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Investigation of Total Phenolic, Flavonoid Contents and Antioxidant Activity from Extracts of *Azadirachta indica* of Bundelkhand Region

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ABSTRACT

Azadirachta indica common name Neem is a very useful traditional medicinal plant in the sub-continent and each part of the tree has some medicinal properties. It has many therapeutic properties as it boosts the immune system, beneficial in treating acne, provides relief from bad breath, helps to protect against diabetes, effective in treating malaria symptoms, lowers the risk of cancer and cardiovascular disease. The plant is native to Asia and its sub-continents. Therefore, the aim of the present study was to investigate the phytochemical constituents present in leaves and bark of neem. The antioxidant activity, total phenolic, and flavonoid contents were also evaluated. Neem leaves and bark were collected from the Bundelkhand University Campus, Jhansi. It was cleaned with tap water and finally with distilled water and was air dried at room temperature and crushed. TLC was done for the determination of bioactive compounds present in the leaves. A qualitative phytochemical analysis was performed and we found that alkaloids, reducing sugar, flavonoids, glycosides, tannins, phenolic compounds, saponins are present in neem leaves and bark. TLC results shown total 8 spots in the methanolic leaves extract having different Rf values. The total antioxidant capacity of *A. indica* leaves shown the dose dependent activities. The mean values of total phenolic contents and flavonoids are 70 mg GAE/g & 119 mg QE/g respectively. Thus in the present study, the presence of flavonoids and phenolic compounds in the neem leaves extract could be considered responsible for conferring antioxidant ability.

Key-words: Phytochemical, *Azadirachta indica,* Antioxidant Activity, Total Phenolic Content (TPC), Total Flavonoids Content (TFC)

INTRODUCTION

Azadirachta indica commonly known as Neem is a fast-growing tropical evergreen tree found mainly in India, Africa and America. In Sanskrit, it is called '*arishtha*' a word that means 'perfect, complete and imperishable and reliever of sicknesses. India has encouraged scientific investigations of neem tree as a part of its program to revitalize Indian tradition and also to increase its commercial interest [1].

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It is called by various names in India such as "Divine tree", "Wonder tree", "heal all", "Materia Medica", "Free tree of India", Nature's drugstore", "Village Pharmacy", "Panacea for all diseases" [2-4]. Indians have used neem from centuries for various means like grimy skin disorders with neem leaf juice, taken neem tea as a tonic, clean teeth with neem twigs, neem leaves to keep away troublesome bugs, etc. Its benefits have been described in ancient documents like 'Charak Samhita and Susruta Samhita.

The tree is now grown in most tropical and sub-tropical areas of the world for shade, for reforestation programmes and in plantations for the production of the compound which have toxic, anti-feedant and repellent properties against insects. Many biological activities of oil of seed kernels of *A. indica* extract have been demonstrated. From the crude tetranortriterpenes,

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including nimbin, nimbinin, nimbidinin, nimbolide, and nimbidic acid have been isolated. The dose dependent anti-inflammatory activity in a model of carrageenan induced acute paw edema in rats as well as formalin induced arthritis has been reported with Nimbidin and sodium nimbidate. Further, Gedunin isolated from neem seed oil has been shown to possess both antifungal and anti-malarial activities. Mahmoodin belongs to the gedunin class of limonoids, isolated from neem oil showed antibacterial activity against some strains of human pathogenic bacteria. The antioxidant potential of neem seed extract has been associated with low levels of lipooxygenase activity and lipid peroxides [5]. The chloroform extract of stem bark is effective against carrageenan induced paw edema in rat and mouse ear inflammation [6]. Extracts of leaf, oil and seed kernels are effective against certain human fungi [7]. Oil from the leaves, seeds and bark possesses a wide spectrum of antibacterial activity against Gram-negative and Gram-positive microorganisms [8,9]. *A. indica* is perhaps the most useful traditional medicinal plant in India. Further, various medicinal applications and biological activity of the neem compounds has been achieved during last five decades [10,11]. The *A. indica* leaves are widely used among the various tribes of India, Africa and Burma, to cure ailments of skin, and other parts of the body [12]. It is the most important medicinal plant that has been declared as the "Tree of the 21st century" by the United Nations.

Pharmacological studies have acknowledged the value of medicinal plants as a potential source of bioactive compounds [13]. Phytochemicals from medicinal plants serve as lead compounds in drug discovery and design [14]. Both the bark and leaves also contain biologically active molecules but not high levels of azadirachtin which is found mainly in the seed kernels. Phenolic compounds and flavonoids are very important plant secondary metabolites. These compounds have numerous defense functions in plants, and thus several environmental factors, such as light, temperature, humidity, and internal factors, including genetic differences, nutrients, hormones, etc., contribute to their synthesis [15]. Similarly, other factors, such as germination, degree of ripening, variety, processing, and storage, also influence the content of plant phenolics [16]. It is reported that the phenolics are responsible for the variation in the antioxidant activity of the plant [17]. Plant phenolics showed antioxidant activity by preventing decomposition of hydroperoxides into free radicals or inactivating lipid free radicals [18,19] or chelate metal ions and protect against pathogens and predators [20]. Keeping in view of the above beneficial effects of the *A. indica* plant, we sought to analyze the photochemicals present in methanolic as well as in aqueous extract. Anti-oxidant activity was also evaluated. Also, total phenol and flavonoid content were estimated. Further, TLC was conducted to monitor the number of bioactive components (spots) present in the extracts.

MATERIALS AND METHODS

Collection of Plant Materials- Leaves and bark of *A. indica* were collected from the Bundelkhand University campus, Jhansi, in the month of January 2017. Firstly soaked the plant material in normal water for 2-5 minutes to remove the soil and then washed with de-ionized water for two times. After washing, it was kept in the dark for drying at room temperature and under the constant observation to avoid any contamination. Finally, it was crushed with the help of electric grinder. Powdered sample was stored in airtight bottles for further study.

Extraction Procedure- Extraction was done by two methods i.e. Aqueous and Methanolic extraction.

Aqueous Extract- Different concentration of dry powder of *A. indica* leaves i.e. 5 gm and 10 gm was taken in conical flasks with equal amount (100 ml) of de-ionized water. Both the flasks were kept in the water bath for 1 hour at 90°C. After 1 hour flasks were left at room temperature for cooling, and filtered with the help of filter paper and stored at 4°C.

Methanolic Extract- Soxhlet apparatus was used for extraction purpose with 80% methanol. Different concentration of plant material and solvent were taken. After filling the soxhlet apparatus with plant material and solvent it was run at 60°C until it gets colorless and continuous flows of water to cool down the condenser. Finally, the extract was collected in airtight bottles and stored at 4°C.

Phytochemical Analysis- Detailed phytochemical analysis was carried out for all the extracts as described elsewhere [21] with some of modifications.

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Thin layer chromatography- The methanolic extracts were tested using TLC analytical plates coated with silica gel-G of 0.2 mm thickness. The solvent system used a mixture of Butanol-acetic acid-water (4:1:1 v/v) as described by Singh and Kumar [21] with some modifications. This mixture migrates on the silica-coated plates by the capillary action. Fully developed coated plate was air-dried followed by heating for 20-25 minutes. The plate was sprayed with 0.2% freshly prepared ninhydrin solution to detect the spots.

The movement of the spots was expressed by its retention factor (Rf).

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

Antioxidant activity- The total antioxidant capacity of the methanol extract of *A. indica* leaves were evaluated by the phosphomolybdenum reduction assay method according to the procedure described by Prieto *et al*. [22]. 0.1 mL of various concentrations of the extract was combined with 1 mL of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. A typical blank solution contained same volume of methanol in place of extract and the appropriate volume of the same solvent used for the samples/standard. The calibration curve was prepared with the respect of different conc. of ascorbic acid (µg/ml) in methanol as a standard. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer.

Determination of Total Phenolic Content (TPC)- The total phenolic content was determined by using the Folin- Ciocalteu method [23]. Gallic acid was used as a standard. 100 µl of different dilutions were mixed with 500 µl of water and then with 100 µl of Folin-Ciocalteu reagent and allowed to stand for 6 minutes. Then 1ml of 7% sodium carbonate and 500 µl of distilled water were added to the reaction mixture. The absorbance was recorded after 90 minutes at 760 nm spectrometrically. The total phenolic content was calculated as gallic acid equivalents (mg GAE/g). All the experiments were performed in triplicate.

Determination of Total Flavonoid Content (TFC)- The protocol was optimized from the study of Piyanete *et al.* [24]. Aluminium chloride complex forming assay was used to determine the total flavonoid content of the extracts. Quercetin was used as standard and flavonoid content was determined as quercetin equivalent. 100 µl of the quercetin dilution was mixed with 500 µl of distilled water and then with 100 µl of 5% sodium nitrate and allowed to stand for 6 minutes. Then 150 µl of 10% Aluminium chloride solution was added and allowed to stand for 5 minutes after which 200 µl solution of 1M Sodium hydroxide was added sequentially. The absorbance of this reaction mixture was recorded at 510 nm on UV spectrophotometer. The total flavonoid content was calculated as quercetin equivalents (mg QE/g). All the procedures were performed in triplicate.

RESULTS

The phytochemical analysis of the aqueous and methanolic extract of *A. indica* leaves and bark reveals the presence of various secondary metabolites (Table 1). Presence and absence of the phytochemical constituents depend on the test applied for the qualitative detection of secondary metabolites. In humans and other animals, alkaloids have many important physiological effects. All tests for alkaloids shown positive results for both extracts except Hager's test which shown negative results for aqueous & methanolic extract of neem leaves. Molish's and Barfoed's tests were done for the presence of carbohydrate. Molish's and Barfoed's test shown absence of carbohydrates. Presence of reducing sugar was monitored by Fehling's and Benedict test. Reducing sugar are absent in aqueous extract of leaves while Benedict's test shown positive results for all the extract except aqueous extract of bark. Flavonoids are very important plant secondary metabolites and have numerous defense functions in plants. We observed very interesting results regarding flavonoids i.e. leaves contain it whereas it is absent in the bark extract. Many drugs and poisons derived from plants are glycosides. Different tests for glycosides had shown its presence in all the extract except Borntrager test, which is negative in aqueous and methanolic extract of neem leaves. Cardiac glycosides have been shown to have anticancer activities during various stages of carcinogenesis.

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Phenolic compounds are closely associated with the sensory and nutritional quality of fresh and processed plant foods. Plant-derived tannins are the basis of the tanning industry for many years. Tannins bind to salivary proteins, producing a taste which humans recognize as astringency. Many types of tannin are extremely astringent and render plant tissues inedible. Many mammals avoid eating plants with high tannin contents. We used different tests for the qualitative estimation of tannin and phenolic and almost all the tests showed the presence of these metabolites with little exception. Saponins are glucosides with foaming characteristics. Saponins consist of polycyclic aglycones attached to one or more sugar side chains. Saponins have many health benefits such as the beneficial effects on blood cholesterol levels, cancer, bone health and stimulation of the immune system. Saponin was present in all the extracts whereas amino acids and proteins are absent. Terpenoids and steroids constitute the largest known group of plant secondary metabolites. Terpenoids are the largest class of natural products and are used for various purposes like perfumery, cosmetics, spices, flavors, fragrances, etc in the industrial sector. Terpenoids and steroids are absent in all the extracts.

Table 1: Qualitative phytochemical analysis of the aqueous and methanolic extracts of *A. indica leaves* and barks

S. No	Phytochemical Tests	Neem Bark				Neem Leaves		
		Aqueous			Methanolic	Aqueous		Methanolic
		5 gm	10 gm	Soxhlet		5 gm	10 gm	
	Test for alkaloids							
1	(a) Mayer's test	+ ve	+ve	+ve	+ve	+ve	+ve	+ve
	(b) Wagner' test	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	(c) Hager's test	+ve	+ve	+ve	+ve	-ve	-ve	-ve
2	**Test for carbohydrate**							
	(a) Molisch test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	(b) Barfoed's test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3	**Test For Reducing Sugar**							
	(a) Fehling's test	+ve	+ve	+ve	+ve	-ve	-ve	+ve
	(b) Benedict's test	-ve	-ve	+ve	+ve	+ve	+ve	+ve
	Test For Flavonoids							
4	(a) Alkaline reagent	-ve	-ve	-ve	-ve	+ve	+ve	+ve
	(b) Lead acetate	-ve	-ve	-ve	-ve	+ve	+ve	+ve
	(c) Ammonia test	-ve	-ve	-ve	-ve	+ve	+ve	-ve
	Test For Glycosides							
5	(a) Borntrager test	+ ve +	+ve	+ve	+ve	-ve	-ve	-ve
	(b) Legal's test	ve	+ve	+ve	+ve	+ve	+ve	+ve
	(c) 10% NaOH test	+ ve	+ ve	+ve	+ve	+ve	+ve	+ve
6	**Test of Cardiac Glycosides**							
	(a) Keller killani test	+ve	+ve	+ve	-ve	+ve	+ve	-ve

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	Tannin & Phenolic Test							
	(a) Ferric chloride test	+ve	+ve	+ve	+ve	+ve	+ve	+ve
7	(b) Lead acetate test	-ve	-ve	-ve	-ve	+ve	+ve	+ve
	(c) Dilute iodine test	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	(d) Ferric chloride10%	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	(e)Hydrolysable tannins	+ve	+ve	-ve	-ve	-ve	-ve	-ve
8	**Test For Saponin**							
	(a) Saponin test	+ve	+ve	+ve	+ve	+ve	+ve	+ve
9	**Amino acid and protein**							
	(a) Ninhydrin test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	(b) Biuret test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
10	(a) Test for terpenoids	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	(b) Test for steroid	-ve	-ve	-ve	-ve	-ve	-ve	-ve

(+) indicates presence while, (–) indicates the absence of the components

Thin layer chromatography- The thin layer chromatography of sample shows +ve result for the methanolic extracts of neem leaves. Total 8 spots were present in the methanolic leaves extract having Rf values 0.22, 0.31, 0.45, 0.51, 0.63, 0.75, 0.90, 0.95 respectively (Fig. 1).

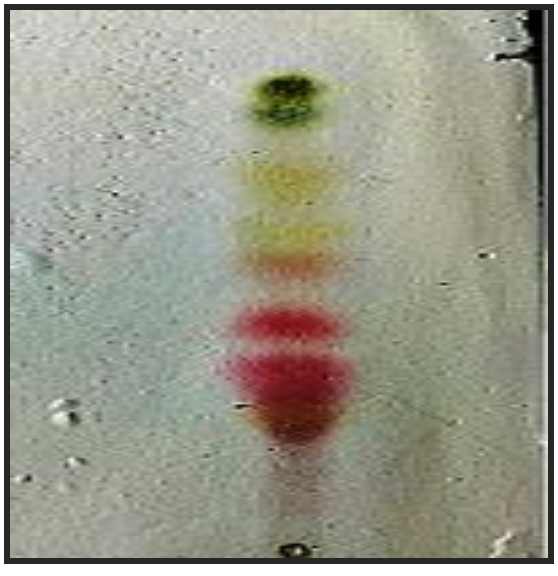

Fig. 1: TLC Plate showing spots having different Rf values (0.22, 0.31, 0.45, 0.51, 0.63, 0.75, 0.90, 0.95) of Methanolic extract of *A. indica* leaves

Antioxidant capacity, total phenolic & flavonoid contents- Total antioxidant capacity of *A. indica* leaves was determined and it has shown the dose-dependent activities. Since we observed the antioxidant activities in the neem leaves extracts we sought to analyze the total phenolic and flavonoid contents. The mean values of total phenolic content and flavonoids are 70 mg GAE/g & 119 mg QE/g respectively. (Fig. 2, Table 2).

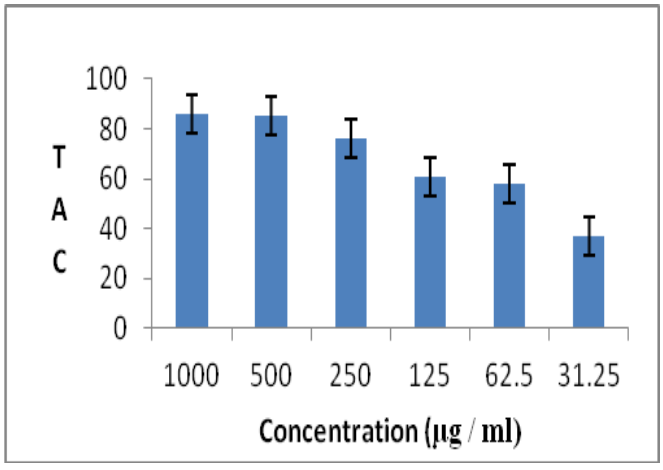

Fig. 2: Total antioxidant capacity (TAC) of *A. indica* leaves

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Table 2: Total Flavonoid & Phenolic Content of methanolic extract of *A. indica* leaves

Conc. of extract (µg/ml)	Total Flavonoid Content (TFC mg QE/g)	Conc. of extract (µg/ml)	Total Phenolic Content (TPC mg GAE/g)
1000	95.48	150	86.79
500	119.76	120	76.41
250	143.52	90	73
125	117.44	60	71.35
62.5		30	40.23
Mean value	119.05		69.55

DISCUSSION

Medicinal plants and their products have been used extensively and safely for the treatment of medical problems [25]. Traditionally, medicinal plants play a vital role in developing countries for basic health needs [26]. However, herbal remedies have been used in developed countries since ancient times [27]. Because of their medicinal importance, plants and their products continue to be a rich source of therapeutic agents. The sources of many of the new drugs available in the world and active ingredients of medicines are derived from natural products. Those active ingredients play a vital role in the treatment of diseases. The drug industry has used medicinal plants for manufacturing new drugs for the treatment of different diseases and illness. Phytochemical and biological studies have already been performed on a large number of plants by scientists all over the world. Therefore, our interest was to carry out a phytochemical screening of neem leaves and bark that are available in Jhansi. Since the District Jhansi is located in the Bundelkhand region, where less rainfall is reported and climate as well as soil is different from other parts of the country. The bark and neem leaves were collected from Bundelkhand University, Campus and aqueous as well as methanolic extraction were carried out. In the preliminary phytochemical screening of two different extracts shows most of the secondary metabolites. Results revealed the presence of various phytochemicals and these phytochemicals independently or in combination may be responsible for the medicinal properties. Most of the plant-derived drugs in the world are of alkaloid-containing. Alkaloids are organic nitrogenous substances (alkaline in nature) having remarkable physiologic and pharmacologic properties like stimulant, spasmolytic, vasodilator, anti-asthmatic, anti-arrhythmic etc. It is established that anthraquinone and related glycosides exert their action by increasing the tone of the smooth muscles. As *Azadirachta indica* showed positive results for both tannins and glycoside, it is quite obvious that it has pronounced astringent and antimicrobial properties. Moreover, *A. indica* also showed positive results for saponins. Saponins show anti-fungal, antibacterial and anti-protozoal effects. Therefore, the very high medicinal potential of neem may be due to the presence of these metabolites. Further, *A. indica* also contains flavonoids and phenolic compounds which have been reported to be associated with anti-oxidative action which provides protection against free radicals that damage cells and tissues. The phytochemicals like alkaloids, glycosides, flavonoids and saponins are antibiotic of plants and act as the defensive mechanism of plants against different pathogens. Due to such activities of metabolites, these are beneficial for further medicinal use. Thin layer chromatography was also performed of the methanolic extracts of neem leaves for the determination of bioactive components present and the Rf values were determined.

The different parts of the neem plant are considered as a valuable source of unique natural products for the development of medicines against various diseases [28,29]. Phytochemical extracts from neem plant are potential sources of antiviral, antitumor and antimicrobial agents [30]. Many researchers have evaluated antibacterial,

antisecretory, antihemorrhagic, insecticidal activity of *A. indica* based drugs to meet the health care needs [31,32]. Various pharmacological activities and medicinal applications of different parts of neem are well known [33,34]. Biological activity of the crude extracts and their different part such as leaf, bark, root, seed, etc have been used as traditional medicine for the treatment of various diseases ranging from the teeth decay, ulcers, swollen liver, malaria, dysentery, diarrhea, skin infections, blood morbidity, biliary afflictions, itching, skin, ulcers, burning sensations and pthysis control leprosy, intestinal helminthiasis, respiratory disorders, constipation and also as a general health promoter, etc [35-39]. The antioxidant activity of neem seed extract has been demonstrated *in vivo* condition during horse grain germination, which is associated with low levels of lipooxygenase activity and lipid peroxides [40]. An antioxidant principle has also been isolated, which is a potent inhibitor of plant lipooxygenases.

Phenolic acids and flavonoid compounds have been reported to be the main phytochemical responsible for the antioxidant capacity of fruits and the antioxidant capacities of fruits and vegetables are due to primarily non-vitamin-C phytochemicals [41]. Flavonoids are major compounds occurring ubiquitously in dietary plants as glycosides and contain several phenolic hydroxyl groups on their ring structures. Many flavonoids are found to be strong antioxidants capable of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups [42]. It is interesting to observe the correlation between the phenolic content and the antioxidant activity in plant extracts since phenolic compounds contribute directly to antioxidant activity [43]. The phenol content of a plant depends on a number of intrinsic (genetic, extracting solvent) and extrinsic (environmental, handling and development stage) factors [44]. We observed the good amount of total phenolic compounds and flavonoids in the neem leaves extracts. In this study, the antioxidant activity observed is may be due to the phenolic acids and flavonoid compounds.

CONCLUSIONS

Numerous phytochemical and pharmacological studies have been conducted on different parts of *Azadirachta indica.* The present study conducted at Bundelkhand University, Jhansi supports the potential of *Azadirachta indica* as a medicinal plant. The phytochemicals present in neem leaves and bark are alkaloids, reducing sugar, flavonoids, glycosides, tannins, phenolic compounds, saponins since this plant had been used in the treatment of different ailments such as malaria, cancer, skin burn, various infections, etc. The plant extracts show the variability in presence of the phytochemicals as the different solvent is used but when the concentration has changed no effect was observed. Methanolic solvent extraction shows the presence of more phytochemicals as compare to the aqueous solvent extraction. We determined the total phenolic and flavonoids content in the leaves extract. Phenolic compounds in general and flavonoids, in particular have the ability to provide protection against oxidative stress.

Thus in the present study, the presence of flavonoids and phenolic compounds in the neem leaves extract could be considered responsible for conferring antioxidant ability.

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CONTRIBUTION OF AUTHORS

This study was designed and the manuscript is written by Dr. Ramesh Kumar. The experimental work was carried out by Ms. Smrati Sharma and Ms. Laxmi Devi.

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