)^{b}$	$(0.0\pm0.0)^{a}$
4mg/ml	$(0.0\pm0.0)^{a}$	$(0.0\pm0.0)^{a}$	$(14.0\pm0.18)^{b}$	$(0.0\pm0.0)^{a}$
5mg/ml	$(0.0\pm0.0)^{a}$	$(8.50\pm0.48)^{b}$	$(18.0\pm0.22)^{c}$	$(0.0\pm0.0)^{a}$
7.5mg/ml	$(0.0\pm0.00)^{a}$	$(10.30\pm0.67)^{b}$	$(30.0\pm0.51)^{c}$	$(0.0\pm0.00)^{a}$
10mg/ml	$(8.90\pm0.67)^{b}$	$(12.67\pm0.48)^{b}$	$(33.0\pm0.48)^{c}$	$(0.0\pm0.0)^{a}$
20mg/ml	$(10.70\pm0.48)^{b}$	$(14.56\pm0.52)^{b}$	$(34.0\pm0.23)^{c}$	$(0.0\pm0.0)^{a}$
30mg/ml	$(12.30\pm0.60)^{b}$	$(15.80\pm0.33)^{b}$	$(34.0\pm0.10)^{c}$	$(0.0\pm0.0)^{a}$
40mg/ml	$(14.78\pm0.62)^{b}$	$(17.67\pm0.67)^{b}$	$(34.0\pm0.138u)^{c}$	$(0.0\pm0.0)^{a}$
50mg/ml	$(16.00\pm0.67)^{b}$	$(18.70\pm0.72)^{b}$	$(34.0\pm0.29)^{c}$	$(0.0\pm0.0)^{a}$
60mg/ml	$(20.58\pm0.48)^{b}$	$(24.50\pm0.51)^{b}$	$(34.0\pm0.41)^{c}$	$(0.0\pm0.00)^{a}$
100mg/ml	$(22.31\pm0.72)^{b}$	$(27.28\pm0.44)^{b}$	$(35.0\pm0.23)^{c}$	$(0.0\pm0.0)^{a}$

Values with different superscript along horizontal row indicate significance ($\alpha_{0.05}$); Mean values (± S.E.M.) bearing different superscript letters within the same row (i.e., compared across the four treatments at a given concentration) differ significantly by one-way ANOVA and Duncan's Multiple Range Test ($\alpha_{0.05}$); values sharing at least one common letter are not significantly different. Superscripts were not compared down columns (i.e., not across increasing concentrations of the same treatment).

Minimum Inhibitory Concentrations (MIC) of Aqueous and Ethanol Extracts against *K. pneumoniae* strains

The minimum inhibitory concentration of *A. indica* leaf ethanol extract against *K. pneumoniae* strains was 5 mg/ml (Figure 3), while MIC of aqueous extract was 10 mg/ml (Figure 4 and Table 3).

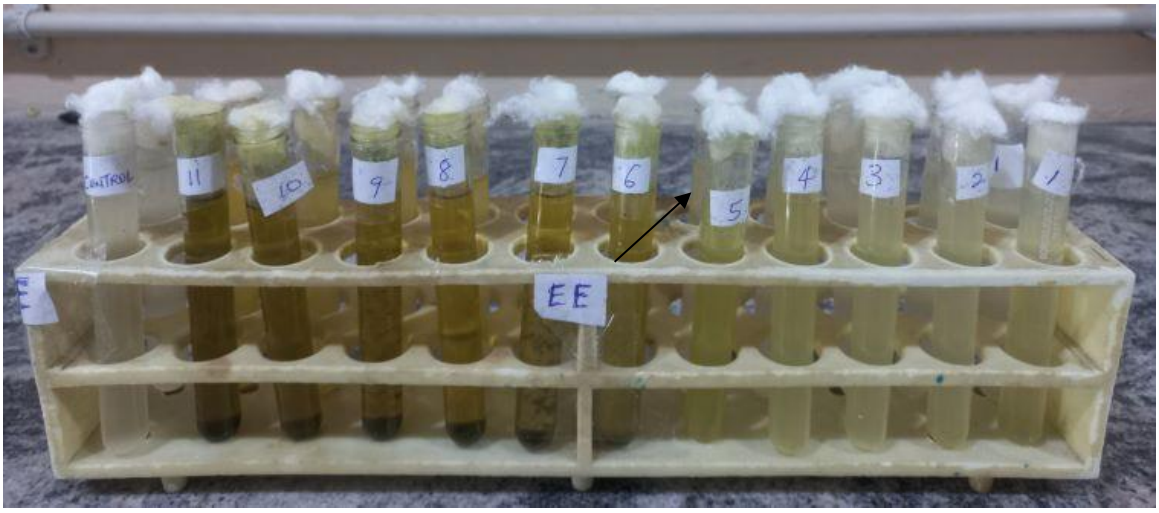

Fig. 3: Minimum inhibitory concentration (5.0mg/L) of *A. indica* leaf ethanol extract (black arrow) against *Klebsiella pneumoniae* strains.

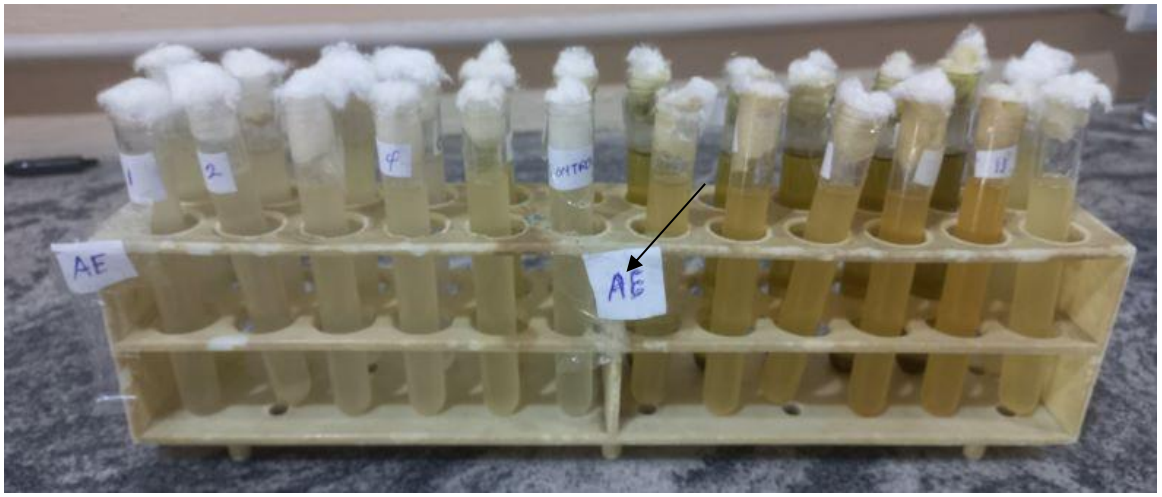

Fig. 4: Minimum inhibitory concentration (10.0mg/L) of *A. indica* leaf aqueous extract (black arrow) against *Klebsiella pneumoniae* strains.

Table 3: Minimum Inhibitory Concentrations (MIC) of Aqueous and Ethanol Extracts against *K. pneumoniae* (1.5 x 10^7cfu/ml) strains

Minimum inhibitory concentration in mg/ml	Ethanol Extract	Aqueous Extract	Ciprofloxacin	Normal saline
0.625mg/ml	+	+	-	+
1.25mg/ml	+	+	-	+
2.50mg/ml	+	+	-	+
5.00mg/ml	MIC	+	-	+
7.50mg/ml	-	+	-	+
10.00mg/ml	-	MIC	-	+
12.50mg/ml	-	-	-	+
15.00mg/ml	-	-	-	+
17.50mg/ml	-	-	-	+
20.00mg/ml	-	-	-	+
22.50mg/ml	-	-	-	+

+ = positive, - = negative

Plants have been used for centuries in alternative medicine, natural medicine, pharmacology, and food preservation. To raise the standard of healthcare, it is imperative to do phytochemical analysis on the plants that are traditionally utilized in medicine. Certain chemical compounds that are present and have a clear biological function on the human and animal body are what give plants their therapeutic significance. Plant extracts are potential sources of new antimicrobial chemicals, particularly those that are effective against bacterial infections. Although they are not a single molecule but a combination of metabolites, the beneficial therapeutic effects of plants mostly come from secondary products found in the plant (Ogidi *et al*., 2021).

In this study, phytochemicals like *flavonoids*, reducing sugars, phenols, alkaloids, saponins, tannins, terpenoids, steroids, and glycosides were present in varying quantities of ethanol and aqueous extracts of *A. indica* leaf. Quantitatively, flavonoids and terpenoids were significantly ($\alpha_{0.05}$) greater in ethanol extract than in aqueous extract, while saponins and alkaloids were significantly ($\alpha 0.05$) greater in water extract than in ethanol extract.

Moreover, in this study, the phytochemical analysis results of *A. indica* leaf ethanol and aqueous extracts were similar to the reports of Nagano and Batalini (2021). Different solvent polarities may cause the observed variation in phytochemical presence (Hikaambo *et al*., 2022). Mallikharjunah *et al*. (2007) stated that identifying bioactive compounds may pave the way for the advancement and discovery of novel drugs. One of the bioactive principles in *A. indica* is flavonoids, a vital group of polyphenols that are extensively dispersed in abundance throughout the plant flora. Their existence may provide the rationale behind the ethno-medical usage of *A. indica* leaf ethanol and

aqueous extract for the treatment of wounds (Osunwoke *et al*., 2013). The phytochemicals present in *A. indica* leaf extracts have been reported to have different pharmacological effects in the mammalian system.

Flavonoids have been reported to work as antioxidants and antimicrobials as well as nutritional supplements (Swanson, 2003). The phenolic and flavonoid compositions of *A. indica* leaf extracts have high antioxidant potential. The rich flavonoids, which are 60% of the phytochemicals in *A. indica* leaf, are responsible for the strong antioxidant, antibacterial, and, in particular, anti-inflammatory effects exhibited by the plants (Ugoeze *et al*., 2021). Flavonoids function as antioxidants, defending against the damage that free radicals do to cells and tissues. The flavonoid quercetin contains a glycoside called rutin. Rutin encourages effective communication between the skin layers by lowering the damage caused by free radicals to the basement membrane. Additionally, rutin is well-known for its vasoactive qualities and anti-inflammatory properties (Casa *et al*., 2000). Rutin binds to the iron ion (Fe^{2+}) in humans and prevents it from joining with hydrogen peroxide, which would otherwise result in the formation of a highly reactive free radical that may cause harm to cells.

Glycosides are complex, active molecules made up of carbon, hydrogen, and oxygen. They influence the contractile forces of the heart muscle in a distinctive manner (Pandey *et al*., 2014). Tannins aid in the wound-healing process and work well for colitis, peptic ulcers, and diarrhea. Therefore, the alkaloids, glycosides, flavonoids, and saponins found in *A. indica* leaf extracts are antimicrobial building blocks. The defense mechanism of *A. indica* leaf extract against many diseases is essentially based on these antimicrobial principles (Pandey *et al*., 2014).

Lupeol is a pharmacologically active terpenoid that is present in many different parts. *A. indica* offers a number of therapeutic benefits, including anti-inflammatory ones. The pharmacology of humans indicated that lupeol exhibited antiprotozoal, antibacterial, anti-inflammatory, and chemo-preventive effects (Pandey *et al*., 2014). Ferulic acid is a natural phenol in *A. indica* leaf, which is an antioxidant that reduces the production of thymine dimers in the skin and oxidative stress when combined with ascorbic acid and vitamin E for topical treatment (Swanson, 2003). Ellagic acid is an /effective phenol, free radical scavenger, and antioxidant that reduces lipid peroxidation. Additionally, it shields the skin from chromosome damage brought on by radiation. Polyphenols, which are known to have antioxidant and free radical-scavenging capabilities, are also abundant in the leaf (Abireh *et al*., 2020). Alkaloids, flavonoids, and glycosides are described as possessing various special biological functions, which include anti-allergic, antioxidant, antimicrobial activity, anti-viral, anti-inflammatory, anti-diabetic, anti-leprosy, and anti-cancer activities (Dash *et al*., 2017).

A long history of usage and the renewable and antibacterial properties of medicinal plants make them more acceptable and affordable than antibiotics. They are typically free of side effects, better tolerated by patients, and relatively less expensive (Sherein *et al*., 2014). Antibiotic susceptibility tests of *K. pneumoniae* isolates were done, and the zone of inhibition diameter was determined. The findings demonstrated that *A. indica* leaf extracts in both aqueous and ethanol form had in-vitro antibacterial effects on all *K. pneumoniae* isolates. However, the *A. indica* ethanol extract had a significant ($\alpha_{0.05}$) greater zone of inhibition (12.67±0.48) than the aqueous extract (8.90±0.67) and was therefore expected to be more effective. The antimicrobial effectiveness of the *A. indica* leaf aqueous and ethanol extracts was consistent with the results of Itelima *et al*. (Itelima *et al*., 2016), which indicated that the A. *indica* leaf ethanol extract had a significantly ($\alpha_{0.05}$) higher zone of inhibition (4 mm) against *Klebsiella* sp. compared with the A. *indica* leaf aqueous extract (0.04 mm) at a concentration of 50 mg/ml. However, Olusola *et al*. (2017) reported that the *A. indica* leaf aqueous extract had a significantly ($\alpha_{0.05}$) higher zone of inhibition against *Aeromonas hydrophila* at a concentration of 66.7 mg/ml than the ethanol neem leaf extract. Additionally, Muhammad *et al*. (2019) observed that *A. indica* ethanol leaf extract showed a zone of inhibition (12.0±0.02 mm) against *K. pneumoniae* isolates at a 50 mg/mL concentration. The findings of this particular study were similar to those of Olusola *et al*. (Olusola *et al*., 2017), Muhammad *et al*. (2019), and Abalaka *et al*. (2015) in that neem leaf extracts in both aqueous and ethanol demonstrated antibacterial activity and reduced the development of all *K. pneumoniae* strains. *A. indica* leaf extracts in both aqueous and ethanol solutions showed extremely high potential as antibacterial agents. The MIC of 5 mg/ml and 10 mg/ml of *A. indica* leaf ethanol and aqueous extract against *K. pneumoniae* observed in this study was lower to 50 mg/ml reported by Muhammad *et al*. (2019) against *Escherichia coli*. The study's findings demonstrated that neem leaf extracts had intriguing inhibitory effects on all strains of *K. pneumoniae*. It was established that the antimicrobial characteristics of these extracts, whether in vivo or in vitro, are influenced by the incidence of many antimicrobial active ingredients present in the leaves, including sitosterol, quercetin, and desactylimbin (Olusola *et al*., 2017). It is helpful to justify the usage of this plant in regional government veterinary clinics since *Azadirachta indica* leaves have good antibacterial activity, demonstrating the

high potential of bioactive chemicals (Maragathavalli *et al*., 2012). The secondary metabolites present in the plant might be accountable for the extract's reported antibacterial activity against the test organisms (Isaka *et al*., 2017). Alkaloids, glycosides, flavonoids, and saponins are phytoconstituents that are also plant-based antibiotics. Actually, plants use these antibiotic principles as a defense mechanism against many diseases (Muhammad *et al.* 2019). The MIC results further displayed that the ethanol extract exhibited greater antibacterial effectiveness against *Klebsiella pneumoniae* when compared with the aqueous extract, depicting that ethanol was more efficient in extracting bioactive antibacterial compounds from the plant material (Anyanwueze, 2026).

Phytochemical screening revealed the presence of alkaloids, tannins, saponins, flavonoids, steroids, terpenoids, glycosides, and reducing sugars in *A. indica* extracts. These phytochemical constituents are known to contribute to the natural defense mechanisms of plants and may be responsible for the observed antimicrobial activity against the test organism (Anyanwueze, 2026).

Conclusion

The phytocompounds present in *Azadirachta indica* leaf ethanol and aqueous extracts indicated the presence of flavonoids, tannins, phenols, saponins, terpenoids, alkaloids, steroids, reducing sugars, and glycosides in varying concentrations. The crude ethanol extract showed lower MIC and a higher in-vitro anti-bacterial effect against *Klebsiella pneumoniae* strains from farmed *Clarias gariepinus.*

Contribution by Authors

All the authors contributed equally to writing the manuscript. The final manuscript was read by all authors and consented to publication.

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Conflict of Interests

There is no conflict of interest.

Data Availability Statement

The datasets used and analysed during the current study are available from the corresponding author on reasonable request

Ethics Approval and Consent to Participate

Ethical approval was received from the University of Ibadan, Animal Care and Use Research Ethics Committee (UI-ACUREC) with the assigned number UI-ACUREC/056-0621/10

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