Phytomedicinal and Nutraceutical Benefits of the GC-FID Quantified Phytocomponents of the Aqueous Extract of \textit{Azadirachta indica} leaves

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Abstract

Background: The continued human disruptive exploitation of the ecosystem has given room to sustained health hazard due to saturated environmental free radicals culminating in the emergence of disease states borne out of low immune systems and damaged body cells orchestrated by environmental free radicals causing the diminished quality of life and life-span. Health system approach to therapy exploiting and utilizing the rich antioxidants and other useful phytocontituents of plant origin as free radical scavengers will go a long way to boost the quality of life and immune system as well as lessen morbidity and mortality rates.
Aim: The present study aimed to quantify the phytocomponents of the aqueous extract of *Azadirachta indica* leaves and highlighting their phytomedicinal and nutraceutical benefits.

Materials and Methods: Aqueous extract was obtained from 100 g of powdered air-dried leaves of *Azadirachta indica*. Its phytochemical constituents were identified and quantified using a gas chromatographic device fitted with a flame ionization detector (GC-FID).

Results: Lunamarin, an alkaloid was at the highest level followed by sapogenin, a saponin. Various flavonoids ranked third, then the phenols. Though, several phytochemicals such as the steroids, phytate, oxalate, tannins and resveratrol were also identified and quantified. Over 35% of the phytochemicals identified were of the flavonoids.

Conclusion: The aqueous extract of *Azadirachta indica* leaves contains very essentials phytochemicals with useful phytomedicinal and nutraceutical benefits.

Keywords: Aqueous extract; *Azadirachta indica* leaves; Phytomedicinal; Nutraceutical; Phytocomponents; GC-FID

Introduction
Phytomedicine could be described as herbal-based medication that could be offered for therapeutic, healing or alleviation of diseases in humans. Its origin is as old as human evolution [1]. Sheng Nongs Herbal Book is recognised as one of the earliest foundations of traditional folk information founded on the usage of herbs in China and dates back to around 3000 BC. It embraces the particulars of virtually 365 plants, animals and minerals that catch a place in medicine. More than 420,000 kinds of plants are found on planet earth with most still lacking precise and comprehensive information about their possible diverse applications, hence, prompting the need for their continuous evaluations. The exploitation of plants for human benefit falls within the three basic components for: food, medicine (folk and traditional) and research (phytochemical analysis) [1,2]. The word “nutraceutical” was devised from two words: “nutrient” (a nutritious food constituent) and “pharmaceutical” (a medicinal drug). It was made up in 1989 by Stephen DeFelice, forerunner of the Foundation for Innovation in Medicine located in Cranford, New Jersey [3,4]. The idea behind nutraceuticals is to emphasize on prevention, agreeing to the saying by a Greek physician, Hippocrates, “Let food be your medicine”. Nutraceuticals refer to a wide-ranging household word defining any food-based preparation with additional functional value for the well-being of humankind. The supplementary gains are based on their physiological aids by providing protection from or treatment for chronic disease [2,4-7]. They could be viewed as alternatives to pharmaceuticals regarded as dietary supplements. They can be measured as non-specific organic remedies employed in enhancing the overall health, regulate signs and inhibit malignant progressions [8,9]. Their part in the sustenance of the mortal system is one of the utmost essential areas of exploration, with a wide range of outcomes for end-users, health-care workers, regulators, food producers and distributors [4,10]. Nutraceuticals can be classified based on their origin,
pharmacological actions and chemical composition. However, they are mostly grouped as dietary supplements, functional food, medicinal food and farmaceuticals [6]. A dietary supplement embodies a preparation that comprises nutrients resulting from food products and is often concentrated in liquid, capsule, powder or tablet form. Though dietary supplements are controlled by the FDA as foods, their guideline varies from drugs and other foods. Dietary supplements are not intended to treat or cure diseases [11,12]. Functional foods are commonly recognized as whole foods which are fortified, enriched or boosted dietary constituents that may lessen the risk of protracted disease and offer a health-benefit further than the traditional nutrients it contains [13]. Medical food is constituted to be taken, under the direction of a competent physician. Its intended use is a precise dietary treatment of an infection for which distinguishing nutritious necessities are recognized by the medical assessment (based on standard scientific code) [14]. In another way, farmaceuticals are medically valuable constituents manufactured from improved crops or animals. The term is a combination of the words “farm” and “pharmaceuticals”. Advocates of this perception are persuaded that using crops (and possibly even animals) as pharmaceutical factories is much more cost-effective than conventional methods, with higher revenue for agricultural producers. For several years nutraceuticals have gained a good amount of recognition due to their prospective nutritious, security and healing effects [1,14-17].

These nutraceuticals have been employed in several conditions such as antioxidant defences, gene expression, cell multiplying and safeguarding of mitochondrial integrity. In these cases, they are employed to advance health, inhibit long-lasting diseases, defer the ageing process, or just to provide support for the various functions and integrity of the body. They have also been known to inhibit life menacing ailments such as diabetes, cancer, eye disorders, cardiovascular, renal and gastrointestinal disorders and provide immunity against infections [8,14,15]. Generally, phytomedicine focuses on the usage of vegetation to cure and alleviate human diseases. Though contemporary medicine seems to have replaced herbal medicines in managing illnesses in humans, the application of herbal substances has improved in the contemporary years globally with the assumption that they are safer, with fewer or no side effects compared to modern medicines. Herbal preparations are ordinarily given as an extract of the entire herb, like herbal tea or fresh juice. At sundry times, the complete herb is used up either fresh or in the dry and pulverized form. The use of herbal therapies in many advanced countries has been carried along with complementary and alternative medicines (CAMs) and it has become conventional in the United Kingdom (UK) and the rest of Europe, North America and Australia [18-20]. Referring to the World Health Organization (WHO), herbal therapies is one of the popularly used forms of treatments after primary health-care for around 3.5-4 billion people through the globe. The WHO estimates that close to 70 and 95% of the populace living in various emerging countries still depend more on herbal-based medicines for their major medication against diseases [2,21]. Phytomedicine, in combination with various other health-care fields, has certainly modernized and reinforced the base of the present health-care system and takes up a key position in the industrial sector with over 35,000 kinds of plants that are presently being utilized in herbal treatments and formulas [22]. However, only 20% of the over-all go through the phase of phytochemical analysis while 10% get to the biological investigation phase. The outstanding still require several extents of assessment through up-to-date
technologies. The prospect of medicinal plant-based medications, therefore, appears to have a remarkable opportunity for determining some new and novel beneficial approaches and medicinal substances [23-25].

*Azadirachta indica* (*A. indica*) popularly called neem is a traditional medicinal plant used in many parts of the world for therapeutic purposes in wounds, cuts and other skin infections. It is further employed widely by numerous kinfolks. Therapeutic values of its leaves such as antioxidant and antimicrobial actions were attributed to its phytoconstituents. The flavonoids present in them act as antioxidants which safeguard against free radicals that impair cells and tissues and also the tannins support wound healing [26-28]. Extracts from neem plant have been highly applied in Ayurveda, Unani and Chinese treatments to hinder and manage a diversity of conditions [29,30]. Each component of the plant possesses certain organic and therapeutic relevance, hence it has persisted as a treasured base for natural therapeutic ingredients [31].

Extracts gotten from neem offer free radical scavenging activities due to its rich content of antioxidants [32]. It has been documented that *A. indica* has various phytoconstituents such as nimbin, nimbidin, nimbolide and limonoids and these are valuable in the management of numerous disorders by modulation of some inherent pathways and other activities. The initial polyphenolic flavonoids extracted from fresh leaves of neem were quercetin and ß-sitosterol which were acknowledged to retain antifungal and antibacterial activities [33]. Rich organic and pharmacological properties have been recorded and comprise antibacterial [34], antifungal [35], and anti-inflammatory. Preceding investigators have recognized their role as anti-inflammatory, anti-arthritis, antipyretic, hypoglycemic, anti-gastric ulcer, antifungal, antibacterial and anti-tumour properties [36-39]. The wound healing properties of the ethanolic or methanolic extracts of neem leaves have been documented [40-42]. In a recent study using hydroxyproline as a biochemical marker for wound healing, the aqueous extract of the leaves of *A. indica* was found to possess wound healing properties with minimum wound healing effective concentration determined at 1.5% w/v of its extract [43].

With the earth’s rich plant life and their robust phytochemical compositions retaining diverse phytomedicinal and nutraceutical potentials, it has become very necessary to engage in a consistent exploration of these natural deposits of nature especially in this era of availability of innovative technologies to elucidate and interpret the phytocomponents of materials. The exposure of these useful constituents of vegetative origin has equally become necessary following the alarming rate of strange illnesses resulting from the ravaged ecosystems due to the detrimental environmental activities of man following urbanization which culminate in the emergence of disease states that have to do with low body immunity orchestrated by increased environmental free radicals which have continued to cause damage to human body cells, reducing the quality of life and life-span. Increased investigations and revelation of the rich antioxidants and other useful phytoconstituents of plants around us in addition to the continuous exposure of the need to make use of these will enhance the quality of life, boost body immunity and reduce morbidities and mortalities. The aim of the present study was to quantify the phytoconstituents of the aqueous extract of *A. indica* leaves and to broadly highlight their phytomedicinal or nutraceutical benefits.
Materials and Methods

Materials

In this study, the following materials were employed as procured and include ethanol (96%), anhydrous sodium sulphate, potassium hydroxide (Sigma-Aldrich, USA), n-hexane (BDH, England).

Methods

Sourcing and preparation of plant samples

Fresh *A. indica* leaves collected from the medicinal plant garden of the Faculty of Pharmaceutical Sciences, University of Port Harcourt was identified by a Taxonomist. It has been deposited in the University of Port Harcourt herbarium (voucher no. EH/P/070). A 100 g of the pulverized air-dried leaves was macerated in 1 L of distilled water at ambient temperature and shaking intermittently for 48 h. The filtrate was clarified and concentrated under a reduced temperature to obtain the extract.

Quantification of the phytochemical components by GC-FID

One gram (1g) of the aqueous extract of *A. indica* leaves [44] was conveyed into a test tube, adding 15 ml of ethanol and 10 ml of 50% w/v potassium hydroxide and leaving the contents of the test tube to react in a water bath at 60 °C for an hour. The outcome of the reaction was emptied into a separatory-funnel. The tube was rinsed in turn with 20 ml of ethanol, 10 ml of cold water, 10 ml of hot water and 3 ml of n-hexane, with each transferred to the separatory-funnel. These extracts were combined together and rinsed three times with 10 ml of 10% v/v ethanol-water solution which was dried with anhydrous sodium sulfate and evaporating the solvent. The sample was dissolved in 1000 µl of n-hexane and 200 µl of it was placed in a vial for analysis [45]. The analysis of phytochemicals was performed on a BUCK M910 gas chromatography fitted with a flame ionization detector (GC-FID). A RESTEK 15 meter MXT-1 column (15 m × 250 um × 0.15 um) was used. The injector temperature was 280 °C with a splitless injection of 2 ul of sample and a linear velocity of 30 cms⁻¹, Helium 5.0 was the carrier gas with a flow rate of 40 ml min⁻¹. The oven was operated initially at 200 °C. It was heated to 330 °C at a rate of 3 °C min⁻¹ and was maintained at this temperature for 5 min. The detector was run at a temperature of 320 °C. Phytochemical content was assessed by the ratio between the area and mass of internal standard and the area of the identified phytochemicals. The concentration of the various phytochemicals existing in the extract was stated as µg/g of the extract [44, 45].

Results

The results of the GC-FID quantitative analysis of the aqueous extract of *A. indica* leaves are shown in Table 1. It shows the presence of flavonoids (38.63%), tannins (1.85%), Saponins (15.79%), alkaloids (24.84%), other phenolics (7.04%) and steroids (3.88%) and anti-nutrients (7.97%). The lunamarin, an alkaloid had the highest concentration (41 µg/ml or 21.36%). This is followed by sapogenin (30.37 µg/ml or 15.79%), a saponin and naringenin (17.29 µg/ml or 8.99%), a flavonoid.
Table 1: Composition of the phytochemical components of the aqueous extract of *A. indica* leaves quantified with the GC-FID

| Type of Phytochemical | Phytochemical    | Retention | Area     | Height   | Conc. (µg/ml) | % Composition |
|-----------------------|------------------|-----------|----------|----------|--------------|---------------|
| Flavonoids (38.63%)   | Proanthocyanin   | 0.21      | 5426.71  | 354.14   | 5.61         | 2.92          |
|                       | Anthocyanin      | 6.02      | 18234.49 | 1020.75  | 14.82        | 7.70          |
|                       | Naringenin       | 10.37     | 19625.31 | 1096.78  | 17.29        | 8.99          |
|                       | Flavonones       | 22.73     | 9583.12  | 539.39   | 5.39         | 2.80          |
|                       | Flavone          | 34.60     | 6059.41  | 344.11   | 4.27         | 2.22          |
|                       | Rutin            | 44.17     | 10547.78 | 596.53   | 7.74         | 4.02          |
|                       | Kaempferol       | 29.86     | 5484.79  | 311.59   | 2.68         | 1.39          |
|                       | Epicatechin      | 27.54     | 11538.70 | 649.86   | 10.18        | 5.29          |
|                       | Cathechin        | 39.20     | 10239.63 | 576.28   | 6.35         | 3.30          |
| Tannins (1.85%)       | Tannin           | 15.46     | 4978.26  | 282.66   | 3.55         | 1.85          |
| Saponins (15.79%)     | Sapogenin        | 17.96     | 11351.01 | 641.44   | 30.37        | 15.79         |
| Alkaloids (24.84%)    | Ribalinidine     | 7.47      | 8483.30  | 480.62   | 4.88         | 2.53          |
|                       | Lunamarin        | 2.39      | 12419.30 | 701.55   | 41.09        | 21.36         |
|                       | Spartein         | 12.97     | 6253.00  | 354.93   | 1.83         | 0.95          |
| Other Phenolics (7.04%)| Resveratrol      | 42.28     | 3510.21  | 199.55   | 2.58         | 1.34          |
|                       | Phenol           | 20.31     | 12766.14 | 680.81   | 10.96        | 5.70          |
| Steroids (3.88%)      | Steroids         | 25.65     | 10090.10 | 570.42   | 7.47         | 3.88          |
| Anti-Nutrients (7.97%)| Phytate          | 32.99     | 14337.08 | 803.99   | 1.65         | 0.86          |
|                       | Oxalate          | 36.88     | 6996.18  | 394.00   | 13.67        | 7.10          |

Discussion

Previous report of the qualitative phytochemical evaluation of the aqueous extract of *A. indica* showed that it contains alkaloids, flavonoids, glycosides, tannins, saponins and phenols [41,43,46-48]. These abundant phytochemicals found in the aqueous leaf extract of *A. indica* possess several pharmacological, antioxidant, immunological and antimicrobial actions, enzyme and hormone modulation, reduction of platelet aggregation and anti-cancer properties [49-51].

The phytomedicinal and nutraceutical benefits of the various phytocomponents of the aqueous extract of *A. indica* leaves

Flavonoids
Flavonoids consist of natural substances with variable phenolic groups which are found mostly in vegetables and in some grains, stems and flowers. They are well known for their valuable health benefits especially with their attributable anti-oxidative, anti-mutagenic, anti-inflammatory, anti-carcinogenic properties and enzyme modulatory functions [52-54]. In the present study, the flavonoids identified include proanthocyanin, anthocyanin, naringenin, flavonones, flavone, rutin and kaempferol. Naringenin (17.29 µg/ml or 8.99 %) had the highest concentration while kaempferol (2.68 µg/ml or 1.39 %) was of the lowest concentration (Table 1). Proanthocyanin and anthocyanin are widely distributed pigments in land plants where they serve as stress protectants and health-promoting components because of their potent antioxidant activity [55,56]. Naringenin, which is the most abundant flavonoid in the extract is found mainly in citrus fruits and tomatoes and have been shown to be beneficial in the management of cancer, cardiovascular diseases and osteoporosis [57,58]. Recently, it has been shown to cause a significant reduction in the accumulation of collagen fibres in liver injury in rats [59]. Other beneficial properties of naringenin include its ability to reduce oxidative stress [60], anti-inflammatory [61], anti-diabetic [62], anti-hyperlipidaemia [63], antioxidant [64] and antidepressant properties [65]. Flavones and flavanones are other important forms of flavonoids. While flavones are mostly present in leaves, flowers, fruits, celery, parsley and red peppers, flavonones are found in all citrus fruits such as lemons, grapes and oranges [53]. Flavones are known to possess anti-microbial and anti-fungal activities. They are also able to interact with proteins, binding with human serum albumin for easy transportation through plasma [66]. On the other hand, flavonones are known to possess antioxidant, anti-hyperlipidemic and anti-inflammatory properties [53]. Rutin, another flavonoid is found majorly in plants such as passion-flower, buckwheat, tea and apple. Some of the pharmacological activities of rutin include antioxidant, cytoprotective, vasoprotective, cardioprotective, neuroprotective and anti-carcinogenic properties [67,68]. Similarly, kaempferol is another flavonoid commonly found in a variety of vegetables and other plants such as grapes, green tea, potatoes, onions and cucumber. Just like other flavonoids, they can possess anti-diabetic, anti-cancer, anti-inflammatory activities [69]. It has been reported to modulate certain key elements in cellular signal transduction pathways linked to apoptosis, angiogenesis, inflammation, and metastasis, hence their ability to inhibit cancer cell growth and angiogenesis by inducing cancer cell apoptosis [70]. Cathechin and epicatechin are also among the flavonoids present in the aqueous extract of A. indica leaves. While cathechins are found in various foods and herbs like apples, grapes, berries and tea, epicatechins are majorly found in both green tea and black tea with the highest epicatechin content found in cocoa [71,72]. Cathechin possesses enormous health benefits such as anti-obesity, anticancer, hepatoprotective, anti-diabetic and neuro-protective effects while epicatechins are known to possess cardio-protective, antioxidant, anti-diabetic and anti-cancer activities. Epicatechin rich green tea has also been shown to exhibit in vivo platelet anti-aggregation [73] and enhanced insulin sensitivity [74]. Generally, these protective actions of flavonoids in organic systems are attributed to their ability to transfer electrons to free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals and prevent oxidases [75,76] since oxidative stress and inflammation are the common responses which contribute in the development of tumour via stimulating defected cells to go through promotion and progression of tumours, initiating direct damage to genomic nucleic acids, initiating abnormal cell growth and modifying intracellular signalling [77]. The rich flavonoid content of the aqueous extract of A. indica leaves confers on it many
pharmacological activities such as anti-inflammatory, antipyretic, hypoglycemic, antifungal, antibacterial, and anti-tumour and wound healing properties [36-39].

Tannins
Tannins are mostly water-soluble polyphenols that are present in a variety of plant foods. They are found in tea, cocoa, vegetables, legumes and some unripe fruit [78]. Aqueous extract of A. indica leaves was found to contain low amounts of tannins (3.55 µg/ml) comprising of 1.85 % of the total phytochemicals (Table 1). Tannins have played a key role in the Asian traditional medicine where plant extracts containing tannins are used as astringent as well as a diuretic. They have also been used in the treatment of diarrhoea, gastrointestinal ulcers and tumours. They also possess anti-inflammation and antioxidant activities [79-81]. However, tannin-rich food is usually considered to be of lower nutritional value as they have been reported to be responsible for decreases in feed intake and efficiency in experimental animals. They have been thought to inhibit the conversion of absorbed nutrients into new body substances [82,83]. Tannin-protein complexes may cause digestive enzymes inactivation and protein digestibility reduction caused by protein substrate and ionizable iron interaction [84].

Saponins
Saponins comprise of a group of structurally related natural occurring compounds either containing a steroid or triterpenoid aglycone (sapogenin) found mostly in plants and other lower marine animals, including some bacteria. They occur both in wide plants and cultivated crops with the triterpenoid saponins more predominant as they are found in many legumes like soya beans, beans and peas. They are also found in oats, ginseng, yam and tomato seed. Some of the pharmacological effects ascribed to saponins include immunomodulatory, anti-inflammatory, anti-fungal, antiviral, antibacterial, hypercholesterolaemic and anti-carcinogenic properties [85-87], thus making them very essential in human and animal nutrition. The aqueous extract of A. indica leaves contains significant quantities of sapogenin (30.37 µg/ml) comprising of 15.79 % of the total phytoconstituents obtained (Table 1). While Sapogenins have been noted for their many beneficial properties, other harmful properties have also been documented. For example, their haemolytic and cytotoxic actions have been observed [88-90]. They have also been observed to significantly impair the digestion of protein and the absorption of vitamins and minerals in the small intestine leading to hypoglycaemia [85].

Alkaloids
Plant alkaloids remain one of the largest groups of natural products made up structurally of diverse and biogenetically unrelated molecules. They possess a wide range of pharmacological activities and have been used as a component of many herbal remedies [91]. They include narcotic analgesics, morphine and codeine. Also, they have been shown to possess potent antimalarial, antimicrobial and antiprotozoal properties [92]. The result of the present study showed that the aqueous extract of A. indica leaves contains a significant amount of quinoline alkaloids (24.84%) with lunamarin (41.09 µg/ml or 21.36%) having the highest concentration, followed by ribalinidine (4.88 µg/ml or 2.53%) and then, spartein (1.83 µg/ml or 0.95%) (Table 1). Lunamarin and ribalinidine have been reported to have radical scavenging function [93]. Also, lunamarin possess anticancer,
immunomodulatory, anti-estrogenic, anti-amoebic properties [94-96]. These alkaloid contents could be attributed to some of the pharmacologic properties of the extract of *A. indica* leaves [29, 31, 48].

**Anti-Nutrients: Phytates and Oxalates**

While nutrients are associated with beneficial effects in human health, anti-nutrients, on the other hand, interfere with the absorption of minerals and hence are thought of as not so beneficial, though, some have valued health benefits. Their interference with nutrient absorption has been known to cause headaches, rashes, nausea, bloating and nutritional deficiencies [97]. While some anti-nutrients will bind to essential micronutrients to prevent the body from absorbing them, others may inhibit the optimal functioning of digestive enzymes, hence preventing the proper break down of food. Anti-nutrients are mostly of organic or synthetic structure and are highly reactive, hence capable of toxic effects. Phytates and oxalates are some of the well-known anti-nutrients found to be contained in the aqueous extract of *A. indica* leaves in concentrations of 0.86 and 7.10% respectively (Table 1). Phytate (myo-inositol hexaphosphate) is found in varieties of foods such as nuts, seeds and whole grains. They are also found in substantial amounts in roots and tubers. It is held that phosphorylated inositol, particularly phytic acid have parts in the secretion of insulin by the beta cells of the pancreas. Phytic acid has also been proposed to obstruct the beginning of plaque development and lower serum cholesterol and triglycerides [98-100]. Oxalates on the other hand are known to interfere with calcium absorption and utilization by forming calcium oxalate crystals which lead to the formation of kidney stones. They have also been reported to cause irritation and swelling in the mouth and throat as well as able to form tissue crystals creating arthritis-like symptoms [101-103]. Oxalates are commonly distributed in raw, cruciferous vegetables like kale and broccoli, as well as spinach, soybeans, black pepper and chocolate.

**Conclusion**

The aqueous extract of *A. indica* leaves contains alkaloids, flavonoids, glycosides, saponins, tannins and phenols. Lunamarin, an alkaloid was in the highest level followed by sapogenin, a saponin. Various flavonoids ranked third, then the phenols. Though, several phytochemicals such as the steroids, phytate, oxalate, tannins and resveratrol were also identified and quantified, over 35% of the phytochemicals belong to the flavonoids. Aqueous extract of *A. indica* leaves contains very essentials phytochemicals with useful phytomedicinal and nutraceutical benefits to human health.

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