RESEARCH ARTICLE

Total Phenolics and *In-vitro* Antioxidant Activities in Methanol Extracts of Raw, Ripe and Overripe Neem (*Azadirachta indica* A. Juss) Seeds

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Abstract

The harvesting of fruits at different developmental stages until maturity may influence their antioxidant activities. Therefore, the present study aimed to investigate the changes in total phenolics, flavonoid contents, and *in-vitro* antioxidant activities in methanol seed extracts of *Azadirachta indica* A. Juss at different maturity stages (raw, ripe and overripe). All the tested biochemical parameters varied significantly with maturity stages ($p < 0.05\%$). Total phenolics [mg gallic acid equivalent (GAE)/g dw] and flavonoids contents [mg quercetin equivalent (QE)/g dw] were highest in ripe (33.7 and 0.28, respectively) followed by raw (19.4 and 0.16, respectively) and least in overripe seeds (12.9 and 0.15, respectively). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2-Azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) activities in methanol seed extracts [µM ascorbic acid equivalent (AAE)/g dw] varied between 83.6–106.5, 68–93.8 and 44.8–177.9, respectively. IC50 values (mg/mL) for overripe, raw and ripe seed methanol extract were 0.60, 1.11, and 2.10, respectively. The study concludes that seeds of A. *indica* were rich in natural phenolics and also possess significant antioxidant activities. Thus the present study suggested that fruit industries should harvest ripe fruit seeds of A. *indica* to meet out the need of natural phenolics for health benefits of local people.

Keywords: IC50, *In-vitro* assays, Maturity stages, Natural antioxidants, Seed extracts.

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Introduction

Under various conditions such as temperature, salinity, heavy metals, UV, ozone, water loss, pest assault, etc., phenolic compounds, the most prevalent category of secondary metabolites discovered in plants, are in charge of many crucial biological processes (Bartwal *et al*., 2013; Kumar and Sharma, 2018). The most well-known and frequently discussed function of phenolic compounds is their antioxidant properties and ability to stabilize blood vessel capillary walls, which are especially crucial in the prevention of cancer and cardiovascular disorders (Khalid *et al*., 2016). The incidence of cancer and heart disease was found to be inversely correlated with the consumption of polyphenolic substances in epidemiological research (Taguchi *et al*., 2020). Aside from their role in plant defense, phenols have been extensively researched and found to have a variety of bioactivities that may benefit human health. They have been associated with a reduction in the risk of cancer, heart disease, diabetes, inhibition of plasma platelet aggregation, cyclooxygenase activity, histamine release, and antibacterial, antiviral, anti-inflammatory, and antiallergenic properties. (Yao *et al*., 2004; Oak *et al*., 2005). These benefits are due to the antioxidant characteristic of phenols; therefore, quantification, identification, and evaluation of their antioxidant activities in phenols-rich plant extracts are essential.

Azadirachta indica A. Juss, is a tropical and subtropical evergreen tree that is indigenous to India. It has been shown to have ethno-medicinal benefits and is significant for both agriculture and the pharmaceutical industry. This ancient medicinal tree, often known as the "wonder tree," is said to be a chemical factory producing a wide range of intricate and complicated compounds with a wide variety of structural scaffolds that are exceedingly challenging to replicate through chemical synthesis. A wonderful repertory of functional features, including a wide range of biological activity and distinctive modes of action against specialized and generalist diseases and pests, is produced by such diverse chemical diversity. More than 400 distinct chemicals, including significant bioactive secondary metabolites like azadirachtin, nimbidin, nimbin, nimbolide, gedunin, etc., have so far been identified from various neem components. (Priyadarsini *et al*., 2010; Chen *et al*., 2011, Elumalai *et al*., 2012). *A. indica* plants also possess antimicrobial, antimalarial, insecticidal, antiviral, anti-inflammatory, analgesic, hypoglycaemic, antiulcer, antipyretic, anticarcinogenic, hepatoprotective, antioxidant, antifertility, anxiolytic, molluscicidal, acaricidal, and antifilarial properties (Alam *et al*., 2012; Naz *et al*., 2022; Baby *et al*., 2022).

It is well established that differences in genotype in addition

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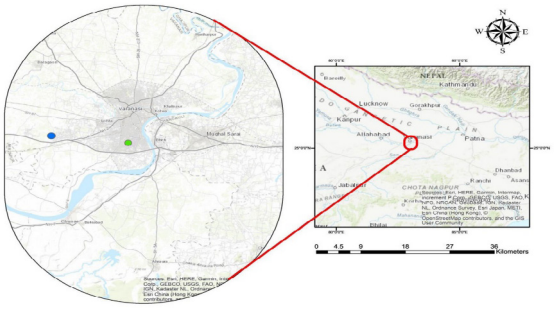

Fig. 1: Map showing location of sampling sites in rural and suburban areas of Varanasi

to environmental factors can cause differences in phenotype among organisms, leading to inter-specific variation. In general, higher genetic variation can be seen in populations of species that range over a large geographic area. Spatial variation and level of environmental pollution have a significant impact on the architecture of plants, the flowering and fruiting of plants, the phytochemical composition and antioxidant properties of plants, and in-situ competition with other species (Sharma *et al.*, 2012; Kumar *et al.*, 2017)

Although numerous studies have summarised the therapeutic value of various elements of A. *indica* plants. The present study focused on the effects of maturity stages and growing areas on total phenolics and flavonoid contents and the antioxidant potential of A. *indica* seeds collected from rural and suburban areas of Varanasi, Gangetic plains of Northern India.

Materials And Methods

Sampling and Processing

Fruits of A. *indica* at three ripening stages i.e. raw, ripe and overripe were collected from two sites namely; Mehndiganj (25.2602° N, 82.8466° E, 82.84 m) and Sushwahi (25.2677° N, 82.9913° E, 80.71 m), located in rural and suburban areas of Varanasi, India (Fig. 1). These sites differ in terms of pollution levels and population density. The fruits were brought into the laboratory and peeled out for seeds. Seeds were air-dried and were powdered using a stainless steel blender and the powder was passed through a 2 mm mesh-sized sieve and stored at room temperature for further analysis.

Chemicals

All the chemicals such as 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-Azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,4,6-tripyridyl-S-triazine (TPTZ), Folin Ciocalteu Phenol Reagent, gallic acid, quercetin, ascorbic acid, sodium carbonate, methanol, aluminium chloride, etc., used in the current study were purchased from Merck, Pvt. Ltd, India. All the chemicals used in the analysis were of analytical grade.

Preparation of Methanol Extract

Dry powder of seeds (10 g), was soaked in 100 mL of 80% aqueous methanol (v/v) and were kept in an orbital shaking incubator at 25°C for 72 hours at 100 rpm. The mixture was then filtered through Whatman No. 1 filter paper and the filtrates were centrifuged at 3354 g for 10 minutes. The supernatants were collected in a conical flask, and the pellets were again extracted as per the procedure described above. The extraction procedure was repeated twice using 50 mL and 25 mL of methanol, and all supernatants were combined. The supernatant (175 mL) were evaporated to 10 mL at a constant temperature using a rotary evaporator (Rotary Vacuum Evaporator, Shivam, India). The concentrated extracts were stored at 4°C in a refrigerator till further analysis.

Determination of Total Phenolics and Flavonoids Content

Total phenolics and flavonoids contents in the methanol extracts of raw, ripe, and overripe seeds of A. *indica* was determined using the methodology of Wolfe *et al.* (2003) and Ordonez *et al.* (2006), respectively. A reaction mixture was prepared by using double distilled water (0.5 mL), seed methanol extract (125 µL) and folin ciocalteu phenol reagent (150 µL). After an incubation of 6 minutes at room temperature, 1.25 mL of an aqueous sodium carbonate solution (w/v) was added to the reaction mixture, Final volume of the reaction mixture was made 3 mL using double distilled water. The resulting mixture was then left to stand at room temperature for 90 minutes until the blue colour appeared properly and the absorbance was taken using a spectrophotometer (Systronics, India) at 760 nm. A standard curve was prepared with gallic acid (0–0.05 mg/mL), and the data was expressed in mg gallic acid equivalent (GAE)/g dw. For the quantification of total flavonoids, 1-mL of methanol extract was mixed with 1-mL of 2% (w/v) $AlCl_3$ and shaken thoroughly. The sample mixture was allowed to stand for 60 minutes at room temperature. The absorbance of the golden yellow colour was measured at 420 nm using a spectrophotometer (Model no.166, Systronics, India). Different concentrations of quercetin ranging between 0–0.05 mg/mL were used for the preparation of the standard curve. Total flavonoids content in methanol extracts was expressed as mg quercetin equivalent (QE)/g. A standard curve was formulated with quercetin (0–0.05 mg/mL) and data was expressed as mg QE/g dw.

In-vitro Antioxidant Assays

The *in-vitro* antioxidant activities in the methanol extracts of raw, ripe and overripe seeds of A. *indica* plants were measured using *in-vitro* models, namely 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-Azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and ferric radical antioxidant power (FRAP) assays as described by Liyana-Pathirama and Shahidi (2005), Re *et al.* (1999) and Benzie and Strain (1996), respectively. DPPH radical assay was performed by mixing methanol extract with 0.135 mM DPPH. The reaction mixture was then kept in the dark at room temperature for 30 minutes and absorbance was recorded at 517 nm using an UV-vis spectrophotometer (Systronics, India). A stock solution of 7 mM ABTS and 2.4 mM potassium persulphate were prepared in methanol to measure ABTS cation scavenging activity in the methanol extracts. The working solution i.e. production of ABTS radicals was prepared using an equal volume of 7 mM ABTS and 2.45 mM potassium persulphate. The methanol extract was mixed ABTS (0.706 ± 0.001 at 734 nm) and shaken properly. The absorbance of the reaction mixture was recorded at 734 nm using a spectrophotometer (Systronics, India). For FRAP assay, a

Table 1: Effects of variables such sites, maturity stage and their interaction on tested biochemical attributes methanol extracts of A. indica seeds (results of 2-Way ANOVA test).

Parameters	*Variables*		
	Site	*Maturity stage*	*Site × Maturity stage*
TPC	29.11***	325.05***	4.82**
TFC	13.54**	45.76***	4.08*
DPPH	2.32NS	41.28***	2.04 NS
ABTS	1.12 NS	108.16***	4.85**
FRAP	2.76 NS	815.28***	12.53***

Level of significance * $p < 0.05$; ** $0.05 < p > 0.01$; *** $p < 0.001$; NS-not significant

fresh working solution (a 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3.6H_2O$ mixture) was prepared from a stock solution [300 mM acetate buffer (pH 3.6) and 10mM TPTZ in 40mM HCl and 20mM $FeCl_3.6H_2O$] and the temperature was raised to 37°C. A reaction mixture was prepared using methanol extract and FRAP working solution and incubated in the dark for 30 minutes. The reaction mixture's absorbance was measured at 593 nm using an UV-vis spectrophotometer (Systronics, India).

The inhibition of DPPH (%), ABTS (%) radicals and FRAP activities (µM Fe (II)/g) in methanol extracts of seeds of A. *indica* were converted to mM ascorbic acid equivalent (AAE) /g dw using an equation, derived from the relationships of ascorbic acid (mM) with DPPH ($y = 1.672x$; $R^2 = 0.96$), ABTS ($y = 0.977x$; $R^2 = 0.99$) and FRAP ($y = 1.776x$; $R^2 = 0.96$), respectively.

Estimation of Inhibition Concentration (IC_{50}) Values

An IC_{50} value is the concentration of methanol extract required to inhibit 50% of DPPH radical activity. The IC_{50} value for seed extract was calculated using a simple line equation derived from the relationship between the increasing concentrations of kernel extracts and their DPPH inhibition potential and expressed in mg/mL. The obtained IC_{50} values for each extract were compared with that of synthetic antioxidants such as benzene hexa toluene (BHT) and ascorbic acid (AA).

Statistical Analysis

The results were presented as a mean ± standard error of three independent analyses. Duncan's multiple range tests revealed significant differences between the treatment means at $p \leq 0.05$. 2-way ANOVA test was also performed to assess the effects of variables such as sites and maturity stages on tested biochemical parameters. All the statistical analyses were carried using statistical software (SPSS, Version 16).

Results And Discussion

Total Phenolics and Flavonoids Content

Polyphenols are important plant-derived secondary metabolites, including hydrolysed tannin (acid ester polyphenols) and condensed tannin (flavanols polyphenols or proanthocyanins). Several studies have shown that the different parts i.e. bark, leaves, fruits, etc. of A. *indica* plants are rich in polyphenols and antioxidants which are responsible for many health benefits (Kharwar *et al.*, 2020; Rashmi and Negi 2020; Khan *et al.*, 2022).

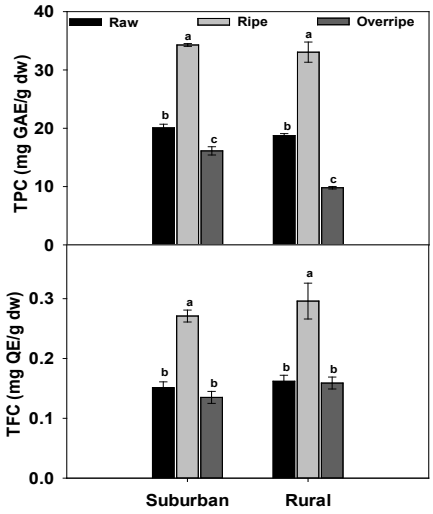

Fig. 2: Total phenolics (TPC) and flavonoids (TFC) contents in methanol extracts of A. *indica* seeds at different maturity stages collected from rural and suburban areas of Varanasi. Bars are mean ± SE of three replicates. Bars adhered with different alphabets in group are statistically significant at $p \leq 0.05$ (Duncan's Multiple Range Test)

Data pertaining to total phenolics and flavonoids contents in methanol extracts of raw, ripe and overripe seeds of A. *indica* plants are shown in Fig. 2. The results showed that total phenolics concentration varied from a minimum of 9.81mg GAE/g dw in overripe seeds of rural area to a maximum of 34.29 mg GAE/g dw in ripe seeds of suburban area. Whereas, total flavonoids contents in methanol extracts varied from 0.151 mg QE/g dw in overripe seeds of suburban areas to 0.30 mg QE/g dw in ripe seeds of rural areas (Fig. 2). In the present study, total phenolics were found significantly higher in ripe seeds as comparison with raw and overripe at both rural and suburban areas. The results showed that total flavonoid content in ripe seeds was also slightly higher than raw and overripe seeds in suburban and rural areas, respectively (Fig. 2).

Variations in phenolics and flavonoids content greatly depend on the extraction processes, various abiotic and biotic stresses, maturity stages, processing of samples, spatial factors, etc., (Kumar and Sharma 2021; Jiang *et al.*, 2021; Derakhshan *et al.*, 2018; Sharma *et al.*, 2017). In the present study, it has been investigated that both total phenolics and total flavonoids were found to be lower in raw and overripe than the ripe seeds. This may be due to the late synthesis of some particular phytochemicals and their degradation/bioconversion in overripe fruits which affects total of phenolics and flavonoid contents. Sharma *et al.* (2017) studied the variations of phenolics and flavonoid contents in methanol extracts of Olea ferruginea Royal seed where they reported higher contents of phenolics and flavonoids in ripe seeds over the raw seeds. Rebey *et al.* (2019) analyzed total phenolics and flavonoid contents in Pimpinella anisum L. plants and found their higher concentration at the intermediate maturity stage. Moulehi *et al.* (2012) reported higher phenolic contents in immature fruits (41.39 mg GAE/g) and total flavonoid contents (36.21 mg CE/g) in semi ripen fruits. Results of 2-way ANOVA test also showed significant effects of

Table 2: *In-vitro* antioxidant activities in methanol extracts of A. *indica* seeds at different maturity stages

In vitro assays (μM AAE/g dw)	*Sampling locations*	*Methanol Seed Extracts*		
		Raw	Ripe	Overripe
DPPH assay	Suburban	92.56±2.63b	106.46±0.29a	83.61±0.61c
	Rural	91.84±0.31a	99.02±3.38a	83.97±1.20b
ABTS assay	Suburban	79.37±0.12b	93.19±1.00a	74.74±3.37b
	Rural	81.45±0.12b	93.75±0.00a	67.98±0.48c
FRAP assay	Suburban	50.28±0.49c	173.95±0.80a	73.72±3.98b
	Rural	58.70±2.55b	151.25±0.22a	44.77±6.32c

Values are mean ± SE of three replicate analyses. Values adhered with different alphabets in each row are statistically different at $p \leq 0.05$ (Duncan's Multiple Range Test)

site, maturity stages and their interaction on both total phenolics and flavonoids in A. *indica* seeds (Table 1)

In-vitro Antioxidant Activity

Organic acids, aromatic rings with linked hydroxyl groups, and acetylated sugars make up polyphenols. Polyphenols, distinguished by their unique structures, possess a high level of antioxidant activity and can thwart the production of free radicals in either a direct or indirect manner. (Borges *et al.*, 2010). The flavan-3-ols, flavonols, phenolic acids, anthocyanins, and hydroquinones are the most prevalent polyphenols in plants (Brahem *et al.*, 2017). There are various chemical assays that can be used to measure the antioxidant efficacy of polyphenols in plants, and each assay relies on a distinct mechanism. These *in-vitro* spectrophotometry-based tests comprise tests for ferric reducing antioxidant capacity, 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), and 2, 2-diphenyl-1-picrylhydrazyl radical scavenging power (Amorati *et al.*, 2015).

The results of antioxidant activities in the methanol extracts

Table 3: IC_{50} of methanol extracts of A. *indica* seeds at different maturity stages

Methanol extracts/ Standards		*IC_{50} (mg/mL)*	
		Suburban	*Rural*
Raw		1.45 ± 0.60b	1.24 ± 0.61b
Ripe		0.83 ± 0.02c	0.65 ± 0.05c
Overripe		2.05 ± 0.11a	2.23 ± 0.17a
AA	0.027		
BHT	0.060		

Values are mean ± SE of three replicate analyses. Values adhered with different alphabets in each row are statistically different at $p \leq 0.05$ (Duncan's Multiple Range Test)

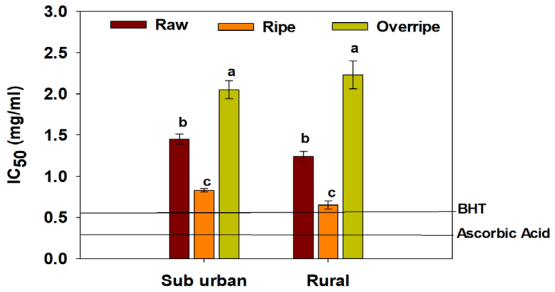

Fig. 3: IC50 value of synthetic antioxidants and methanol extracts of A. *indica* seeds at different maturity stages, collected from rural and suburban areas of Varanasi. Values of synthetic antioxidant i.e. benzene hexa-toluene (BHT) and ascorbic acid are 10 times higher than the original value. Bars are mean ± SE of three replicates. Bars adhered with different alphabets in group are statistically significant at $p \leq 0.05$ (Duncan's Multiple Range Test).

of A. *indica* seeds are given in Table 2. The results showed the highest antioxidant activities in methanol extracts obtained from ripe seeds followed by raw and the least in overripe seeds of both suburban and rural areas. The DPPH radical scavenging activity in tested seeds ranged between 83.6 μM AAE/g dw (overripe) to 106.5 μM AAE/g dw (ripe) in suburban area and ABTS activity from 68 μM AAE/g dw (overripe) to 93.8 μM AAE/g dw (ripe) in rural areas (Table 2). FRAP assay showed a minimum activity in raw seeds (50.3 μM AAE/g dw) from suburban area to a maximum in ripe seeds (174 μM AAE/g dw) in suburban area. (Table 2). Sharma *et al.* (2013) reported that DPPH and FRAP activities in methanol extracts of ripe fruits were higher as compared to raw fruits, and higher FRAP activity in raw fruits in comparison to O. ferruginea plants. Further, Sharma *et al.* (2017) also reported that DPPH and FRAP activities were higher in ripe seeds of O. ferruginea than raw seeds. On the other hand, ABTS activity was higher in raw seeds as compared to overripe seeds of O. ferruginea. Duda-Chodak *et al.* (2011) studied the changes in the antioxidant activities of apple fruits during storage and maturity and reported maximum ABTS activity at ripe stage and cold storage. Moghaddam *et al.* (2015) showed a positive correlation of the antioxidant efficacy of the essential oils with phenolic contents, which increased at intermediate and premature stages in cumin (*Cuminum cyminum* L.) plants.

The synthesis or breakdown of specific phytochemicals during the particular development stage due to certain biotic and abiotic stimuli may cause a variance in the antioxidant capability of the examined seeds. Because of delays in process of phytochemical production at various stages of maturation, rawer seeds may exhibit reduced antioxidant capacity. The antioxidant potential of the examined A. *indica* seeds may also be influenced by extraction procedure, plants under various stressors, types of extraction solvents, etc. Additionally, both polyphenol concentrations are positively associated with the antioxidant potential of plants (Kumar and Sharma, 2021).

IC_{50} Values of Seed Extracts

The results further showed that IC_{50}, a minimum concentration of extract required to scavenge 50% of free radicals, was lower in ripe A. *indica* seeds. In the tested seeds the IC_{50} values was found lowest for the ripe seeds (0.64 mg/mL) of rural areas and

highest in overripe seeds (2.31 mg/mL) of suburban area (Fig. 3 and Table 3). Higher IC_{50} value *indica*ted the lower antioxidant potential of the tested seeds and vice-versa. Moulehi *et al.* (2012) reported the lowest antioxidant capacity in IC_{50} by beta carotene bleaching assay in semi ripen citrus fruits.

Conclusion

A. indica (neem), a tropical and subtropical evergreen tree native to the Indian subcontinent was selected for the present study due to their industrial and ethno-medical uses. The present study aimed to assess the impacts of maturity stages of seeds (raw, ripe and overripe) on total phenolics and total flavonoids content in seed methanol extracts of A. *indica* and their antioxidant activities. The present study showed that A. *indica* seeds are rich in polyphenolics and possess immense antioxidant properties. Further, it has been summarized that the maturity stages of the seeds play a major role in the synthesis of natural phenolics and affect the antioxidant properties. The present study concluded that ripe seeds have higher phenolics content and antioxidant potential than raw and overripe seeds. Thus utilization of ripe seeds of A. *indica* plants can be promoted to fulfil the industrial needs of natural antioxidants and for the health benefits of local people.

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