

Effects of temperature and storage on the antioxidant potential, polyphenols and Vitamin-C contents of *azadirachta indica* leave aqueous extract

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Abstract: The aim of the study was to investigate the effects of effects of storage and temperature on the antioxidant potential, vitamin-C contents, total as well as selected individual phenolic acids and flavonoids of fresh aqueous leaves extract of *Azadirachta Indica*. The antioxidant activity of *Azadirachta Indica* leaves aqueous extract was determined by scavenging of DPPH free radical, while the phenolic compounds and vitamin-C contents by HPLC method. The analyses were carried out on crude extract of fresh leaves and after storage time of 1, 2 and 3 month at temperature of 20, 30 and 50°C. Storage for longer duration and rise in temperature caused decreasing the phenolic acids and vitamin C contents as well as antioxidant potential. Vitamin C contents were decreased up to 91% upon storage for 3 months at 50°C, while the anti-oxidant potential was decreased 29 %. The effect of storage time and temperature on individual phenolic acid and flavonoids were also remarkable, except ferulic acid which increased upon storage and rise in temperature.

Keywords: Storage, Antioxidant potential, Vitamin-C, HPLC, Phenolic Compounds

INTRODUCTION

Azadirachta indica locally known as Neem of Meliaceae family, it is native to Pakistan, India, Bangladesh, Thailand and Nepal and growing will in tropical and sub tropical regions (Ghimera et al., 2009). It has been declared worldwide as the “tree of the 21st century” by united nation (Negi et al., 2002). It has been extensively used to treat various infections such as gastro intestinal problems, urinary tracks problems, hair problems, oral problems, ulcer, blood pressure and diabetes (Janovska et al., 2003; Morales et al., 2008).

The investigation and finding of new valuables drugs from various plants attracted more researchers due to their potent pharmacological actives. It is estimated by the world health organization that 80% of the population of developing country relies exclusively on these traditional medicinal plant for curing their primary health (Hossain et al., 2013). The finding of natural antioxidants from plant source replaced the synthetic antioxidant because of green medicines are accepted and as to be very safe, these are not associated with side effect. This situation forced the researcher to search for new antioxidants from various medicinal plants (Chanwitheesuk et al., 2005). Some of the active phenolic compounds were isolated and identified in crude extract of *Azadirachta Indica* and currently is used as a natural antioxidant and antimicrobial agents in the formulation of different medicines. These phenolic compounds are determined by different techniques, the total phenolic compounds are

determined by spectrophotometric method, while the characterization and quantification were done by the different chromatographic techniques such as TLC, HPLC, UHPLC, GC-MS etc (Belajova and Suhaj, 2004; Ma et al., 2008; Ye et al., 2012).

Fresh young leaves of *Azadirachta Indica* are soaked in water for few hours and the extracts are use traditionally to cure oxidative disorder, like inflammation and skin diseases, arthritic, rheumatic disorders, treatments of diabetics, diarrhea, amoebic dysentery and fever. These extracts are also used as a bitter tonic to treat malaria fever and stimulate the gastric secretion. These extracts are reported having antimicrobial, anti inflammatory, antiulcer, antioxidant, antipyretic, anti parasitic activities (Aliakbarian et al., 2012; Deba et al., 2008; Kumar and Navaratnam, 2013; Sokmen et al., 2004). The aim of the study was to investigate antioxidant potential, vitamin-C contents, total as well as selected individual phenolic acids and flavonoids of fresh aqueous leaves extract of *Azadirachta Indica* and the effects of storage and temperature on the stability of these compounds. The previous literature reveals that still no work has been done on the fresh juice extract of *Azadirachta Indica* leaves.

MATERIALS AND METHODS

Chemicals and reagents

Standards of phenolic acids (Gallic, vanillic, syringic, caffeic, ferulic), flavonoids (quercetin, luteoline, rutin, hesperidins) were purchases from Sigma (Aldrich Germany), o-phosphoric acid, methanol, Acetonitrile were HPLC grade from Scharlau Spain, while Citric acid

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(Merck Germany). All reagents and de-ionized water were filtered through 0.45µm polyamide membranes and degassed for 20 minutes using ultra sonic cleaner Ceia (model CP-104 Italy) and filtration assembly (model Rocker-300).

Preparation of leaves extract

The *Azadirachta Indica* leaves were collected from the Botanical garden of Islamia College Peshawar, Pakistan washed with plenty of water. 500g of leaves were chopped and extracted with 1 litter water at 30°C for 24h at orbital shaker. The flasks were properly coated by aluminum foil. The mixture was filtered and concentrated to half of its volume by rotary evaporator at 35°C and were stored at 10, 30, and 50°C for duration of 1, 2 and 3 month.

Determination of Antioxidant activity

The Antioxidant potential of the crude aqueous extract of *Azadirachta Indica* leaves was determined using DPPH free radical scavenging method reported by (Sokmen, *et al.*, 2004). 1ml of extract was diluted with 9 ml methanol, centrifuged at 6000 rpm and the supernatant was decanted. 1ml of supernatant was added with 2 ml of DPPH solution (0.004% w/v prepared in 95% Methanol) and incubated for 30 min. The absorbance was measured at 517nm using UV-VIS spectrophotometer. The % radical scavenging activity was determined using following equation.

$$\% \text{ Scavenging Activity} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of the control}} \times 100$$

Determination of total phenolic content (TPC)

The TPC in aqueous *Azadirachta Indica* leave extract was determined (Lachman *et al.*, 2010). 2ml of Folin-Ciocalteu reagent was added to the extract and gallic acid standards. Five concentration of gallic acid (100, 200, 300, 400, and 500µg/ml) were prepared for calibration curve. After 10min sodium carbonate saturated solution was added to each and allow for 30 minutes at room temperature. After incubation the mixture was measured at 760nm against blank on a UV spectrophotometer, and the calibration curve of absorbance vs concentration was plotted. The total phenolic content was calculated from calibration curve of gallic acid: $Y=0.143x + 0.221$, R_2 and expressed as gallic acid equivalent (mg/l).

Determination of total flavonoids contents (TFC)

The TFC of water extract of *Azadirachta Indica* was determined by (Kumar and Navaratnam, 2013) method. For the calibration curve five different concentration solutions of catechin (50, 100, 150, 200 and 250µg/ml) were prepared 1:2 with water from standard solution (500 mg/100ml methanol). Standard solutions (0.5ml) and sample was mixed with 2ml of deioized water and 150µl of 5% sodium nitrate. After 5 min at room temperature 150µl of 10% aluminium chloride was added and allowed for 6 min. Then added 1 ml of 1M sodium hydroxide and

adjust the total volume to 5ml. The absorbance was read at 510nm. The TFC were determined from the calibration curve of catechins: $Y=0.062x + 0.059$, R_2 and expressed as catechin equivalents (mg/l)

HPLC determination of vitamin C

The extraction and determination of vitamin-C was carried out by (Almeida *et al.*, 2011) method. 5 ml of extract was mixed with 5 ml of mobile phase Methanol and buffer (potassium dihydrogen orthophosphate - meta phosphoric acid). The quantification was done by Hitachi-2000 HPLC using Intersil ODS-3 C18 column and the detection wavelength was set at 240 nm.

HPLC determination of phenolic acids and flavonoids

Determination of polyphenolic constituents in *Azadirachta Indica* extract was carried out by (Proestos *et al.*, 2006) method. 5 ml of extract were further extracted twice with 5ml of diethyl ether, and centrifuged at room temperature at 5,000 rpm for 10min. the supernatant was separated and evaporated to dryness with nitrogen gas stream. The residue was dissolved in water-methanol (1:1) and 20µl aliquots injected to HPLC (Hitachi D-2000) equipped with dual pumps (L-2130), auto injector (L-2200) with UV-VIS detector (L-2420). The chromatographic separations of polyphenol were carried out by analytical column ODS-3 C18 (5µm, 250×4.6mm GL Science Inc. Tokyo Japan). For the separation of phenolic acid and flavonoids in extract the mobile phase Water: Acidic acid: methanol (88:2:10) as solvent A and Water: acidic acid: Methanol (8:2:90) as solvent B in a gradient elution program was run at 0 min, 100% A-0% B; 15 min 85% A-15%; 25min 50% A-50% B and 34 min 30% A-70% B with a flow rate of 1.0ml/min. The phenolic acids and flavonoids were identified by comparing with the retention time of UV spectra of spiked samples of appropriate standard. Quantification of phenolic acids was carried out at 280 nm using external standard method.

STATISTICAL ANALYSIS

Statistical analyses of presented data were performed using Statistica 6.1 and the difference between fresh and stored extracts were evaluated by the Duncan test at 5 % level of significance.

RESULT

Determination of TPC, TFC, vitamin-C contents and antioxidant activity

The TPC, TFC and vitamin-C found in aqueous extract of fresh *Azadirachta Indica* leave were determined by spectrophotometric method and are presented in (table 1). The % scavenging activity of extract determined by DPPH method was 69%, while (Almeida, *et al.*, 2011; Chanwitheesuk, *et al.*, 2005) reported 71% in dry leave

Table 1: Changes in antioxidant activity, TPC, TFC, and vitamin-C contents in aqueous extracts of *Azadirachta Indica* leaves during storage of 3 months at 20, 30 and 50°C

Storage (month)	% Scavenging			Content mg/L								
	(DPPH)			Vitamin-C			TPC			TFC		
	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C
0		69.3a			121a			486a			281a	
1	68.3a	63.4a	61.7b	101a	96.8b	49.3c	461a	434a	381b	264a	259a	231b
2	64.0a	60.7a	57.6b	87.4b	64.3b	33.9d	401a	392a	323c	253b	247b	214c
3	59.3b	56.0b	50.4c	51.8b	23.5d	09.7d	381b	361c	279d	230b	201c	183d

a-d: Means with different letters are significantly different according to Duncan's multiple rang test at $P \leq 0.05$. 3 determination were done for DPPH scavenging, Vitamin-C, TPC and TFC

Table 2: Changes in contents of phenolic acids in *Azadirachta Indica* leaves extracts during 3 month storage at 20, 30 and 50°C

Storage (month)	Phenolic acids mg/L											
	Gallic			Ferulic			syringic			Caffeic		
	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C
0		7.89a			2.71a			2.42a			5.13a	
1	7.83a	7.53a	7.11a	2.55a	3.42b	3.04a	2.42a	2.15a	1.82b	5.03a	4.97a	4.78
2	7.11a	6.46b	6.25b	3.0a	3.36b	3.96b	2.16a	1.63b	1.30c	4.94a	4.54b	4.36c
3	6.36b	5.93c	5.22c	3.98c	4.12d	4.34d	1.72b	1.11c	0.6d	4.76b	4.39c	3.72d

a-d: Means with different letters are significantly different according to Duncan's multiple rang test at $P \leq 0.05$. 3 determination were done for Gallic, Ferulic, Syringic and Caffeic

Table 3: Changes in content of flavonoids in aqueous extract of *Azadirachta Indica* leave extract during 3 month storage at 20, 30 and 50°C.

Storage (month)	Flavonoids mg/L											
	Quercetin			Luteolin			Rutin			Hesperitin		
	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C
0		60.9a			51.6a			09.9a			76.6a	
1	57.3a	56.5a	52.3b	51.6a	50.7a	43.1b	09.1a	09.3a	09.6a	76.4a	73.9a	73.6a
2	54.4a	51.2b	49.7b	49.8a	46.9b	41.3b	09.5a	08.1a	07.5b	74.3a	70.0a	69.7b
3	48.2b	45.0c	41.8c	40.2c	40.1c	38.6c	08.6a	07.0b	07.0b	68.3b	65.7b	63.8c

a-d: Means with different letters are significantly different according to Duncan's multiple rang test at $P \leq 0.05$. 3 determination were done for Quercetin, Luteolin, Rutin and Hesperitin

crude extract. The TPC and TFC were found 486 and 281mg/L while the value of TPC and TFC reported by (Ghimeray, *et al.*, 2009) were 2431 and 1543 mg/L in crude organic extract of *Azadirachta Indica* leaves.

On the other hand (Tsimidou *et al.*, 1992) added that the yield of these compounds depend on the selection of solvent and extraction methods. 121mg/L of vitamin C was found. Although this value becomes zero in crude extract, because vitamin C is very sensitive to heat and light.

Determination of flavonoids and phenolic acid by HPLC

By the using aforementioned procedure, the phenolic acids and flavonoids content in aqueous extract of *Azadirachta Indica* leave were separated and quantified by using HPLC with UV-Vis detection.

Most the phenolic compounds show intense absorbance in UV-Vis region of the spectrum (Almeida, *et al.*, 2011;

Belajova and Suhaj, 2004). The applied (Lachman, *et al.*, 2010) method is simple, easy and effective for identification and quantification of major phenolic compounds. From the data presented (table 2) It is clear that among the phenolic acids the gallic acid was found maximum in fresh extract yield 7.89 mg/l, while ferulic, syringic and caffeic were found in the range of 7-2mg/l. The reported method (Wijngaard *et al.*, 2012) followed for the content of free phenolic acids due to simplicity.

The flavonoids content of aqueous extract of *Azadirachta Indica* leaves are shown (table 3). The Hesperitin was found in maximum yield (76.65mg/l) in fresh extract. Quercetin, luteolin and rutin were found 60.98, 51.62 and 09.98mg/l respectively. From the previous reported data the value of flavonoids and phenolic acids were found too high in dry extract (Choudhary and Swarnkar, 2011; He and Xia, 2011) due to the concentration of extract and extraction procedure. The yield of phenolic compounds

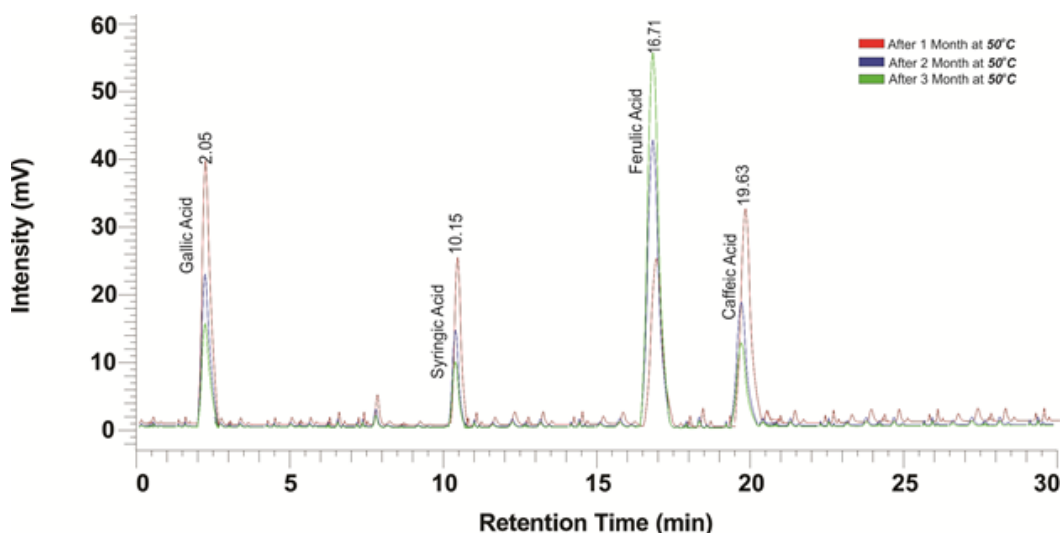


Fig. 1: Chromatogram of Phenolic Acids at 50°C at Storage of 1, 2 and 3 Months

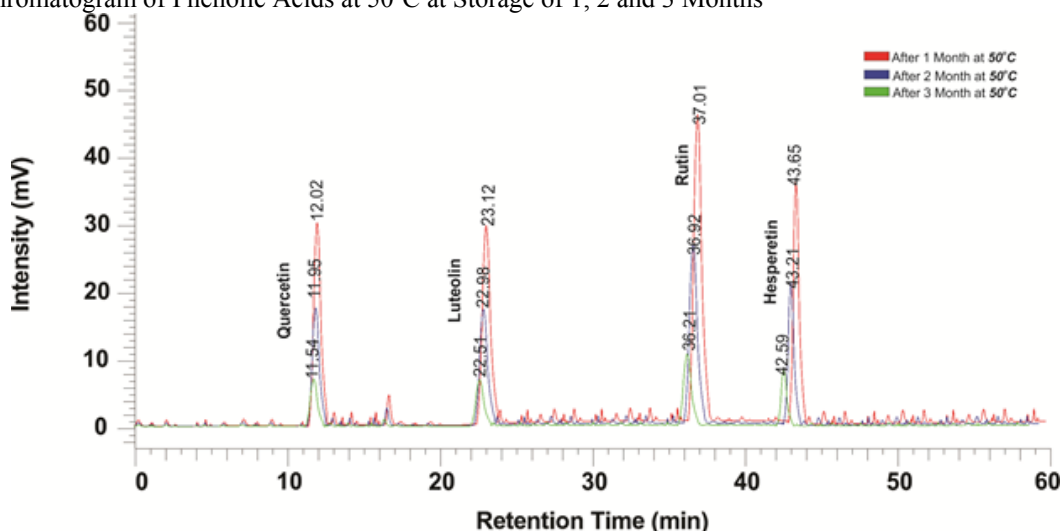


Fig. 2: Chromatogram of Flavonoids at 50°C at Storage of 1, 2 and 3 Months

depends on the extraction solvents and procedures. But the aqueous extraction and procedure applied in current research followed the traditional use of fresh extract..

DISCUSSION

Effect of storage and temperature on antioxidant Potential and Vitamin-C contents

From the data presented in table 1 showed a clear decrease in % scavenging of DPPH, 69% of free radical of DPPH scavenged by fresh aqueous extract while this scavenging decreased to 18% during storage of 3 month at 50°C. The content of Vitamin-C rapidly and strongly effect by the temperature, oxygen, and light. From previous research (Almeida, *et al.*, 2011) it also proved that Vitamin-C in different juices sample decrease very rapidly during storage. It also very sensitive to temperature, so temperature also degraded the value of vitamin-C.

Among the entire tested compounds vitamin was found to be most affected by storage and temperature. The actual content of vitamin-C determined in extract and presented in (table 1). It is clear that initial fresh juice contents was 121.2mg/l which was very clearly reduced by high temperature and storage which stored at 20°C reduce the risk of reduction after storage of 3 months. The content of vitamin-C reduces 57, 80, and 91% at 20, 30, and 50°C for storage of 3 months.

Effect of storage and temperature on phenolic acids and flavonoids

The TPC and TFC in fresh aqueous extract was 486 and 281mg/l, while these values decreased to 279 and 281 when stored at 50°C for 3 months. Because of most of the phenolic compounds are thermostable. The changes in individual phenolic acid and flavonoids were also observed and tabulated in (table 3) which showed also remarkable changes due to storage and temperature. The

maximum reduction was found in quercetin 36%, while minimum in Hesperitin 18%. The rest of two luteolin and rutin were found to be reduced 27 and 22% during storage for maximum 3 months at maximum temperature of 50°C. Influence of storage time and temperature on the content of free phenolic acid was also investigated (Kumar and Navaratnam, 2013). In all phenolic acids decreases was observed except ferulic acid which shows increase from 2.71 to 4.34mg/L during storage of 3 month at 50°C (fig. 2). It was result of release from bonded or from their ester forms which are enclosed agreement with data obtained by (Tsimidou, *et al.*, 1992). From the storage of total and individual flavonoids observed, which explain high stability of these compounds during storage and temperature.

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