IN-VITRO SCREENING OF *NYCTANTHES ARBOR-TRISTIS* FLOWERS FOR ANTIOXIDANT ACTIVITY AND IDENTIFICATION OF POLYPHENOLS BY RP-HPLC

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Summary

Free radicals are highly reactive species produced in the body during normal metabolic functions or introduced from the environment, which are capable of causing tissue injury and have been implicated in the pathology of various human diseases. Antioxidants act as a major defense against radical-mediated toxicity by protecting against the damages caused by free radicals. A number of plants and plant isolates have been reported to protect free radical-induced damage in various experimental models. *Nyctanthes arbor-tristis* flowers were widely used for the treatment of Constipation, Intestinal worms, Piles. The different solvent extracts of the dry and fresh flowers was studied for its free radical scavenging activity with different methods *viz* lipid peroxidation assay, reducing activity and hydrogen peroxide scavenging assay. Along with these various levels of enzymatic and non-enzymatic antioxidants were also estimated. Further, polyphenols were also identified by RP-HPLC. The results showed that methanol extracts of dry flowers exhibit high phenolic content and antioxidant activities, interestingly aqueous extracts showed high enzymatic antioxidants.

Key words: RP-HPLC, Free radical, hydroxyl radical, lipid peroxidation, nitric oxide, enzymatic and non-enzymatic antioxidants, polyphenols.

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Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are various forms of activated oxygen and nitrogen, which include free radicals such as superoxide ions (O_2^-), hydroxyl (OH) and nitric oxide radicals (NO), as well as non-free radicals such as hydrogen peroxide (H_2O_2) and nitrous acid (HNO_2)[1]. In living organisms ROS and RNS can form in different ways. Normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells[2]. Some exogenous sources of free radicals are tobacco smoke, ionizing radiation, organic solvents and pesticides[3]. Free radicals can cause lipid peroxidation in foods that leads to their deterioration[4].

Oxidation does not affect only lipids. ROS and RNS may cause DNA damage that could lead to mutation[5]. In addition, ROS and RNS have been implicated in more than 100 diseases, including malaria, heart disease, stroke, arteriosclerosis, diabetes and cancer[6]. When produced in excess, ROS can cause tissue injury, whilst, tissue injury can itself cause ROS generation[5]. Nevertheless, all aerobic organisms, including human beings, have antioxidant defenses that protect against oxidative damage and numerous damage removal and repair enzymes to remove or repair damaged molecules[7]. However, the natural antioxidant mechanisms can be inefficient, hence dietary intake of antioxidant compounds becomes important[6]. Although there are some synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are commonly used in processed foods, it has been reported that these compounds may have side effects[8]. In addition, it has been suggested that there is an inverse relationship between dietary intake of antioxidant-rich foods and the incidence of a number of human diseases[9]. Therefore, research into the determination of natural antioxidant sources is important.

Nyctanthes arbor-tristis were widely used in Ayruveda and folk medicine in India, to treat varoius diseases including renal disease, haemorrhoids etc and are tend to posses anti-inflammatoy activity, antimicrobial activity[10,11,12]. The flowers of *N. arbor* are used in *Pyleena capsule* which is used for the treatment of Constipation, Intestinal worms, Piles.

However, studies on polyphenolic compounds and their properties in these flowers are little known. Our objectives in the present study were to determine: 1.The antioxidant properties and its efficiency in the aqueous, methanol, ethanol flower (both fresh and dry) extract of *Nyctanthes arbor-tristis*; 2. The evaluation of different levels of enzymatic antioxidants and non-enzymatic antioxidant levels 3. In addition, the qualitative analysis of polyphenols in the above mentioned flower extracts were assessed using standard polyphenols by RP-HPLC.

Materials and Methods

Chemicals:

Methanol HPLC grade from Merck, Acetonitrile HPLC grade from Sd. Fine chemicals. Catechin, Caeffic acid, *p*-Coumaric acid and Quercetin of HPLC grade from Sigma chemicals.

Flower collection and preparation of extracts:

Flowers of *N. arbor* were collected from the local markets of Visakhapatnam district, Andhra Pradesh during the months of July-September and authenticated by Dr. M. Venkaiah, Botany department, Andhra University. The collected flowers were cleaned, dried under shade at room temperature, then grounded and extracted. For fresh extracts fresh flowers were extracted immediately with out drying procedure.

Aqueous extracts: Aqueous extracts was prepared according to modified method[13]. A 10g of flower sample were soaked in distilled water, at the rate of 1:4 (fresh) and 1:20 (dry) w/v, after 1 day, the homogenized solution was squeezed through a cheese cloth and the liquid was filtered through whattman filter paper. This filtrate (1: 4 w/v or 1:20 w/v) was designed as standard(s). The supernatants were recovered and used for analysis immediately.

Ethanol and Methanol extracts: Extraction of fresh flower was prepared according to modified method[14].10 grams of flower material were soaked separately in 100ml (twice i.e., 2×100ml) of methanol and ethanol for 8-10 days at room temperature in dark conditions, stirring every 18h using a sterile rod. The final extracts were filtered using a Whatman No 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure at 40°C using a rotary evaporator (Buchi rotavapour-114) and stored at 4°C for

further use. Each extract was resuspended in the respective solvent (methanol and ethanol) to yield a 40 mg/ml stock solution.

Assay of Enzymatic antioxidants:

The assay of superoxide dismutase was carried by the method of [15] based on the reduction of Nitroblue tetrazolium (NBT). To 0.5 ml of plant extract, 1ml of sodium carbonate, 0.4ml of NBT and 0.2 ml of EDTA were added. The reaction was initiated by adding 0.4ml of Hydroxylamine hydrochloride. Zero time absorbance was taken at 560nm using spectrophotometer (Hitachi, Germany) followed by recording the absorbance after 5 min at 25^{o}C. The control was simultaneously run without plant extract. Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed in terms of units per milligram of protein.

The Catalase activity was assayed by the titrimetric method described by[16]. Briefly, 2.5 ml of 0.1M phosphate buffer, pH 7.5 and 2.5 m1 of 0.9% Hydrogen peroxide (v/v) in the same buffer were taken and 0.5 ml of the plant extract was added and incubated at room temperature for 3 min. The reaction was then arrested by adding 0.5 ml of 2N Sulphuric acid and the residual hydrogen peroxide was titrated with 0.1N potassium permanganate solution. A blank was carried out similarly with boiled enzyme extract. Units of enzyme activity were expressed as ml of 0.1 N potassium permanganate equivalents of hydrogen peroxide decomposed per min. per mg of protein.

Assay of Peroxidase activity was carried out according to the procedure[17]. 3.5 ml of phosphate buffer, pH 6.5, was taken in a clean dry cuvette, 0.2ml of plant extract and 0.l ml of freshly prepared O-dianisidine solution was added. The temperature of assay mixture was brought to 28-30^{o} C and then placed the cuvette in the spectrophotometer set at 430nm. Then, 0.2ml of 0.2M H_2O_2 was added and mixed. Read the initial absorbance and then, at every 30sec intervals up to 3min. A graph was plotted with the increase in absorbance against time. From the linear phase, read the change in absorbance per min. The enzyme activity was expressed per unit time per mg of protein or tissue weight. Water blank was used in the assay.

Estimation of Non-Enzymatic antioxidants:

Ascorbic acid was determined colorimetrically[18]. The brominated samples (ml) and standards (10-100 μg/ml) were taken and make up to 3ml with distil water then add 3ml of DNPH reagent followed by 1-2 drops of thiourea, mix them thoroughly and incubated at 37°C for 3h. After incubation, dissolve the orange-red osazone crystals formed by adding 7ml of 80% sulfuric acid and measure the absorbance at 540nm.

Reduced glutathione was determined by the method[19]. Briefly, 1.0 ml of the plant extract was treated with 4.0 ml of precipitating solution containing 1.67g of glacial metaphosphoric acid, 0.2 g of EDTA and 30 g of NaCl in 100ml water. After centrifugation, 2.0ml of the protein free supernatant was mixed with 0.2ml of 0.4M disodium hydrogen phosphate and 1.0ml of DTNB reagent. Absorbance was read at 412 nm within 2 min. GSH concentration was expressed as n mol per mg protein.

The total phenolics were determined using the Folin Cio-calteau reagent as reported by [20]. To 50 µl of the plant extract, 2.5ml of diluted Folin Cio-calteau reagent and 2.0 ml of 7.5% (w/v) sodium carbonate was added and incubated at 45°C for 15 min. The absorbance values of all samples were measured in a spectrophotometer at 765 nm. The results were expressed as mg of Gallic acid equivalent per gm weight.

Total flavonoids content was measured by aluminum chloride colometric assay [21]. 1ml of extracts or standard solution of catechin was added to 10 ml volumetric flask containing 4 ml of distilled water. To above mixture, 0.3 ml of 5% $NaNO_2$ was added. After 5 min, 0.3 ml of 10% $AlCl_3$ was added. At 6^{th} min, 2 ml of 1M NaOH was added and the total volume was made up to 10 ml with distill water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510nm.

The tannins were determined using the Folin Phenol reagent as reported by [22]. Briefly, 0.1 ml of the sample extract is added with 7.5 ml of distilled water and add 0.5 ml of Folin Phenol reagent, 1 ml of 35% sodium carbonate solution and dilute to 10 ml with distilled water. The mixture was shaken well, kept at room temperature for 30 min and absorbance was measured at 725 nm. Blank was prepared with water instead of the sample. A set of standard solutions of tannic acid is treated in the same manner as

described earlier and read against a blank. The results of tannins are expressed in terms of tannic acid in mg/g of extract.

Total monomeric Anthocyanin was estimated by pH-differential method of[23]. Turn on the spectrophotometer. Allow the instrument to warm up at least 30 min before taking measurements. Zero the spectrophotometer with distilled water at all wavelengths that will be used (λvis-max and 700 nm). Prepare two dilutions of the sample, one with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5. Let these dilutions equilibrate for 15 min. Measure the absorbance of each dilution at the λ vis-max and at 700 nm (to correct for haze), against a blank cell filled with distilled water.

Absorbance *(A)* = (*A* λ vis-max – *A*700) pH 1.0 – (*A* λ vis-max – *A*700) pH 4.5

Monomeric anthocyanin pigment (mg/liter) = (*A*×MW×DF×1000) / (ε×1).

Where MW is the molecular weight of *cyanidin-3-glucoside is 449.2*, DF is the dilution factor and ε is the molar absorptivity is *26,900*.

Antioxidant ability assays:

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method according to the procedure [24]. Briefly, 0.3 ml of plant extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing reaction solution were incubated at 95°C for 90 min. then the absorbance of the solution was measured at 695nm using spectrometer against blank after cooling to room temperature. Ascorbic acid was used as reference standard. The antioxidant activity is expressed as the number of equivalents of ascorbic acid (AscAE).

The ability of the extracts to reduce iron (III) was assessed by the method of [25]. One ml of plant extract was mixed with 2.5 ml of 0.2 M phosphate buffer, pH 6.6, and 2.5 ml of 1% aqueous potassium hexacyanoferrate [$K_3Fe(CN_6)$] solution. After 30 minutes of incubation at 50°C, 2.5 ml of 10% trichloroacetic acid was added, and the mixture was centrifuged for 10 minutes. Finally, 2.5 ml of the upper layer was mixed with 2.5 ml of water and 0.5 ml of 0.1% aqueous $FeCl_3$, and the absorbance was recorded at 700 nm. The mean absorbance values were plotted against concentration, and a linear regression analysis was carried out.

The results were expressed as Ascorbic acid equivalents (AscAE) in milligrams of ascorbic acid per gm of extract. Butylated hydroxy Toluene (BHT) and Ascorbic acid were used as positive controls.

The ability of extracts to reduce hydrogen peroxide was assessed by the method of [26]. A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). 1.0 ml of sample was added to a 0.6 ml of hydrogen peroxide solution (40mM). Absorbance of hydrogen peroxide at 230nm was determined after 10 min. against a blank solution containing phosphate buffer solution with out hydrogen peroxide. BHT and ascorbic acid were used as positive controls. The percentage scavenging of hydrogen peroxide of samples was calculated using the following formula:

$$\% \text{ scavenged } [H_2O_2] = [(A_0-A_I)/A_0] \times 100$$

Where A_0 was the absorbance of the control, and A_I was the absorbance of the sample.

The lipid Peroxidation was induced by FeSO4 – ascorbate system in sheep liver homogenate by the method of [27]. The reaction mixture consisting of 0.1ml each of 25%(w/v) sheep liver homogenate in 40 mM Tris-HCl buffer, p^H7.0, 30mM KCl, 0.16mM ferrous iron ($FeSO_4$), plant extract and positive controls, and 0.06 mM ascorbic acid. Appropriate controls for each of the plant extracts and positive controls were maintained. The reaction mixture was then incubated at 37^{o}C for 1h. After incubation, 0.4 ml of the above reaction mixture was taken and treated with 0.2 ml of sodium dodecyl sulfate, 1.5 ml of TBA, and 1.5 ml 20% acetic acid solution, then adjusted to P^H 3.5. The total volume was then made up to 4.0 ml by adding distilled water and the reaction mixture was kept in a water bath at 95^{o}C for 1 h. To the pre cooled reaction mixture, 1 ml of distilled water and 5 ml of n-butanol and pyridine (15:1 ratio v/v) was added and was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken, and its absorbance at 532 nm was measured. Inhibition of lipid peroxidation was determined by the OD of the extract with that of the control. The percentage of inhibition of lipid peroxidation was determined by comparing the results of the plant extract with those of controls.

% of inhibition (I) = (Absorbance of control –Absorbance of test / Absorbance of control) x100.

Qualitative analysis of HPLC:

Preparation of standards

Polyphenolic standards including Catechin, Quercetin, Caeffic acid and *p* Coumaric acid were dissolved in mobile phase as 1mg/ml concentrations. The solutions were filtered through a 0.45 µm membrane filter and stored in darkness. Standards are prepared freshly and immediately injected to HPLC column. Evaluation of each standard was repeated three times.

Extraction of the sample: All above mentioned flower extracts was prepared according to modified method [28].

Aqueous, ethanol and methanol extracts: The extractive solutions of fresh and dry flowers of *C. guianensis* fresh were prepared by maceration in water, ethanol and methanol. The plant: solvent ratio of 1:20 was employed for all extracts. All extracts were filtered through filter paper and concentrated with Rotary evaporator at 50˚C; the concentrated extracts were made up to 500ml with the mobile phase. The solutions were filtered through a 0.45 µm membrane filter. Evaluation of each sample was repeated three times.

Separation of phenolic compounds by HPLC:

Separation of the plant phenolic compounds in all samples was performed with help of isocratic Varian system, equipped with Sperisorb column 5mm (C 8, Hypersil MOS, 5µm, 200 × 2,1 mm, Hewlett Packard) 4.6 x 250mm and two detectors namely Refractive index and Photo Diode Array (PDA) type. The detection wavelength applied was in the range of 200-600nm. The mobile phase consisted of Acetonitrile: Water (70:30, v/v) and 1% of formic acid (v/v). The amount of the sample injected into the column is 10µl and the flow rate of the sample was adjusted 1mL/min. All separations were performed at the temperature of 25˚C. Identification of the phenolic compounds was carried out by comparing their retention times with known standards (caffeic acid, *p*-coumaric acid, catechin and quercetin).

Statistical analyses

Experimental results are presented as the mean standard deviation (SD) of three parallel measurements. Probability values of less than 0.05 were regarded as significant. All statistical analysis is performed using Excel 2007 and STATISTICA computer software packages.

Results

Assay of enzymatic antioxidants:

The inevitable generation of ROS in biological system and the oxidative damage is counterpoised by an array if enzymatic defense system. The levels of enzymatic antioxidants assessed in *N. arbor* flowers in different extracts are collectively represented in table-1.

Table 1: Enzymatic antioxidant levels of *N. arbor* (*NA*) flowers

[Values represent average of three determinations and expressed as mean ± S.D]

	Flowers	Extracts	Catalase U/mg	Peroxidase U/mg	SOD U/mg
NA	Dry (D)	Aqueous (A)	3.575±0.14	0.244±0.1	1.82±0.1
		Methanol (M)	0.64±0.1	0.031±0.004	1.6±0.004
		Ethanol (E)	1.06±0.08	0.13±0.01	1.1421±0.01
	Fresh (F)	Aqueous (A)	4.58±0.6	1.02±0.035	5.58±0.035
		Methanol (M)	0.77±0.4	0.099±0.08	2.67±0.08
		Ethanol (E)	2.43±0.4	0.29±0.09	2.285±0.09

The highest activity of antioxidant enzymes were observed in fresh flower extracts than dry flower extracts. The activity of superoxide dismutase, catalase and peroxidase is high in aqueous extracts than methanol and ethanol extracts. Superoxide scavenging effect of alcoholic extracts was reported earlier in mangrove plants [29].

Table 2: Non-Enzymatic antioxidant levels of *Nyctanthes arbor* (*NA*) flowers
[Values represent average of three determinations and expressed as mean ± S.D]

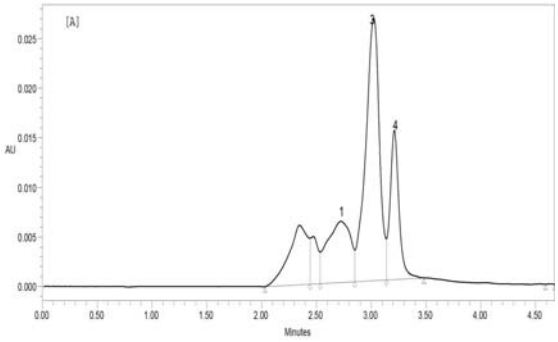
	Flowers	Extracts	Phenols mg GAE	Flavonoids % Catechin	Tannins mg TAE	Anthocyanins mg cyn3glu	GSH n mol/mg ptn	Vit-C mg AscAE
		Aqueous (A)	4.55±1.06	0.205±0.035	5.75±0.35	0.062±0.0442	125±35.5	11±1.414
	Dry (D)	Methanol (M)	4.725±0.88	0.75±0.24	9±0.7	0.2±0.001	122.65±0.001	21.25±0.002
NA		Ethanol (E)	4.6±0.14	0.275±0.07	6.75±0.35	0.187±0.021	709.99±3.3	17.5±0.001
		Aqueous (A)	0.5±0.012	0.05±0.01	1±0.01	0.01±0.014	72.7±4.81	1.2±0.001
	Fresh (F)	Methanol (M)	1.44±0.88	0.137±0.017	5.2±0.5	0.0939±0.04	64±0.01	9.75±3.53
		Ethanol (E)	0.75±0.7	0.072±0.002	1.5±0.01	0.06±0.01	251.42±0.001	8.75±1.76

Dermination of total phenols, flavonoid, anthocyanin and tannin content:

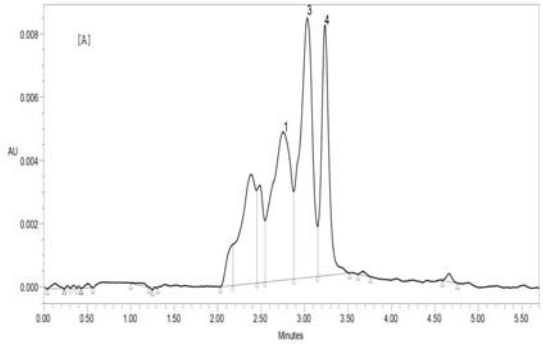
Polyphenol compounds are essential for the anti-oxidation process and for bioactivities in plants [40, 41]. The total polyphenol, flavonoid, anthocyanin and tannin content of the *N. arbor* dry and fresh flowers are shown in Table 2. The total polyphenol content is expressed as mg of gallic acid equivalent per mg of dry/fresh weight. The total phenol content of the *N. arbor* flowers was ranged from 4.725±0.88 to 0.5±0.012 mg GAE/gm, and decreased in the following order: *NA*DM>*NA*DE>*NA*DA>*NA*FM>*NA*FE>*NA*FA.

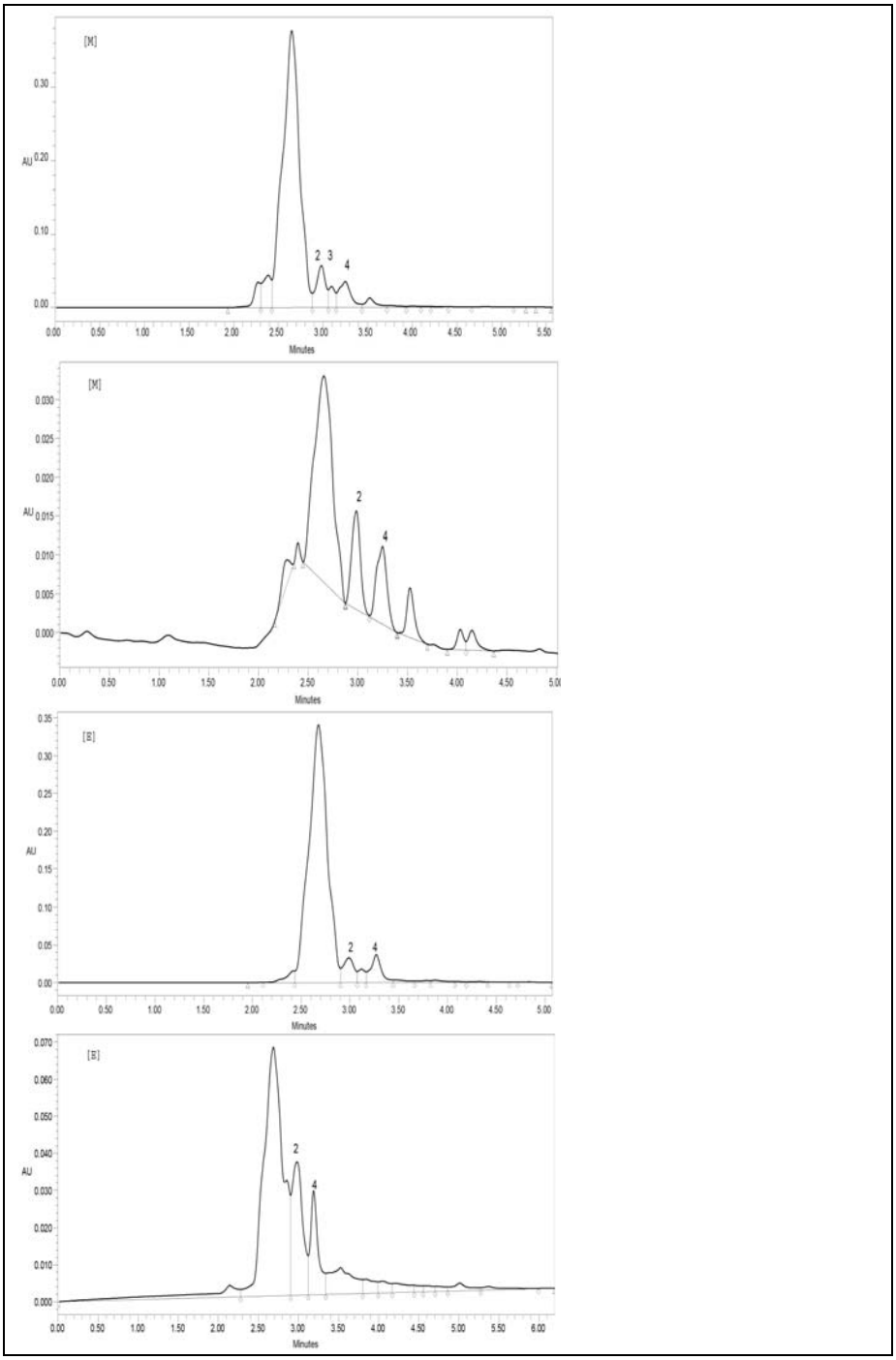
Fig 1: HPLC profiles in dry and fresh flowers of *N. arbor* analyzed: Aqueous (A), methanol (M), ethanol (E). Peaks: 1-catechin, 2-quercetin, 3-caffeic acid, 4-*p*-coumaric acid.

N. arbor dry flowers



N. arbor fresh flowers

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The methanol extracts of the *N. arbor* dry flowers had higher polyphenol content than the ethanol and aqueous extracts. Similar results were reported earlier in day lily flowers [32]. The total flavonoid content was expressed as % of catechin equivalent per gm. The total flavonoid content of the extracts of *N. arbor* dry and fresh flowers ranged from 0.75±0.24 to 0.05±0.01% catechin Eq/gm, and decreased in following order *NA*DM> *NA*DE> *NA*DA>*NA*FM> *NA*FE> *NA*FA. The methanol extracts of the *N. arbor* dry flowers had higher flavonoid content than other extracts. The total monometric anthocyanin content was expressed as cyanidin-3-glucosideequivalent per gm. The monomeric anthocyanin content of the extracts of *N. arbor* dry and fresh flowers ranged from 0.2±0.001 to 0.01±0.014 mg cyanidin-3-glucoside Eq/gm. There is no significant difference is observed between the dry and fresh flowers of *N. arbor* flowers. The total tannin content was expressed as tannic acid equivalent per gm. The total tannin content of the extracts of *N. arbor* dry and fresh flowers ranged from 9±0.7 to 1±0.01 mg TAE/gm, and decreased in the following order: *NA*DM> *NA*DE> *NA*DA> *NA*FM> *NA*FE> *NA*FA. The methanol extracts of the *N. arbor* dry flowers had higher tannin content than the ethanol and aqueous extracts. Similar results were reported in roots of *Areca Catechu* L. plant [33].

Estimation of GSH and Vitamin C content: Glutathione peroxidase acts as a radical scavenger, membrane stabilizer[34] and precursor of heavy metal binding peptides[35]. The content of GSH and Vit-C of the *N. arbor* dry and fresh flowers were shown in Table 2. GSH was found to be maximum in ethanol extracts of dry flowers ranging from 709.99±3.3 to 64±0.01 n moles/mg protein and decreased in following order *NA*DE>*NA*FE> *NA*DA>*NA*DM>*NA*FA>*NA*FM, and was observed that fresh flowers had higher reduced glutathione content than dry flowers in methanol extracts. Vit-C content was expressed as ascorbic acid equivalents per gm. The total Vit C content of the extracts of *N. arbor* dry and fresh flowers ranged from 21.25±0.002 to 1.2±0.001 mg Asc AE/gm, and decreased in following order *NA*DM, *NA*DE> *NA*DA> *NA*FM> *NA*FE>*NA*FA. The methanol extracts of the *N. arbor* dry flowers had higher Vit C content than other extracts.

In above all observations the dry flower extracts yields more content than fresh flowers. Fresh plant extracts may contain lower amounts of bioactive principles due to a water content of typically 75 to 95%, resulting in a marked dilution effect[36]. Recent empirical research on greater celandine extracts indicated that fresh plant tinctures contain less total alkaloid content than dried counterparts [37].

Antioxidant Potential:

Total antioxidant capacity:

The total antioxidant capacity of the *N. arbor* flowers is given in table-3. The results of total antioxidant capacity are expressed as equivalents of ascorbic acid. The phosphomolybdenum method is an important antioxidant assay based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of green phosphate/Mo (V) complex with a maximal absorption at 695nm and antioxidant capacity of flowers is expressed as the number of equivalent of ascorbic acid. The *NA*DM (137.5±3.53 Asc AE) showed high antioxidant capacity followed by *NA*DE, *NA*DA, *NA*FM, *NA*FE and *NA*FA this is due to presence of high content phenols in methanol extracts, as polyphenols plays an important role as antioxidants in living systems due to the presence of hydroxyl groups in *ortho*- and *para*- positions [38, 39].

Iron(III) to Iron(II) reducing activity:

The reducing ability of a compound generally depends on the presence of reductants[40], which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom[41]. The presence of deductants in *N. arbor* flowers causes the reduction of the Fe^{3+}/ ferricynide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation fof Perl's Purssian blue at 700nm. Table-3 shows the reductive capabilities of the flower extract compared to ascorbic acid.

Table 3: Antioxidant capacity of *Nyctanthes arbor (NA)* flowers
[Each value is an average of triplicates±S.D]

	Flowers	Extracts	TAA	% of Inhibition		Fe^{2+}-Fe^{3+}
			(Asc AE)	H_2O_2 Scavenging activity	Lipid peroxidation	(Asc AE)
		Aqueous (A)	24±0.01	51.635	78.57±4.95	26.12±3.7
	Dry (D)	Methanol (M)	63.33±7.07	59.33	66.66±6.01	32±2.47
NA		Ethanol (E)	41.66±3.53	55.81	79.65±3.5	11.6±5.12
		Aqueous (A)	9.3±0.001	51.22	74.99±5	12.5±0.07
	Fresh (F)	Methanol (M)	20.83±1.17	59.46	51.66±11.7	22.8±0.72
		Ethanol (E)	17.5±3.53	55.7	79.165±4.98	10±1.97
Positive controls		Asc.A(100µg/ml)		92.94	96.42	
		BHT (100µg/ml)		69.409	95.83	

The reducing power of methanol extract of *N. arbor* dry flowers was very potent compared to other extracts. reducing power of *N. arbor* flowers ranging from 56±3.3 to 8.125±1.5mg Asc AE/gm, and decreased in following order *NA*DM > *NA*DA > *NA*FM > *NA*DE > *NA*FA > *NA*FE.

Scavenging activity:

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly, and inside the cell, H_2O_2probably reacts with Fe2+, and possibly Cu2+ ions to form hydroxyl radical which may be the origin of many of its toxic effects[42]. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The scavenging activity of the different extract fractions from dry and fresh flowers is shown in table-3. Ascorbic acid and BHT were used as positive controls. *N. arbor* flowers scavenged $H_2O_2$and this may be attributed to the presence of phenols, which could donate electrons there by neutralizing it into water. It was observed that methanol extracts of *N. arbor* flowers exhibit slight high inhibition than ethanol and aqueous extracts ranged from 60.75 to 50.69% inhibition and also seen that fresh and dry flowers do not show much significant difference in inhibition of H_2O_2. All tested extracts can inhibit H_2O_2 but lesser when compared to reference standards ascorbic acid and BHT. Similar results were reported in *Carissa carandas* and *Pergularia daemia* root extracts[43].

Lipid peroxidation assay: The lipid peroxidation has been broadly defined as the oxidation deterioration of polyunsaturated lipids. The initiation of peroxidation sequence in membrane or polyunsaturated fatty acids is due to abstraction of a hydrogen atom from the double bond in the fatty acids. The free radicals tends to be stabilized by a molecular rearrangement to produce a conjugated dienes, which then easily react with an oxygen molecule to give a peroxy radical. Peroxy radical can abstract a hydrogen atom from another molecule or they can abstract hydrogen atom to give lipid hydroperoxide, ROOH. A probable alternative fat of peroxy radicals is to form cyclic peroxidase; these cyclic peroxidase, lipid peroxidase and cyclic endoperoxidase fragment to aldehyde including MDA and polymerization product. MDA is the major product of lipid peroxidation and is used to study the lipid peroxidation process[44]. Determination of lipid peroxidase content was carried out indirectly by means of derivatizing MDA with TBA at high temperature and acidic condition, CG has strongly inhibited the lipid peroxidation a shown in the Table-3. All tested extracts can inhibit lipid peroxidation but lesser when compared to reference standards ascorbic *acid* and BHT.

HPLC analysis:

Qualitative analysis of *N. arbor* dry and fresh flowers with three extracts were carried out using reverse phase HPLC and the chromatographic profiles were compared with the retention times of reference standards. From the chromatographic profiles it was also observed that quercetin, caffeic acid and *p*-coumaric acid was present in methanol extracts and catechin, caffeic acid and *p*-coumaric acid in aqueous extracts and quercetin and *p*-coumaric acid in ethanol extracts of *N. arbor* dry flowers. In *N. arbor* fresh flowers, HPLC chromatogram showed the presence of quercetin and *p*-coumaric acid were present in ethanol and ethanol extracts and in aqueous extracts HPLC chromatograms showed the presence of catechin, caffeic acid and *p*-coumaric acid.

The selection of these standards is due to their medicinal properties stated in literature; Catechin mainly reduces atherosclerotic plaques[45] and cancer[46]. Quercetin acts as antihepatotoxic and antiangiogenesis [47, 48]. Caffeic acid has antimitogenic, anticarcinogenic and immunomodulatory properties[49]. *p- Coumaric* acid has antioxidant properties and is believed to

`reduce the risk of stomach cancer[50] by reducing the formation of carcinogenic nitrosamines[51].

Discussion

There was a close correlation between the antioxidant capacity and the amount of polyphenols, flavonoids, and flavonols present in the plant. Total polyphenols play a vital role in anti-oxidization as well as in the biological functions of the plant[55]. Other studies have also indicated that the anti-oxidative properties of polyphenols in edible plants and plant products may help prevent diseases[56]. For example, fruits such as blueberry, cranberry and *Sambucus nigra* have been proven to be rich in flavonoids that protect endothelial cells from oxidation, a key factor in the development of cardio-vascular diseases[57]. The methanol extracts of dry flowers have been reported to exhibit high antioxidant activities with high total phenolic content. Phenolic compounds such as flavonoids, phenolic acids possess diverse biological properties such as anti-inflammatory, anticarcinogenic and anti-atherosclerotic activities. These biological properties might be to due their antioxidant activities[58]. Unfortunately, high concentration tannins in alcoholic extracts often cause undesirable gastric disorders. The use of polar and nonpolar solvents in adequate ratio also gives a great opportunity for pharmacological modification and standardization of the preparations originated from *Nyctanthes arbor* flowers. It was observed that unknown phenols were also identified in HPLC chromatograms leads to future investigation of these phenols.

Conclusion

Our results showed that methanolic extracts of *N. arbor* flowers were rich in phenolic constituents and demonstrated good antioxidant activity, whereas aqueous extracts were found to be poor in antioxidant capacity. The chromatographic separation enabled the identification of a wide range of phenolic compounds present in medicinal flowers without time consuming sample preparation or previous fractionation. These flowers, rich in flavonoids and phenolic acids could be a good source of natural antioxidants. Therefore, the quantitative analysis of polyphenols in this flower could be helpful for explaining the relationships between total

antioxidant activity and total phenolic content of the extracts. Obviously, to confirm the beneficial effects of these extracts, it is necessary to carry out further studies about their in vivo activity and bioavailability.

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