COMPARATIVE QUANTITATIVE PHYTOCHEMICALS SCREENING OF AQUEOUS, METHANOL AND HEXANE LEAVES EXTRACTS OF *AZADIRACHTA INDICA* "LIFE GIVING TREE"

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Received: 18th Mar., 2024 Accepted: 18th Apr., 2024 **Published:**

ABSTRACT

Background

The *Azadirachta indica* tree is an incredible plant that has been declared the "Tree of the 21st century" by the United Nations, as the tree for solving global problems, with incredible antibacterial, antifungal and antimalarial activities used traditionally.

Aim: The aim of the study was to evaluate the qualitative and quantitative phytochemicals of aqueous methanol and hexane leaves extract of Azadirachrata indica.

Methodology

The study was carried out to determine the phytochemical content responsible for these activity. Fresh leaves were collected, aqueous, methanolic and hexane extracts of the collected leaves were prepared, and the extracts were screened for phytochemical constituent using standard methods.

Result

Results of the phytochemical screening of all the crude extracts revealed the presence of saponins, tannins, steroids, phenol, alkaloid and flavonoid. In aqueous extract, flavonoid show higher content of 14.53%, followed by alkaloids with 11.03%, methanol with higher content of alkaloid with 11.65%, and hexane with the highest in tepernoid with only 2.37%.

Conclusion

Based on the present study, it can be concluded that the extracts of *Azadirachta indica* are rich source of phytocemicalcs and Flavonoid is found to be most abundant phytochemical presence which could be the reason for pharmacological activity that is used traditionally by many people as an alternative treatment for a variety of health diseases

Keywords: *Azadirachta indica*, Phytochemical, Aqueous, Methanol and Hexane.

INTRODUCTION

Azadirachta indica, also known as Neem, Nimtree, and Indian Lila and dogonyaro in northern parts of Nigeria, is a tree in the mahogany family Meliaceae. It is one of two species in the genus *Azadirachta*, and is native to India and the Indian subcontinent including Nepal, Pakistan, Bangladesh and Sri Lanka, It is typically grown in tropical and semi-tropical regions. Neem trees now also grow in islands located in the southern part of Iran. Its fruits and seeds are the source of neem oil (Venugopalan and Visweswaran, 2013). The latinized name of neem, *Azadirachta indica*, is derived from the Persian. Azad means "free"; dirakht means "tree"; i-Hind means "of Indian origin".

Hence it literally means "the free tree of India" (Kumar and Navaratnam, 2013). The neem tree is an incredible plant that has been declared the "Tree of the 21st century" by the United Nations, The US National Academy of Science published a report in 1992 entitled "Neem: A tree for solving global problems" (Venugopalan and Visweswaran, 2013). *A. indica* has a vital role in various problems associated with human health (Sarkar *et al.*, 2021). The chemical constituents present in the neem plant make it a doctor tree due to its wide scope in biological activities associated with it, and has become a global context today. Various parts of the neem tree have been used as traditional ayurvedic medicine in India from time immemorial (Alzohairy, 2016). The medicinal utilities have been described, especially for leaf, fruit and bark (Subapriya and Nagini 2005). Neem oil and the bark and leaf extracts have been therapeutically used as folk medicine to control leprosy, intestinal helminthiasis, respiratory disorders, and constipation and also as a general health promoter (Senapati *et al.*, 2019). Its use for the treatment of rheumatism, chronic syphilitic sores and indolent ulcer has also been evident (Senapati *et al.*, 2019). Neem oil were used to control various skin infections. Bark, leaf, root, flower and fruit together cure blood morbidity, biliary afflictions, itching, skin ulcers, burning sensations and pthysis (Kumawat and Kumar 2018). *Azadirachta indica* leaves possessed good anti-bacterial activity, confirming the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care (Joshi and Sahu 2019). Neem oil also has definite antiplaque activity (Elavarasu *et al.*, 2012). Neem leaf extract can inhibit the formation of biofilm in Pseudomonas aeruginosa (Harjai, *et al.*, 2013). *Azadirachta indica*, plant is considered to be the richest sources of drugs for traditional medicine, modern medicine, nutraceuticals, food supplements, folk medicine, pharmaceutical intermediates and chemical entities for synthetic drugs (Ujah *et al.*, 2021) and this could be attributed to phytochemicals present in the plant. The objective of this study was to screen for the phytochemical constituents of aqueous, methanol and hexane leaves extracts of *A. indica* and relate it to some of its traditional use.

MATERIALS AND METHODS

Collection of Plant Material

Fresh and mature leaves of *Azadirachta indica* was obtained at Jiddari polo Maiduguri, Borno State. The samples were collected in a clean sterile polythene bag and brought to the herbarium of the Department of Biological Sciences, Nigerian Defence Academy Kaduna, for identification and authentification with Voucher specimen number NDA/BIOH/2023/50. Three different solvent extraction method were employed for the plant material that is polar solvent, intermediate polar and non-polar to determine the extract with higher phytochemical yield, Aqueous for polar, methanol for intermediate polar and hexane for non-polar.

Sample preparation

The fresh plants material were rinsed in water and allow to air dried under shade for three weeks. Dried samples were milled to powder using grinding machine. The samples were stored in sterilized polythene bags prior to use.

Extraction

Preparation of Aqueous Crude Extracts

Fifty grams (50 g) of each of the sample of *Azadirachta indica* was extracted separately with 1500 mL of distilled water in 2000 mL beaker. The soaked samples were stirred and covered with aluminum foil and kept for twenty-four hours. The resultant extract was filtered using muslin cloth and each filtrates were evaporated separately to dry using hot plate set at 40̊ C to obtain crude extract. The crude extract was weighted and stored in refrigerator until use (Patil and Gaikwad, 2010).

Preparation of Methanol Crude Extracts

The powder sample (50 g) were extracted with methanol solvent (500 mL) by using Soxhlet extractor for 72 h. After complete extraction, the methanol solvent was evaporated by using rotary evaporator (Yamato, Rotary Evaporator, model-RE 801) under reduced pressure to obtain methanol crude extract. The methanol crude extract from each sample was suspended in water (60 mL).The crude extracts was filtered separately through Whatman filter paper to remove particles. The particle free crude extract was evaporated completely by using rotary evaporator under reduced pressure to obtain dry crude extracts. The residue left in the separator funnel was re-extracted twice following the same procedure and filtered (Patil and Gaikwad, 2010).

Preparation of hexane Crude Extracts

The extract was prepared in a Soxhlet apparatus using 100 g of the powdered leaves and 1 L of *n*-hexane. The solvent was evaporated at 75 rpm and 64.4 °C in a HB10 rotary-evaporator. The resulting material after solvent evaporation was the crude extract (Costa Cordeiro *et al.,* 2018). Percentage yield was calculated as follows

Percentage yield = $\frac{W_1}{W_2}$ x 100

Where:

W_1= net weight of powdered extract in grams after extraction.

W_2= total weight of powder weighed in grams before extraction

Qualitative Phytochemical Screening of Leaf Extracts

The leaves extracts of the plant were screened for metabolites such as alkaloids, tannins, flavonoids, saponinins, balsams, anthraquinones, cardiac glycosides, glycosides, and steroids.

Test for Alkaloids (Dragendoff's Test)

About 0.2 g of each plant samples was added in to 3 ml of hexane in a test tube. These were mixed, shacked and filtered. Then 5 ml of 2 % HCl was poured in to a test tube containing the mixture of plant extract and hexane. The mixture was Heated and then filtered. Few drops of picric acid was poured in the filtrates. Formation of yellow color precipitate indicates the presence of alkaloids (Wadood *et al.*, 2013).

Test for Tannins (Ferric Chloride Test)

Two milliliters (2 mL) of the extract was added to 2 mL of water, and then 1 to 2 drops of diluted ferric chloride solution was added. A dark green or blue green coloration indicates the presence of tannins (Wadood *et al.*, 2013).

Test for Flavonoids (H_2SO_4 Test)

About 0.5 g of each plant extract was added in to a different test tube containing 10 mL of distilled water, 5 mL of dilute ammonia solution was then added to a portion of the filtrate of each plant extract followed by addition of 1 mL concentrated H_2S0_4. Indication of yellow color shows the presence of flavonoid in each extract (Wadood *et al.,* 2013).

Test for Saponins (Frothing Test)

Few volume of distilled water was added to 1 mg of each plant extract in a test tube. The solution was then shaken vigorously and observed for a stable persistent froth for 20 min; formation of layer of foam indicates the presence of saponins. (Sabri *et al.,* 2012)

Test for terpenoid Steroids (Liebermann Burchard's Test)

Ten milliliters (10 mL) of each extract were evaporated. The residue was then dissolved in 0.5 mL of hot acetic anhydride; 0.5 mL of the filtrate chloroform was added and then treated with Liebermann Burchard's reagent. The appearance of blue-green at the interphase, confirms the presence of steroids (Wadood *et al.,* 2013).

Test for Anthraquinone: (Borntrager's Test)

Two milliters of 10% hydrochloric acid was added to the extract in the test tube and boil for 2 minutes. Equal amount of chloroform was added to test tube and vortexes twice. The chloroform layer was pipetted out and then equal volume of ammonia was added to the chloroform later. A pinkish layer indicates the presence of anthraquinones (Efe *et al.,* 2016).

Test for Glycosides (Fehling's Test)

About 2.5 of 50% sulphuric acid was added to 5ml of the extract in a test tube. The mixture was heated in boiled water for 15min, cooled and neutralized with 10% NaOH and 15mL of Fehling's reagent was added and mixture was boiled. A brick-red precipitate was observed which indicate the presences of glycocides (Ayoola *et al.,* 2008).

Test for Cardiac Glycosides (Keller-Kiliani's Test)

About 2 mL of 0.5% ferric chloride solution was added to 1ml of the extract in a test tube and allowed to stand for 1 min. One milliliter (1 ml) of 10% H_2SO_4 was carefully poured down the wall of the test tube. The reddish-brown ring at middle of the two layers indicates the presence of cardiac glycosides (Gul *et al.,* 2017).

Test for Saponins Glycosides (Fehling's Test)

About 2.5mL of Fehling's reagent was added to 2.5ml of the extract in a test tube. A bluish green precipitate shows the presence of saponin glycosides (Gul *et al.,* 2017).

Test for Volatile oils (HCL Test)

Ninety percent (90%) of HCl was mixed with each extract. A white precipitate confirms the presence of volatile oils (Efe *et al.,* 2016).

Test for Balsams: (Ferric Chloride Test)

The extract 2.5ml was mixed with equal volume of 90% ethanol. Two drops of alcoholic ferric chlorides solution was added to the mixture. A dark green color indicates the presence of balsams (Gul *et al.,* 2017**).**

Quantitative Estimation of Alkaloids

One (1 mg) of the plant extracts were dissolved in dimethylsulphoxide and added 1ml of 2N HCl and filtered. This solution was transferred to a separating funnel, 5ml of bromocresol green solution then 5ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3 and 4ml of chloroform by vigorous shaking and was collected in a 10ml volumetric flask and diluted to the volume with the chloroform. A set of reference standard solutions of atropine (20, 40, 60, 80 and 100µg/ ml) was prepared in the same manner as described already. The absorbance for standard solutions and test solutions was determined on the reagent blank at 470nm with an UV/Visible spectrophotometer. The content of alkaloids was expressed as mg of AE/g of plant extract (Selvakumar *et al.,* 2019).

Quantitative Estimation of flavonoids

Colorimetric assay was used to determine the total content of flavonoid using aluminium chloride for the reaction, the plant extract of 1 ml and distilled water of 4 ml was taken in a 10 ml flask. 0.30 ml of 5 % sodium nitrite and after 5minutes, 0.3ml of 10 % aluminium chloride were mixed in the flask. 5minutes later, 2 ml of 1M NaOH was treated and diluted using 10 ml distilled water. A set of standard solutions of quercetin (20, 40, 60, 80 and 100µg/ml) was prepared. The absorbance was measured for test and standard solutions using reagent blank at 510nm wavelength by UV-Visible spectrophotometer. The total content of flavonoid was expressed as mg of QE/g of extract (Selvakumar *et al.,* 2019).

Quantitative Estimation of Glycosides

Eight (8ml) of plant extract was transferred to a 100ml volumetric flask and 60ml of H2O and 8ml of 12.5% lead acetate was added, mixed and filtered. Fifty millilitre of the filtrate was transferred into another 100ml flask and 8ml of 47% Na2HPO4 was added to precipitate excess Pb2+ ion. This were mixed and completed to volume with water. The mixture was filtered twice through same filter paper to remove excess lead phosphate. 10ml of purified filtrate was transferred into clean Erlyn – Meyer flask and treated with 10ml Baljet reagent. A blank titration was carriedout using 10ml distilled water and 10ml Baljet reagent. This was allowed to stand for one hour for complete colour development. The colour intensity was measured colorimetrically at 495nm with an UV/Visible spectrophotometer (Muhammad and Abubaka, 2016).

Quantitative Estimation Terpenoid

Approximately 100mg plant material in screw capped tubes and freeze in liquid nitrogen at 80^0 C for 1-2 months, the sample tissue was homogenized with 95% (v/v) methanol in pre-cooled teflon adaptors for 5 min at 30 Hz, tungsten carbide was removed with a magnet and the sample was incubated at room temperature for 48h in dark. The sample was centrifuged for 15 min at room temperature and supernatant was collected in a fresh 2 ml micro-tube. 1.5 chloroform was added in each 2 ml micro centrifuge tube. Standard curve 200µl of linalool solution in methanol was added to 1.5 ml of chloroform and serial dilution was done. Linalool solution in methanol was added to 1.5 ml chloroform and serial dilution of 12.965- 1.296µm linalool concentration, dilution total volume of 200 µl was made up, total volume of 200µl was made up by addition of 95% (v/v) methanol. The sample mixture was vortex thoroughly and allow to rest for 3min. 100µl Sulfuric acid (H2SO4) was added to each 2 ml micro centrifuge tube, the assay tube was incubated at room temperature for 1.5-2h in dark. At the end of incubation time a reddish brown precipitation was formed in each assay, all supernatant reaction mixture was gently removed without disturbing the precipitation. The reddish brown precipitation is partially soluble in reaction mixture solution. 1.5 ml of 95% methanol was added and vortex thoroughly until all the precipitation dissolve in methanol completely, the sample assay tube was transferred to colorimetric cuvette 95 %(v/v) methanol was used as blank] and measured at 538nm with an UV/Visible spectrophotometer (Ghorai *et al.,* 2017).

Quantitative Estimation of Saponins

Test extract was dissolved in 80% methanol, 2ml of Vanilin in ethanol was added, mixed well and the 2ml of 72% sulphuric acid solution was added, mixed well and heated on a water bath at 60 0c for 10min, absorbance was measured at 544nm with an UV/Visible spectrophotometer against reagent blank. Diosgenin is used as a standard material and compared the assay with Diosgenin equivalents (Madhu 2016).

Quantitative Estimation of Anthraquinones

The extract (1.00 g) was accurately weighed and distilled water (30 ml) was added. The mixture was mixed, weighed and refluxed on a water bath for 15 minutes. The flask was allowed to cool, weighed, adjusted to the original weight with water and the mixture was centrifuged at 4000 rpm for 10 minutes. Twenty milliliters of the supernatant liquid was transfered to a separatory funnel and acidified with 2 M hydrochloric acid. Fifteen milliliters of chloroform was added, the mixture was extracted and the chloroform layer was discarded. The extraction was done triplicate. The aqueous layer was separated and 0.10 g of sodium bicarbonate was added. The mixture was then shaken for 3 minutes and centrifuged at 4000 rpm for another 10 minutes. Ten milliliters of the supernatant liquid was transferred to a 100 ml flask. The solution of 10.5% w/v ferric chloride hexahydrate (20 ml) was added and mixed. The mixture was refluxed on a boiling water bath for 20 minutes. Concentrated hydrochloric acid (1 ml) was added and the mixture was heated for 20 minutes, with frequently shaking to dissolve the precipitate. The mixture was cooled, transferred to a separatory funnel and shaken with 25 ml diethyl ether (Sakulpanich and Gritsanapan, 2008). The partition was repeated until anthraquinones were exhaustively extracted, tested by the Borntrager‘s reaction. The diethyl ether extracts were combined and washed with 15 ml distilled water twice. The combined diethyl ether was then transferred to a 100 ml volumetric flask and adjusted to volume. Twenty five milliliters of the solution was evaporated to dryness. The residue was dissolved with 10 ml of 0.5% w/v magnesium acetate in methanol yielding a red solution. The UV absorbance was measured at 515 nm (Sakulpanich and Gritsanapan, 2008).

Quantitative Estimation of Tanins

The tannin contents were determined using Folin Denis Reagent. In that method, a standard calibration curve was prepared and the Absorbance (A) against concentration of tannins at specific wave length. Suitable aliquots of the tannin-containing extract (initially: 0.05, 0.2 and 0.5 mL) were pipetted in test tubes, the volume was made up to 1.00 mL with distilled water, then 2.5 mL of sodium carbonate reagent were added. Then, the tubes were shaken and the absorbance was recorded at 725 nm after 40 min. The amount of total phenols was calculated as tannic acid equivalent from the standard curve (Sulieman *et al.*, 2007).

Data Analyses

Data obtained from the study were subjected to statistical analysis using statistical package for social science (SPSS version 25.0.) Analysis of variance (ANOVA) were carried on the data, at 95% level of significant and mean generated from this study were separated using List Significant Difference (LSD).

RESULTS

The percentage yields of the leaves extracts in aqueous, methanol and hexane indicates that aqueous extract yield higher extract with 21.09% then methanol with 9.76% and hexane with lowest yield of 4.29%. The result was presented in table 1.

Table 1: Percentage Yields of the Leave Extracts

Extracts	Aqueous	Methanol	Hexane
Yield (%)	21.09	9.76	4.29

The results of the qualitative analysis of *Azadirachta india* is presented in Table 2. The result reveals the presence of tannins, saponins alkaloids, glycosides, flavonoid balsams, steroids, saponin glycocides and cardial glycocides in aqueous extract, and tannins, saponins alkaloids, glycosides, flavonoid balsams, steroids, saponin glycocides, cardial glycocides and Volatile Oils were detected in methanolic extract, while hexane extract indicate the presence of steroids, Terpenoids, tannins, flavonoid and cardial glycocides and Volatile Oils. Qualitative phytochemical analysis

Table 2: Qualitative Phytochemical Component of Aqueous, methanol and hexane Leaf Extracts of *Azadirachta indica,*

Phytochemical components	Aqueous	Methanol	Hexane
Alkaloids	+	+	-
Saponin glycocides	+	+	-
steroids	+	+	+
Tannins	+	+	-
Terpenoids	+	+	+
Cardial Glycosides	+	+	+
Flavonoid Balsams	+	+	-
Volatile Oils	-	+	+
Saponins	+	+	-
Anthraquinones	+	+	-

Key: - Absent. + Present

The result of the quantitative phytochemical analysis of *A. indica* aqueous, methanol and hexane leaves *extracts* are presented in Table 3. The result showed significant amount of Alkaloids in aqueous and methanol, Terpenoids in all three solvent, Flavonoid in aqueous and methanol, Saponins in aqueous, Anthraquinones in aqueous and methanol leaves extracts and Tannins in aqueous extract.

Table 3: Quantitative Phytochemical Component of Leaf Extracts of *Azadirachta indica* at C (ug/ml).

Phytochemical Component	Aqueous	Methanol	Hexane
Alkaloids (%)	11.03 ± 0.09^{b}	11.65 ± 0.20^{c}	0.00 ± 0.00^{a}
Terpenoids (%)	1.81 ± 0.00^{a}	2.93 ± 0.01^{c}	2.37 ± 0.07^{b}
Flavonoid (%)	14.53 ± 0.60^{c}	2.72 ± 0.33^{b}	0.88 ± 0.14^{a}
Saponins (%)	3.15 ± 0.37^{c}	0.66 ± 0.07^{b}	0.00 ± 0.00^{a}
Glycosides (%)	0.00 ± 0.00^{a}	0.29 ± 0.05^{b}	0.00 ± 0.00^{a}
Anthraquinones (%)	2.00 ± 0.00^{b}	2.25 ± 0.00^{c}	0.00 ± 0.00^{a}
Tannins (%)	2.00 ± 0.14^{c}	0.43 ± 0.08^{b}	0.17 ± 0.09^{a}

Values are mean ± standard deviation of 3 replications, means in a column with different superscripts are significantly different ($P\leq0.05$).

DISCUSSION

Aqueous leave extract of *Azadiracta indica,* indicated that the plants are rich sources of bioactive compounds such as tannins, saponins alkaloids, glycosides, flavonoid balsams, steroids, saponin glycocides and cardial glycocides. Similar bioactive compounds were also earlier observed in the seed, back and leaves of *Azadiracta indica* (Alkali *et al.,* 2018; Niyi, 2011; Padal, *et al.,* 2013; Susmitha *et al.,* 2013 and Abdulrazaq *et al.,* 2020). The most important of these plants bioactive chemical constituents (That is, phytochemicals or infochemicals) are alkaloids; tannins, flavonoids, anthraquinonnes, and phenolic compounds (Okwu, 2001). The result of hexane leave extract of *A. indica* is not in agreement with (Daskum, *et al.,* 2020) and this could be attributed to the location of the plant collected or period. The quantitative phytochemical screening of *A.indica* leaves extract revealed the presence of significant high content of flavonoids with 14.5% in aqueous extract while methanol with 3.72% and low content of hexane 0.88%. Alkaloids with 11.65 and 11.03% methanol, saponins 3.15% and anthraquinones with 2% and 2.25% for aqueous and methanol respectively, tepernoids with 2.93% methanol and 2.37% hexane, aqueous with low content of 1.81%, very low content was recorded in methanol and hexane extract with 0.43% and 0.17% tanins and Glycosides with 0.29% methanol At ($P\leq0.05$) significant. This report is in agreement with (Padal, *et al.,* 2013; Susmitha *et al.,* 2013), that their findings reveals methanolic extract of *A. indica* shows the presence of glycoside having highest concentration, while alkaloids, flavonoids, tannins and sugar having moderate concentration and saponins having low concentration and aqueous extract was found to have maximum number of phytoconstituents in saponins and flavonoid, sugar have low concentration and findings by (Oyun and Oyetayo 2020) that reported hexane extract indicated the presence of tannin, terpenoid and flavonoid with quantitative values of 5.19%, 14.04% and 9.48% respectively. Other reports includes (Ujah *et al.,* 2021; Oyun and Oyetayo, 2020; Ogbonna *et al.,* 2016; Muhammad *et al.,* 2022 and Okanlawon *et al.,* 2020). This also explains the rampage use of *A.indica* by the people.

Conclusion

Based on the present study, it can be concluded that the extracts of *Azadirachta indica* are rich source of phytocemicalcs. Flavonoid is found to be most abundant phytochemical, though alkaloids is in very high concentration as well. The phytochemical screening revealed the presence of bioactive constituents that could be the reason for pharmacological activity that is used traditionally by many people as an alternative treatment for many deceases.

Acknowledgements

The authors wish to acknowledge Mal Kabiru, lab technician department of phamacognocy and drugs production, ABU Zaria, for providing the necessary facilities**.**

Conflict of Interest

There is no conflict of interest.

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