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Comprehensive Analysis of Nutrient Composition: Evaluating Vitamins, Essential Minerals, and Trace Metals in Neem (*Azadirachta indica*) Stem Bark Extract

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Abstract

The current study sought to investigate the nutrients in an aqueous extract of Neem (*Azadirachta indica*) stem bark. The bioactive chemical contents of Neem stem bark were isolated, separated, and studied to determine the presence of vitamins, vital minerals, and trace metals. A fresh Neem sample was collected and the sample was ground into powdered form and prepared via extraction using various solvents (n-hexane, ethyl acetate, and ethanol), and the chemical constituents were separated using the GC/MS technique. The Neem stem sample was later digested with nitric acid and hydrogen peroxide in the ratio 4:1 (acid ratio). The trace metals and important minerals in digested Neem samples were determined using an atomic absorption spectrophotometer (AAS), while Na^+ and K^+ were determined using a flame photometer. The results revealed that calcium is the most prevalent mineral in Neem stem bark, followed by potassium and sodium, but copper, magnesium, iron, zinc, and other minerals are present in trace amounts, while cadmium and lead are virtually missing. Furthermore, according to the results of the vitamin studies, the most abundant vitamins in Neem stem bark are vitamin B3 and vitamin C, but vitamins A, B1, B6, and B12 are present in trace amounts.

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Introduction

Several natural sources, such as plants, have been investigated in an attempt to integrate alternative medicine with evidence-based medicine in both the global health sector and medical practice. Drugs derived from medicinal plants are used to treat a wide range of diseases around the world, and they also serve as important raw materials for the pharmaceutical industry [1]. This therapeutic plant has use in the pharmaceutical, horticultural, and food industries [2]. One such experiment with plants is the usage of *Azadirachta indica* (Neem) [3]. Neem has been demonstrated to be effective as an insect repellent, supplement, anti-inflammatory, diabetic treatment, and cancer fighter [3]. It was recently discovered that bark extract and its nimbin isomers inhibit B-coronaviral infection and replication [4]. The Neem tree is mostly grown in southern Asia and Africa, where it has been utilized in medical folklore for centuries [3]. Many parts of the tree, including the leaves, gum, fruit, oil, and flowers, have been demonstrated to have medical and pharmacological benefits. Alternative medicine is becoming increasingly popular in underdeveloped nations, with estimates ranging from 80% to 90%, but it is also becoming more popular in developed nations as a complementary form of therapy [5]. Several researchers have expressed an interest in extracting Neem from various portions of the plant. However, oil seems to be the most widely used portion [6, 7].

Several bioactive compounds with pharmacological potential have been extracted from Neem plants, which are commonly referred to as "metabolites". Metabolites are divided into two types: primary metabolites (protein, fatty substances, glucose, or sugar derivatives) and secondary metabolites (triterpenes), the latter of which are most commonly used in medicine. Other chemicals include flavonoids [8], saponins, nimbins, and catechins, which have been found in smaller amounts [9]. Other substances include alkaloids, limonoids, tannins, terpenoids, sterols [10], and glycoproteins [11, 12]. Aqueous leaf extracts contained significant levels of saponins, tannins, and glycosides, whereas methanoic extracts included high levels of alkaloids, tannins, and flavonoids [13]. As previously noted, Neem stem bark extract was found to prevent the detrimental effects of three distinct corona viruses in a variety of infection models, including in-vivo and in-vitro systems, indicating its promise as a Pan-B-CoV therapy [4]. Furthermore, due to the worrisome rise in bacterial strain resistance to a variety of antimicrobial agents, research was conducted to determine which antimicrobial agents are effective against pathogenic bacteria that are resistant to or less susceptible to present antibiotics. When compared to numerous other studied extracts including the standard antibiotic

Erythromycin, which had a zone of inhibition of 13-14mm, Neem extracts (ethanol and methanol extracts) shown greater antibacterial activity (20-25mm). The antibacterial activity of Neem bark against numerous gram-positive bacteria has been established [15, 16].

Despite numerous studies on secondary metabolites, little is known about the potential of primary metabolites derived from Neem stem bark, which could also be used as supplements. The goal of this research was to discover the nutrients (vitamins, heavy metals, and other trace elements) contained in the stem bark of *Azadirachta indica*. This study seeks to bridge a knowledge gap in the assessment of nutrients, notably vitamins and minerals, in Neem stem bark.

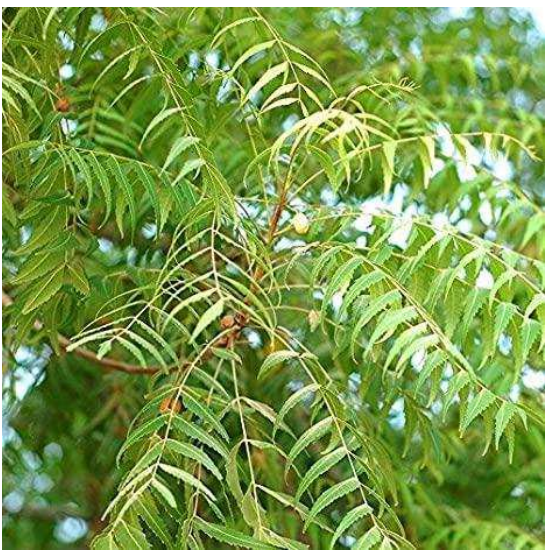

Figure 1. Picture of Neem tree.

Materials and Methods

Sample collection

A fresh Neem stem sample was taken from Shalom Farm in the Ojoo district of Ibadan, Oyo State, Nigeria. Following collection, the exterior section of the stem sample was peeled with a sharp knife, rinsed with water, and air-dried for five (5) weeks. After air drying, the sample was reduced to smaller sizes using a properly cleansed mortar and pestle and then ground into powder using a grinding machine.

Figure 2. Picture of Neem stem bark.

Sample authentication

It was made certain that only Neem stem samples were gathered and not any other plant samples.

Sample preparation

In a container, 100g of the powdered form of the material was extracted using three (3) solvents: n-hexane, ethyl acetate, and ethanol. The sample was extracted with 500ml of n-hexane solvent for 72 hours (3 days), shaking the container at least three times per day to ensure uniform and accurate extraction, and then filtered after 72 hours. This procedure was done for the remaining two solvents, and an extract was obtained from the sample. The extract obtained from the sample was then concentrated using a rotary evaporator, yielding a cream-like concentrate with a weight recorded. The concentrate was then employed in column chromatography, with three solvents (with varying polarity) functioning as the mobile phase and silica gel acting as the stationary phase, yielding approximately 29 fractions. The concentration's weight is listed below.

- **Weight of ethanolic concentrate of Neem stem sample: 21.1g**

Aside from the extraction and column chromatography performed on the powdered form of the Neem stem sample, some other analyses were performed on it. Digestion, heavy metal analysis, vitamin analysis, and phytochemical analysis are among them.

Digestion

The Neem stem sample was digested in a 4:1 acid-to-water ratio with nitric acid and hydrogen peroxide. Each digestion flask was well rinsed, marked, and dried before

adding 1.0g of Neem stem samples as labeled. Each digestion flask received 8 ml of concentrated nitric acid (HNO_3) and 2 ml of hydrogen peroxide (H_2O_2), and the solution was heated on a hot plate in a fume chamber for 2 hours. After 2 hours of heating, the samples were allowed to cool before being filtered into a 100-ml volumetric flask, filled to the 100-ml mark with deionized water, and stored in a polyethylene container. This strategy is consistent with [15] and [18].

Determination of Minerals

The trace metals and essential minerals in digested Neem samples were determined using an atomic absorption spectrophotometer (AAS) model PG 990 at Osun State University in Ejigbo, Osun State, Nigeria, while Na^+ and K^+ were determined using a flame photometer at Obafemi Awolowo University in Ile Ife, Osun State.

Determination of Vitamins

Vitamin A (Retinol) determination

2g of Neem stem sample was weighed into a flat-bottom reflux flask, followed by 10ml of distilled water, and carefully shaken to make a paste. A reflux condenser was fitted, and 25ml of alcoholic KOH solution was added. The aforesaid combination was cooked in a boiling water bath for 1 hour while being shaken frequently. The liquid was swiftly chilled, and 30ml of water was added. The obtained hydrolysate was transported to a separating funnel. The solution was extracted three times, using 250ml of chloroform each time. To eliminate any residues of water, 2g of anhydrous Na_2SO_4 was added to the extract. The solution was then filtered into a 100-ml volumetric flask and marked with chloroform.

By dissolving 0.003g of standard β-Carotene in 100ml of chloroform, the result was a standard solution of β-Carotene (Vitamin A) ranging from 0 to 50 g/ml. The gradients of multiple standard solutions created were determined using absorbance, and the average gradient was used to calculate Vitamin A (β-Carotene in g/100g). The absorbance of the sample and standards was measured using a Spectrophotometer (Metrohm Spectronic 21D Model) at 328nm.

Calculations:

Vitamin A (µg/100g) = *Absorbance of sample* × *Dilution factor* × *Weight of sample*

Conversions:

6 µg of β-Carotene = 1 Retinol equivalent,

12µg of other biologically active carotenoids = 1 retinol equivalent,

1 retinol equivalent of Vitamin A activity = 1µg of retinol,

1 retinol equivalent = 3 IU (International Unit).

Vitamin B1 (Thiamine) determination

1g of Neem stem sample was weighed into a 100ml volumetric flask, and 25ml of 0.1M H_2SO_4 was carefully swirled in. To rinse any sticking sample particles from the flask, 25ml of 0.1M H_2SO_4 was added. To guarantee thorough dissolution of the sample in the acid, the flask was placed in a boiling water bath. The flask was shaken regularly for the first 5 minutes, then every 5 minutes for the next 3 minutes. 5ml of taka-diastase in a 0.5M sodium acetate solution was added, and the flask was placed in cold water to keep the content below 500°C. The flask was sealed and maintained at 45-50°C for 2 hours before being filled to the 100ml mark after properly mixing.

The mixture was filtered through a No 42 Whatman filter paper, with the first 10ml discarded and the remainder saved. 10ml of the residual mixture filtrate was pipetted into a 50ml volumetric flask, followed by 5ml of acid potassium chloride solution, which was thoroughly mixed. Standard thiamine solutions ranging from 100mg/ml to 50mg/ml were made from 100mg/ml stock and treated in the same manner as the sample above. The absorbance of the sample and standards was measured using a fluorescent UV Spectronometer (Cecil A20 model) at 285nm. The following formula was used to compute vitamin B1 in mg/100g:

Vitamin B1 in mg/100g = *Absorbance* × *Average Gradient* × *Dilution Factor* × *Wt. of sample*

Vitamin B2 (Riboflavine) determination

1g of Neem stem sample was weighed into a 250ml volumetric flask, followed by 5ml of 5 NHCl and 5ml of dichloroethene. After shaking the mixture, 90ml of deionized water were added. The mixture was thoroughly agitated before being cooked in a steam bath for 30 minutes to remove all of the riboflavin. After cooling, the mixture was made up to volume with deionized water. It was then filtered, with the first 20ml of the aliquot being discarded. 2ml of the filtrate was pipetted into a new 250ml volumetric flask and filled to capacity with deionized water.

Standard solutions were made by dissolving 0.05mg riboflavin in 100ml of distilled water. To determine the equivalency, different standard solution concentrations ranging from 0 to 5ppm were created. The absorbance, standards, and samples were measured using a Fluorescent Spectrophotometer at 460nm wavelength. The amount of Vitamin B2 in the sample was determined using the formula:

Vitamin B2 (mg/100g) = *Meter reading* × *Standard* × *Dilution factor* × *Weight of sample*

Niacin (vitamin B3) determination

5g of Neem stem sample was mixed, and 100ml of distilled water was added to dissolve any Nicotinic acid or Niacin present. 5ml of this solution was drawn into a 100ml volumetric flask and made up to the mark with distilled water.

Niacin stock solutions ranging from 10 to 50ppm were also produced. A spectrophotometer was used to measure the absorbance of the diluted stock solutions and sample extract at 385nm. To calculate the gradient factor, absorbance at the required wavelength was measured using a spectrophotometer at various concentrations of standard stock solutions. The following formula was used to calculate the amount of Niacin in the sample:

Vitamin B3 (mg/100g) = *Absorbance* × *dilution factor* × *gradient factor of stock solution*

Pyridoxine (Vitamin B6) determination

To extract all of the pyridoxine, 1g of Neem stem sample was weighed into a 100ml beaker, followed by 0.5g of ammonium chloride, 45ml of chloroform, and 5ml of pure alcohol. The mixture was thoroughly combined in a separating funnel for 30 minutes by shaking vigorously.

To clearly separate the aqueous layer from the chloroform layer, 5ml of distilled water was added to the mixture in a separating funnel. The pyridoxine-containing chloroform layer was filtered into a 100ml volumetric flask and brought up to mark with chloroform. The gradient factor was obtained by preparing a 0-10ppm pyridoxine standard from a 100ppm stock standard solution of pyridoxine. The absorbance of a yellow color solution generated was measured at 415nm using a Cecil 505E Spectrophotometer. The formula was used to calculate the amount of pyridoxine in the sample.

Vitamin B6 (mg/100g) =

$$Absorbance\ of\ sample \times Gradient\ Factor \times Dilution\ Factor \times Wt.\ of\ sample \times 100$$

Vitamin C (Ascorbic Acid) determination

In a 100ml volumetric flask, 10g of the sample slurry was weighed and diluted to 100ml with 3% meta-phosphoric acid solution (0.0033 EDTA). The diluted samples were filtered through a Whatman Filter Paper No. 1 filter 3. 10ml of the filtrate was pipetted into a tiny flask and promptly titrated to a faint pink end point with a standardized solution of 2,6-dichlorophenol-in-dephenol. The ascorbic acid content of the sample was determined using the following relationship:

$$\text{Ascorbic acid per 100g Wt sample (mg)} = V \times T \times 100$$

where V is the volume of dye used for titration of an aliquot of diluted sample (ml) and T is the ascorbic acid equivalent of the dye solution expressed as mg/ml of dye. Wt = weight of aliquot titrated sample (g).

Vitamin E (Tocopherol) determination

1g of Neem stem was weighed into a 250ml conical flask and filtered via a reflux condenser. We added 10ml of pure alcohol and 20ml of 1M alcoholic sulphuric acid. Wrapping the condenser and flask in aluminum foil, they were refluxed for 45 minutes and chilled for 15 minutes. After adding 50ml of distilled water to the mixture, it was transferred to a 250ml separating funnel covered with aluminum foil. The mixture's unsaponifiable materials were removed with 5 x 30ml dimethyl ether. The mixed extracts were acid-free washed and dry-evaporated at a low temperature, with the resulting residues dissolved instantaneously in 10ml pure alcohol. Aliquots of sample and reference solutions (0.3-3.0mg vitamin E) were transferred to a 20ml volumetric flask, 5ml pure alcohol was added, and 1ml of Concentrated HNO_3 was carefully added. The flasks were placed in a 900°C water bath for exactly 3 minutes from the time the alcohol began to boil, then quickly cooled under running water and adjusted to volume with absolute alcohol. At 470nm, compare the absorbance to a blank containing 5ml absolute alcohol and 1ml conc. HNO_3 prepared in a similar manner.

Vitamin E (µg/100g) = *Absorbance* × *Gradient factor* × *Dilution factor wt. of sample*.

Results and Discussions

According to mineral analysis, the most abundant minerals in Neem stem bark are calcium, potassium, and sodium, but copper, magnesium, iron, zinc, and other trace elements are found in trace amounts, while cadmium and lead are virtually missing. Calcium is found to be the most abundant element, followed by potassium. This is consistent with [17].

Calcium aids in the formation of strong bones and teeth, the coagulation of blood, the release of hormones and other substances, the squeezing and relaxing of muscles, and so on. Potassium is essential for heart function as well as skeletal and smooth muscle contraction, making it essential for regular digestive and muscular function. It also helps the human body's cells, tissues, and organs work properly. Its importance in the treatment of hypertension, stroke, and arrhythmias cannot be overstated. Sodium aids in fluid balance and contributes to normal muscular contraction and nerve impulse condition. The body also uses it to regulate blood pressure and volume. It is also a key component of both human and animal diets, as well as the transfer of other minerals into and out of cells.

According to the vitamin studies, the most abundant vitamins in Neem stem bark are vitamin B3 and vitamin C, but vitamin A, B1 and B6, and B12 are found in trace amounts. This is consistent with [18], which found that vitamin C is the most abundant vitamin in the extract, followed by vitamins B6, E, A, B1, and B2. Furthermore, based on the many vitamins and components extracted from the extract and its multifunctionality, this extract has the ability to provide important nutrients to hens. The most major advantage of vitamin B3 is that it helps to decrease and control high cholesterol levels in the body. It also helps the brain work normally and maintains excellent blood circulation. It can also be used to treat respiratory and cardiovascular problems. Some studies have also shown that mental derangement and associated disorders such as schizophrenia can be healed by taking Vitamin B3 pills or medicinal drugs. Vitamin C is essential in the cellular oxidation-reduction reaction. It also facilitates in the movement of iron from the blood into the liver, where it is stored as Ferritin, and its importance in promoting quick wound healing (when paired with zinc) cannot be overstated. Vitamin C is also a potent antioxidant that can neutralize damaging free radicals and aids in the neutralization of pollutants and poisons. It also helps to keep teeth and gums healthy by avoiding hemorrhaging and bleeding.

Vitamin A promotes skeletal growth as well as healthy mucous membranes, skin, eyes, and feathers. Vitamin E protects the body's vitamin A stores, tissues, and fat from oxidation and breakdown of red corpuscles, strengthens capillary walls, regulates reproductive period, prevents loss of other vitamins, improves blood flow to the heart, lowers blood cholesterol and fatty acids, and regulates protein and calcium metabolism. Vitamin C is required for the formation of collagen, the absorption of iron, some proteins, and folic acid, the prevention of vitamin oxidation, the metabolism of amino acids and calcium, the prevention of internal bleeding, the strengthening of blood vessels, the maintenance of hard bones and teeth, and the healing of wounds and burns. Vitamin B6 (pyridoxine hydrochloride) aids in protein, carbohydrate, and fat metabolism, as well as cholesterol control, it aids in the chemical balance of blood and tissue, prevents water retention, and the formation of hemoglobin. Thiamine (Vitamin B1) aids in the conversion of sugar and starch into energy, promotes digestion, strengthens heart muscle, promotes chick growth, and prevents weariness and fat deposits in arteries [17]. Vitamin B2 (riboflavin) aids in the release of energy to body cells, the consumption of protein, fat, and glucose, the creation of RBC, and the formation of nucleic acid [17].

Finally, calcium helps create bones, aids in the proper working of the muscles, hearts, nerves, and iron use, aids blood coagulation, controls body temperature, supports nerve function, and bone growth. It aids in the utilization of vitamins B, C, and E, increases mineral absorption and metabolism, activates enzymes for glucose and amino acid metabolism, and inhibits calcium deposits in the ureters. Iron is a component of hemoglobin, which transports oxygen to tissues via blood circulation. Zinc reduces cholesterol deposits, assists in B-vitamin absorption, enzyme and insulin production, and glucose metabolism. It is also necessary for growth, healing, preventing sterility, and keeping feathers glossy and smooth [18].

Sodium's relevance in cellular homeostasis and physiological function cannot be overstated. Element intakes such as macronutrients and micronutrients are required for physiological and metabolic activities. The macro-elements are required in amounts larger than 100mg/dl, whereas the micro-elements must be in amounts less than 100mg/dl [17]. Calcium aids in the formation and maintenance of bone mass and strength, making it a vital dietary component [15]

Table 1. Mineral composition of the Neem Stem bark

Mineral	**Value (ppm)**
Cu^{2+}	0.295
Mg^{2+}	0.105
Mn^{2+}	0.333
Co^{3+}	---
Fe^{2+}	0.564
Zn^{2+}	0.321
Hg^{2+}	N.D
Ca^{2+}	218.0
Se^{4+}	0.251
Cd^{2+}	N.D
Pb^{4+}	N.D
Cr^{3+}	---
K^{+}	211.0
Na^{+}	53.2

Table 2. Vitamin composition of the Neem stem bark

VITAMIN (mg/100g)	**MEAN**
Vitamin B1	0.480
Vitamin B2	0.095
Vitamin B3	4.850
Vitamin B6	1.905
Vitamin B12	0.305
Vitamin C	6.765
Vitamin A	2.876
Vitamin E	0.035
%Total Sugar	3.100

Conclusion

In summary, the evaluation of nutrients in Neem stem bark extract has provided valuable insights into its potential as a source of bioactive compounds with significant health benefits. Through meticulous analysis and examination of various components, this study has highlighted the nutritional richness inherent in Neem stem bark. The findings of this research suggest that Neem stem bark extract is not only a traditional remedy but also a reservoir of essential nutrients that may have applications in pharmaceuticals, nutraceuticals, cosmetics, and drug discovery, among others. The presence of important minerals, trace metals, and vitamins C, A, B3, and B6, among others, underscores the potential health-promoting properties of Neem stem bark, making it a subject of interest for further exploration in the field of chemistry and/or pharmacology.

Furthermore, the outcomes of this evaluation contribute to the broader understanding of the nutritional composition of medicinal plants, emphasizing the need for continued research into the diverse array of bioactive compounds present in natural sources. As we conclude this study, it is evident that Neem stem bark extract holds promise for various applications, and future investigations could delve deeper into its mechanisms of action and potential synergies with other compounds.

In closing, this research not only enhances our comprehension of Neem stem bark as a valuable source of nutrients but also encourages ongoing efforts to harness its potential for improving human health and well-being. The journey undertaken in this study serves as a stepping stone for further scientific inquiry and underscores the significance of nature-derived substances in the pursuit of innovative solutions for health-related challenges.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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