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Title: **Antioxidant Properties of Azadirachta Indica Leave Extracts**

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Antioxidant Properties of *Azadirachta Indica* Leave Extracts

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ABSTRACT

Background: *Azadirachta Indica* is an important medicinal plant commonly found in India, Africa, America, Europe, and many other regions of the world. The current study was designed to investigate the concentrations of important phenolic/flavonoid contents of ethanolic extracts of *Azadirachta Indica* (neem) leaves (Lahore, Pakistan).
Methods: High-performance liquid chromatography (HPLC) analysis revealed the presence of three phenolic compounds (gallic acid, sinapic acid, and caffeic acid) and two flavonols (Myricetin and Kaempferol). The plant extract contained the highest and lowest concentrations of myricetin acid (26.41µg/g) and kaempferol (3.35µg/g) among flavonols, respectively.
Results: Both acids are helpful in the manufacturing of antioxidant medicines. Among phenolics, sinapic acid (45.73µg/g) and gallic acid (1.96µg/g) were present in highest and lowest concentrations in plant leaves, respectively.
Conclusion: Gallic acid, sinapic acid, caffeic acid, myricetin, and kaempferol possess antioxidant and therapeutic potential and are highly beneficial for human health. Human beings can get many benefits and produce more medicines from the leaf extract of neem in the future. Many more advantages can also be taken from different parts of neem (leaves, seeds, and bark).
Keywords: antimicrobial, antioxidant, Ethanolic extract, neem leaves

Highlights

- The ethanolic extracts of *Azadirachta Indica* (neem) leaves were investigated for their phenolic/flavonoid contents, antioxidant, and antimicrobial potential.
- The plant extract contained the highest and lowest concentrations of myricetin acid (26.41µg/g) and kaempferol (3.35µg/g) among flavonols, respectively.

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- From phenolics, the plant material contained sinapic acid (45.73µg/g) and gallic acid (1.96µg/g) at the highest and lowest concentrations, respectively.

1. INTRODUCTION

Plants have been widely investigated throughout the world due to their valuable phytochemical contents [1,2], nutritional [3,4], and medicinal potential [5,6]. *Azadirachta Indica* (Neem or Juss) is thought to be native to the Indian subcontinent and is grown all over the world [7]. This plant remains evergreen, however, sometimes in drought conditions, it sheds off its leaves [8]. Its primary active compounds include tetranotriterpenoid (in seeds), nimbin, melianone, salanin, azadiractol, 14-epoxyiazadiradione, azadirone, meliantrol, vilosinin, gedunin, deacetilasalanin, and nimble [9]. Neem also contains secondary metabolites, for instance, flavonoids, alkaloids, tannins, and terpenoids. which possess medicinal properties [10]. Other secondary metabolites include glycosides, reducing sugars, anthraquinones, and saponins [11]. Some active components (licorice, turmeric, and propolis) of neem play an important role as a herbal agent [12]. *A. indica* is used for the treatment of various medical conditions including heart diseases, hypertension, diabetes, and cancer [13] and is widely employed in traditional medicine, Homeopathic, Yunani, and Ayurveda remedies [14]. Its smooth stems are used as chewing sticks, whereas leaf extract can control airborne pollution [15]. Its leaves, bark, and seed extracts are natural sources of therapeutic agents and demonstrate antioxidant, antimutagenic, anti-inflammatory, anticarcinogenic, antimalarial, antiulcer, and antidiabetic properties [16]. *A. indica* is a good anti-inflammatory agent used in the treatment of metabolic diseases [17]. Neem extract has a strong potential to treat *fungemias* and *Candida albicans* fungi [18] and it also treats numerous viral diseases including vaccinia, chikungunya, coxsackie, and measles [19].

In continuation to previous investigations on the nutritional and medicinal value of plants [20-22], the current study focused on the determination of phenolic/flavonoid contents of *Azadirachta Indica* (neem) leaves (Lahore, Pakistan). Moreover, it also tested their antioxidant potential. The study is important due to the identification of antioxidants from a natural resource, that is, *A. indica* and their health-promoting and therapeutic effects.

2. MATERIALS AND METHOD

The ethanol reagent used in the extraction of plant leaves was of analytical grade. Pyrex origin glassware was used.

2.1. Collection of Leaves Sample

The leaves of *Azadirachta Indica* (neem) (Figure 1) were collected from Walton (Lahore, Pakistan) on March 14th, 2020. The investigated plant was identified by the Department of Biology, Lahore Garrison University, Lahore (Pakistan). Healthy leaves were manually separated from the branches and washed with distilled water. They were then dried under shade for about 2 weeks at room temperature. Finally, they were ground homogenized by using a commercial grinder (TSK-049, West point France) into fine powder which was stored for further use.

2.2. Preparation of Ethanolic Extract

Fine powder (30g) of *A. indica* leaves was mixed with ethanol (150ml) and the mixture was vigorously stirred in a shaker for 24 hours. After filtration, the filtrate was

evaporated with the help of a rotary evaporator to leave behind the solid ethanolic extract. About 0.3g of dried extract was dissolved in 8ml of ethanol in a test tube. Afterwards, 4ml of *n*-hexane was added and the mixture was thoroughly stirred and allowed to stand for 5 minutes. It resulted in the separation of the *n*-hexane layer (on top) containing lipids and waxes. This layer was removed and discarded. Again, fresh *n*-hexane was added into the same solution, the mixture was stirred and after a stay of five minutes, the *n*-hexane layer was again decanted off. The same process (first addition of *n*-hexane and then removal of lipid layer) was repeated five times to ensure maximum removal of fats and waxes [23]. The fat-free ethanolic extract was stored at 4 ^{o}C in an airtight glass container for analysis and other uses.

Figure 1. Leaves on A *Azadirachta Indica* (neem) Plant

2.3. HPLC Separation

HPLC (high-performance liquid chromatography) was performed by LC 10A, Kyoto, Shimadzu, Japan HPLC model. This model consists of two SCL 10A system control units, LC 10 software, 2 LC 10 AS pumps, CTO 10A column oven, rheodyne injector, and SPD 10A UV-visible detector. 20µL of the ethanolic extract was inserted into the ODS-revere phase column. Two-solvent systems were introduced into it, that is, one system contained methanol and acetonitrile with a ratio of 20/80 v/v, whereas the other comprised 3% trifluoroacetic acid. Chromatographic separation was performed at 30 ^{o}C and these two solvents were filtered under elution of mobile phase with the flow rate of 1.00mL min^{-1}. The sample was detected at the wavelength of 360nm. After the identification of flavonoids, their retention times were compared with those of standard flavonoids. From the curves of the standards, the quantitative determination was carried out. The range of dilute solution of the standard was 0.001-0.08mgL^{-1}.

3. RESULTS AND DISCUSSION

Plant materials show antioxidant activities due to the flavonoids [24,25] and phenolics content [26,27]. Flavonoids are responsible for the taste and color of plants, herbs, vegetables, and fruits [27]. Flavonoids are compounds that have low molecular weight and are extracted from different parts of the plant. They possess an

excellent antioxidant potential and play an essential role in human body, for instance, they control the cellular activities in the body and act against radicals formed due to stress or toxins. However, excessive intake of flavonoids may be hazardous for human health since they start the formation of free radicals and disturb the body's hormones. Sometimes, their over dosage may cause death [28]. Phenolic components also show antioxidant properties. Phenolic compounds have aromatic rings which are bound with hydroxyl groups. In addition to their antioxidant potential, they provide many other benefits for human beings, for instance,, they have also been employed as mouth wash too [29]. Gallic acid, caffeic acid, and sinapic acid are the phenolic components that were present in the leaves extract of *A. Indica* as determined by the HPLC (Table 2).

Ethanolic extracts of *A. indica* leaves were subjected to HPLC analysis for the determination of their major phenolic and flavonoid contents. The obtained HPLC data of flavonoids and phenolics is summarized in Tables 1 and 2, respectively. Whereas, they are graphically represented in Figures 2A and 3A, respectively. Figures 2B and 3B display the standard HPLC data for flavonoids and phenolics, respectively.

The investigated extract of *A. indica* leaves has shown the presence of significant amounts of two flavonoids (kaempferol and myricetin), whereas quercetin was not detected at all (Table 1, Figure 2). The most abundant flavonoid was myricetin (26.41µg/g) followed by kaempferol (3.35µg/g). Both the myricetin and kaempferol (flavonols) are good natural antioxidants. Myricetin is a critical nutritive ingredient of diet which is found in vegetables, fruits, wine, and tea. It is beneficial for maintenance of good health and provides immunological protection. Moreover, its therapeutic potential may be owed to its valuable biological properties including antidiabetic, anticancer, hepatoprotective, anti-inflammatory, osteoporosis protection, cardiovascular protection, anti-obesity, antidiabetic, and anticancer. It possesses numerous health benefits due to its impact on different cell processes including osteoclastogenesis, serum protein concentrations, lipid level, energy balance, cell cycle, glycolysis, and apoptosis [30]. Myricetin is also effective in controlling the colon and skin cancer cells by inhibiting the free radicals [31]. Studies have suggested that a myricetin dose of 25 to 100mg/Kg (25 to 100µg/g) is enough to play a stronger anticancer role against the human erythroleukemic cell line (K562) and human lung adenocarcinoma cell line (A549) [32]. Since, the investigated plant extract contains 26.41µg/g of myricetin, it is recommended that the ethanolic extract of *A. indica* (neem) can serve as an anticancer agent for therapeutic purposes.

The HPLC analysis showed the presence of 3.35µg/g kaempferol in the investigated ethanolic extract of *A. Indica* leaves. Kaempferol is a flavanol compound that plays an essential role in human health. It acts as an anti-inflammatory and antioxidant agent and is also effective against chronic diseases, such as cancer [33]. Kaempferol with an amount of 8.04mg/day is essential as part of the nutritional requirements of the body. This compound can also behave as a cardioprotective, antiestrogenic, antidiabetic, anxiolytic, and neuroprotective agent [34].

Table 1. Flavonoid Contents of *A. Indica* Leaves Extract

Sr. No.	Flavonoid Content	Quantity	Retention time
1	Myricetin	26.41µg/g	3.654BB

2	Kaempferol	3.35 µg/g	6.455BB
3	Quercetin	0.00 µg/g	0.00BB

[#]µg is a microgram, BB is Baseline to Baseline

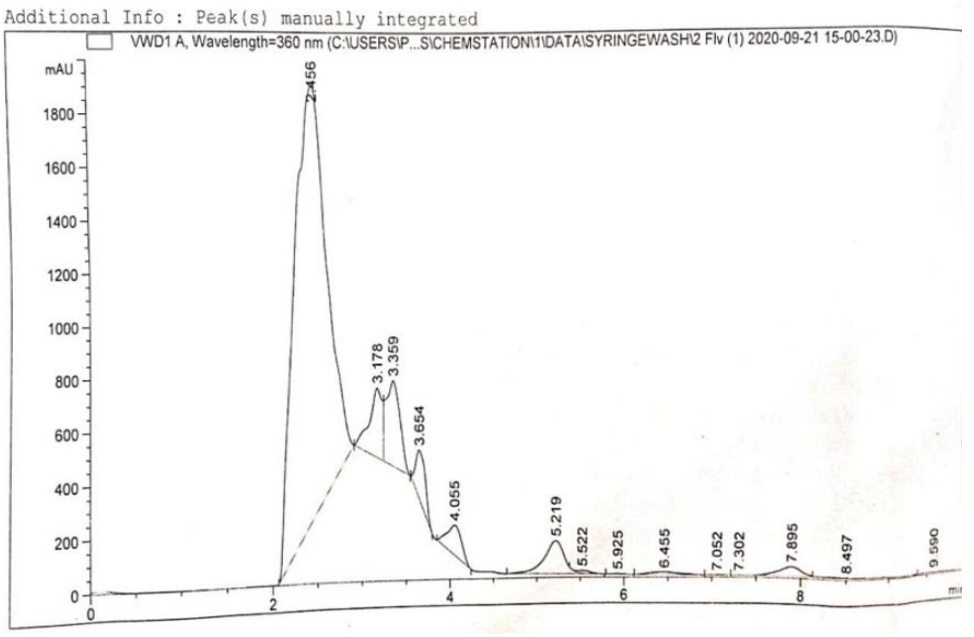

Figure 2A. HPLC Graph Showing the Flavonoid Contents in Neem Leaves

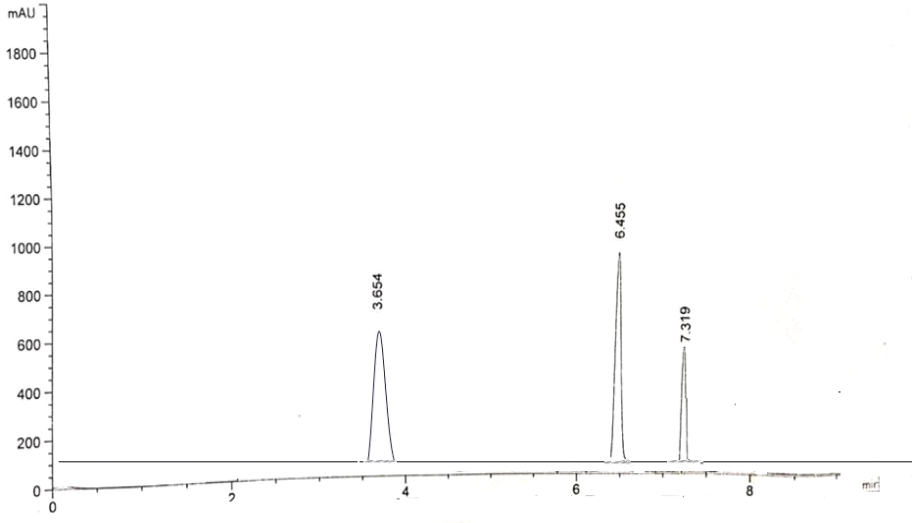

Figure 2B. HPLC Graph Showing the Flavonoid Standards

The ethanolic extracts of *A. indica* leaves have shown the presence of three natural products including sinapic acid, caffeic acid, and gallic acid with the concentrations of 45.73 µg/g, 7.02 µg/g and 1.96 µg/g, respectively (Table 2, Figure 3). Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) is the most abundant phenolic component in the investigated extract of *A. indica* leaves. It is a widespread bioactive phenolic acid in the plant kingdom (medicinal plants, oilseed crops, cereal grains, vegetables, fruits, spices) and is commonly used in human diet. Mainly due to its powerful antioxidant potential, it is used in pharmaceutical preparations, cosmetics, and food processing. Due to its beneficial effects on body functions, it is used in the preparation of functional foods [35]. Sinapic acid is an orally bioavailable phyto-ingredient that has been applied as a medicine in oxidative stress induced diseases and aging and can reduce chemically induced toxicities. Additionally, it also displays antioxidant, antibacterial, neuroprotective, antiglycemic, antimutagenic, anticancer, and anti-inflammatory properties and is extensively present in spices, cereals, oilseeds, cereals, vegetables, berries, and citrus fruits [36]. About 1.5µg/mL amount of sinapic acid is required to form a dose against oxidation. It inhibits the production of ROS (reactive oxygen species) [37].

Caffeic acid is the second most crucial phenolic compound that is primarily found in food. The primary source of caffeic acid is coffee. The investigated ethanolic extract of *A. indica* also contains caffeic acid (7.02µg/g) which shows a substantial antioxidant property and inhibits the formation of free radicals that may be produced as a result of the oxidative stresses [38]. Various supplements of caffeic acid are available which are helpful in weight loss, boost energy levels, and skincare. Caffeic acid prevents photo-damage and photo-aging of skin. It can also be used to treat cancer cells (chemotherapy) without damaging the kidney and liver organs [39].

Gallic acid is a trihydroxybenzoic acid with pale fawn or yellowish-white crystals and is the third most abundant phenolic content in *A. indica*. Gallic acid possesses anticancer, antibacterial, antiviral, antioxidant, and anti-inflammatory properties. Foods containing gallic acid are called powerful antioxidants and are used as natural remedies [40], for instance, the use of aromatic blue berry tea due to its relaxation effects during childbirth and for the purification of blood [41], application of gallnuts from sumac and oak to treat hyperhidrosis, hematochezia, bleeding, and intestinal disorders [42]. Different diseases have been successfully treated over the years by thirty ayurvedic herbs and formulations owing to the presence of gallic acid [43]. The wounds and cuts are prevented from infections by applying hazel balm and tea (containing gallic acid), whereas tea alone is used to treat colds and menstrual issues [41]. Gallic acid shows cytotoxicity activity by repairing the damaged cells without affecting the healthy cells. It also plays a significant role in antiviral and antibacterial activities [40].

Table 2. Phenolic Contents of *A. Indica* Leaves Extract

Sr. No.	Phenolic Content	Quantity µg/g*	Retention time
1	Gallic acid	1.96	6.621BB**

2	Caffeic acid	7.02	9.975BV***
3	Sinapic acid	45.73	11.244BV***

*µg/g is a microgram/gram, **BB is baseline to baseline, ***BV is a baseline to the valley.

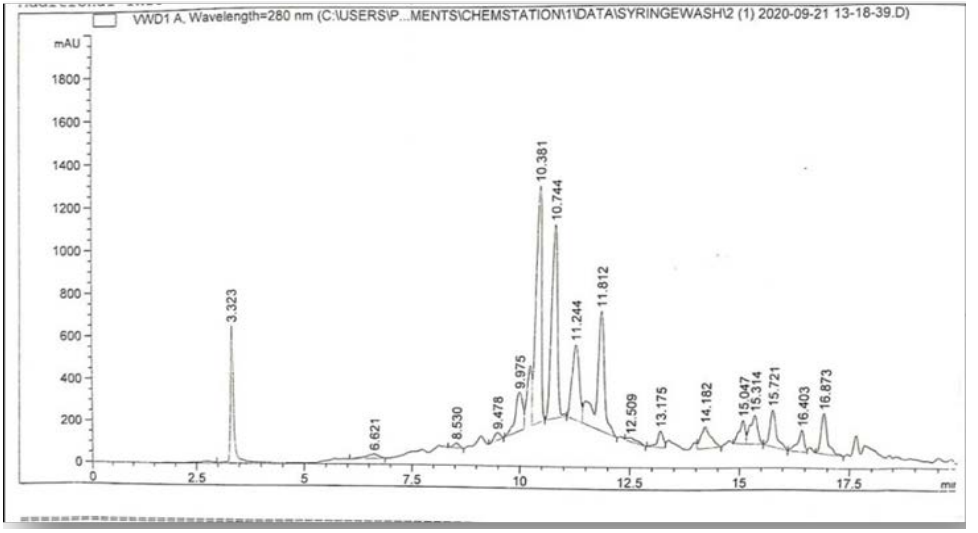

Figure 3A. HPLC Results for Phenolic Content from Leaves Extract of Neem

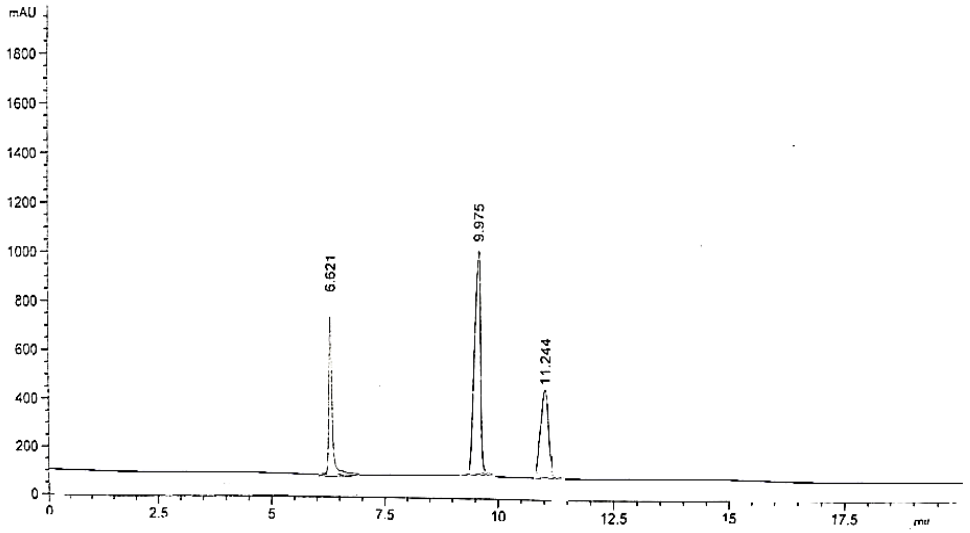

Figure 3B. HPLC Graph Showing the Phenolic Standards

There are strong epidemiological evidences about the presence of an inverse relationship between the excessive use of vegetables/fruits and several health

disorders, for instance, pancreatic, gastric, ovarian, lung or colon cancer [44]. The current investigation has isolated two flavonoids (kaempferol and myricetin) and three phenolics (sinapic acid, caffeic acid and gallic acid) from the ethanolic extracts of *A. indica* leaves. All these natural products are biologically active and possess strong antioxidant potential along with other therapeutic properties. The antioxidant activity of *A. indica* is an important concern that has proven to be very useful for human health. Antioxidant activity is a process that stops the oxidation process in the human body. The oxidation reaction is a chemical reaction that produces free radicals in the body and leads towards a chain reaction. It would be hazardous, that is, it may cause cancer and damage the cell organisms. Antioxidants terminate the long chains of free radicals and protect the human body from different diseases [45].

3.1. Conclusion

HPLC analysis has shown that ethanolic extracts of *A. Indica* (neem) leaves are rich in phenolic and flavanols which are the antioxidant agents and play an essential role in human body. The investigated leaf extracts have shown the presence of three phenolic compounds (gallic acid, sinapic acid, and caffeic acid) and two flavonols (Myricetin and Kaempferol). They contain the highest and lowest concentrations of myricetin acid (26.41µg/g) and kaempferol (3.35µg/g) among flavonols, respectively. Both acids are helpful in the manufacturing of antioxidant medicines. Among phenolics, sinapic acid (45.73µg/g) and gallic acid (1.96µg/g) were present in the highest and lowest concentrations in plant leaves, respectively. Gallic acid, sinapic acid, caffeic acid, myricetin, and kaempferol possess antioxidant and therapeutic potential and are highly beneficial for human health. It is expected that human beings can get many more benefits and produce more medicines from the leaves extract of neem in the future. Even more advantages can be taken from different parts of neem (leaves, seeds, and bark).

CONFLICT OF INTEREST

The author of the manuscript has no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVALIABILITY STATEMENT

The data associated with this study will be provided by the corresponding author upon request.

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