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Research Article

EVALUATION OF ANTIOXIDANT POTENTIAL AND QUALITATIVE ANALYSIS OF MAJOR POLYPHENOLS BY RP-HPLC IN *NYMPHAEA NOUCHALI* BRUM FLOWERS

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ABSTRACT

Medicinal plants, as source of remedies, are widely used as alternative therapeutic tool for the prevention or treatment of many diseases. The recent studies have investigated that the antioxidant effect of plant products is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins etc. these compounds are distributed in different parts of plants such as bark, leaves, fruits and flowers etc. The flowers of *Nymphaea nouchali* Brum have a wide range of applications in ayurveda and traditional medicine. Studies have been taken up to evaluate the enzymatic, non-enzymatic and antioxidant potentials in ethanol, methanol and aqueous extracts of *N. nouchali* dry and fresh flowers. Obtained results showed that the high levels of non-enzymatic antioxidants like phenols, flavonoids, tannins etc. as well as antioxidant potential found to be more in methanol extracts of *N. nouchali* dry flowers. It is very interesting that the levels of enzymatic antioxidants were found to be high in the fresh flower aqueous extracts of *N. nouchali* flowers. Further, studies were also conducted for the identification of phenolic compounds with different solvents using Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) coupled with photodiode array detector.

Keywords - *Nymphaea nouchali*, Antioxidant activity, Total phenols, RP-HPLC, Enzymatic and non-enzymatic antioxidants

INTRODUCTION

Oxygen is a doubled edged sword that can potentially, cause cellular damage through excessive production of reactive oxygen species (ROS). ROS including superoxide radical, hydroxyl radical, singlet oxygen and H_2O_2 have been found to play an important role in the initiation and / or progression of various diseases such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease[1]. Nevertheless, all aerobic organisms, including human beings have multiple mechanisms to protect cellular molecules (DNA, RNA and proteins) against ROS induced damage. These include repair enzymes (DNA glycosylases, AP endonucleases etc), antioxidant enzymes (SOD, Catalase, and Glutathione peroxidase), and intra as well as extracellular antioxidants (glutathione, uric acid, ergothioneine, vitamin E, vitamin C and phenolic compounds) [2]. However, this natural antioxidant mechanism can be inefficient for severe and / or continued oxidative stress.

Based on this idea, there has been a strong demand of therapeutic and chemo preventive antioxidant agents with limited cytotoxicity to enhance the antioxidant capacity of the body and help attenuate the damage induced by ROS. There are mainly two strategies proposed for this, one would be to use the antioxidants with direct radical scavenging activity [3], and the other approach was to identify antioxidants that would increase the expression of antioxidant enzymes [4]. However, there is a paucity of data on natural phytochemicals that modulate the expression of enzymes metabolizing ROS. There are some synthetic antioxidants compounds like butylated hydroxyl toulene, butylated hydroxyl anisole and tetrbutylhydroquinone have commonly used. However, it has been suggested that these compounds are carcinogens[5]. This led to an increased interest in natural antioxidants from plant sources.

Polyphenolic compounds tend to be potent free radical scavengers and their abilities to act as antioxidants mainly depends on their chemical structure, capability to donate/accept electrons, thus delocalizing the unpaired electron with in the aromatic structure and the polyphenols are broadly classified into two categories, flavonoids and phenolic acids[6]. Flavonoids is a large family consisting of more than 4000 ubiquitous secondary plant metabolites, which are further divided into 5 subclasses namely flavonols, flavones, anthocyanins, catechins and flavonones[7]. Flavonols such as Catechin mainly reduces atherosclerotic plaques[8]and cancer[9]and also posses antibiotic properties by disrupting bacterial DNA replication process[10]. Quercetin acts as antioxidant, antiinflammatory, antiulcerative, antihepatotoxic and antiangiogenesis[11, 12]. Other polyphenols like, Caffeic acid act as inhibitor for lipooxygenase enzyme, suppress acute immune and inflammatory response[13] and known to have antimitogenic, anticarcinogenic and immunomodulatory properties[14]. *p- Coumaric* acid has antioxidant properties and is believed to reduce the risk of stomach cancer[15] by reducing the formation of carcinogenic nitrosamines[16].The antioxidant properties of polyphenols depend mainly upon factors such as metal reducing potential, chelating behavior, pH and solubility characteristics[17].

Information on the antioxidant activity and phenolic compounds of traditional medicinal flowers like *Nymphaea nouchali* is scare. Also, these flowers were recommended for traditional healers for treatments of renal diseases, piles and as cardio tonic and also had wide range of applications in ayruveda medicine. The flowers of *N. nouchali* are used in *Chyawanprash Special, Diab-Eaze, Pyleena Capsule*. Therefore, the main objectives of present study were to determine 1. Enzymatic and non-enzymatic antioxidants in aqueous, methanol and ethanol extract of *N. nouchali* dry and fresh flowers 2. Characterize their antioxidant potentials 3. Individual phenolic compounds were identified by HPLC. The difference in the extracts from fresh and dry on antioxidant activities were also compared. Since this may be important for alimentary or pharmaceutical purposes.

MATERIALS AND METHODS

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

Flower collection and preparation of extracts

All flower species 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 a modified method[18]. 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 a modified method[19]. 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 Beauchamp and Fridovich[20] 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 0.4 ml of Hydroxylamine hydrochloride. Zero time absorbance was taken at 560nm using spectrophotometer (Hitachi, Germany) followed by recording the absorbance after 5 min at 25°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 Radha Krishnan and Sarma[21]. 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 of Malik C.P and Singh M.B[22]. 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° 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 30 sec 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 by Sadasivam and Manickam method[23]. 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 Boyne and Ellman method[24]. 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 [25]. 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[26]. 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 6th 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 by Folin and Ciocalteu method[27]. 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[28]. 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.

Calculate the absorbance of the diluted sample (A) as follows:

A = (A λ vis-max – A700) pH 1.0 – (A λ vis-max – A700) pH 4.5

The monomeric anthocyanin pigment:

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 (for example, if a 0.2 ml sample is diluted to 3 ml, DF = 15), and ε is the molar absorptivity is *26,900*.

Antioxidant ability assays

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method[29]. 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 Oyaizu M[30]. 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 Ilhami[31]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). 1.0 ml of sample was added to a 0.6 ml of hydrogen peroxide solution (40 mM).

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:

$$\% \text{ scavenged } [H_2O_2] = [(A_0-A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance of the sample.

The lipid Peroxidation was induced by $FeSO_4$ – ascorbate system in sheep liver homogenate by the method of Bishayee and Balasubramaniyam[32]. 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°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°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[33].

Aqueous, ethanol and methanol extracts

The extractive solutions of fresh and dry flowers of *N. nouchali* 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 a detector namely 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) with 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 analysis

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. nouchali* flowers in different extracts are collectively represented in table-1. 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[34].

Table 1: Enzymatic antioxidant levels of *N. nouchali (NN)* flowers

	Flowers	Extracts	Catalase U/mg	Peroxidase U/mg	SOD U/mg
		Aqueous (A)	0.925±0.06	0.349±0.01	2.66±0.001
	Dry (D)	Methanol (M)	0.117±0.75	0.0149±0.07	0.26±0.07
NN		Ethanol (E)	0.64±0.8	0.023±0.8	0.457±0.8
		Aqueous (A)	3.64±0.3	0.89±0.03	5.366±0.03
	Fresh (F)	Methanol (M)	0.136±0.1	0069±0.14	4.4±0.14
		Ethanol (E)	0.96±0.3	0.041±0.06	1.129±0.06

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

Determination of total phenols, flavonoid, anthocyanin and tannin content

Polyphenol compounds are essential for the anti-oxidation process and for bioactivities in plants[35, 36]. The total polyphenol, flavonoid, anthocyanin and tannin content of the *N. nouchali* 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. nouchali* flowers was ranged from 2.375±0.53 to 1.5±0.35 mg GAE/gm, and decreased in the following order: NNDM > NNDE > NNDA > NNFM > NNFE > NNFA. The methanol extracts of the *N. nouchali* dry flowers had higher polyphenol content than the ethanol and aqueous extracts. Similar results were reported earlier in day lily flowers[37].

Table 2: Non-enzymatic antioxidant levels of *N. nouchali (NN)* flowers

	Flowers	Extracts	Phenols (mg GAE)	Flavonoids (%Catechin)	Tannins (mg TAE)	Anthocyanins (mg cyn3glu)	GSH (n mol/mg ptn)	Vit-C (mg AscAE)
		Aqueous (A)	6.125±1.2	0.262±0.01	4.62±0.17	0.15±0.17	30.9±3.36	7.5±2.12
	Dry (D)	Methanol (M)	26.125±1.23	0.54±0.02	32.625±1.5	0.156±0.13	9.785±1.8	23.75±5.3
NN		Ethanol (E)	24±3.18	0.343±0.02	7.625±0.5	0.125±0.01	106±8.48	8.75±1.76
		Aqueous (A)	1.5±0.35	0.034±0.005	2.25±0.01	0.05±0.01	37.66±3.76	1±0.28
	Fresh (F)	Methanol (M)	9.44±0.23	0.49375±0.44	14.8±1.17	0.25±0.017	36.66±1.56	20±0.001
		Ethanol (E)	2.375±0.53	0.0875±0.001	3.75±0.35	0.156±0.132	60.89±3.74	3.75±1.76

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

The total flavonoid content was expressed as % of catechin equivalent per gm. The total flavonoid content of the extracts of *N. nouchali* dry and fresh flowers ranged from 0.54±0.02 to 0.034±0.005 % catechin Eq/gm, and decreased in following order NNDM> NNDE> NNDA> NNFM> NNFE> NNFA. The methanol extracts of the *N. nouchali* dry flowers had higher flavonoid content than other extracts. Similar results were reported earlier in *Drynaria fortune*[38]. The total monomeric anthocyanin content was expressed as cyanidin-3-glucosideequivalent per gm. The monomeric anthocyanin content of the extracts of *N. nouchali* dry and fresh flowers ranged from 0.156±0.132 to 0.05±0.01mg cyanidin-3-glucosideeq/gm. There is no significant difference is observed between the extracts of *N. nouchali* flowers.

The total tannin content was expressed as tannic acid equivalent per gm. The total tannin content of the extracts of *N. nouchali* dry and fresh flowers ranged from 32.625±1.59 to 2.25±0.01 mg TAE/gm, and decreased in the following order: NNDM> NNDE> NNDA> NNFM>NNFE> NNFA. The methanol extracts of the *N. nouchali* dry flowers had higher tannin content than the ethanol and aqueous extracts. Similar results were reported in roots of *Areca Catechu* L. plant[39].

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 [40]. Recent empirical research on greater celandine extracts indicated that fresh plant tinctures contain less total alkaloid content than dried counterparts[41].

Estimation of GSH and Vitamin C content

Glutathione peroxidase acts as a radical scavenger, membrane stabilizer[42] and precursor of heavy metal binding peptides[43]. The content of GSH and Vit C of the *N. nouchali* dry and fresh flowers were shown in Table 2. GSH was found to be maximum in ethanol extracts of dry flowers ranging from 106±8.48 to 9.785±1.8 n moles/mg protein and decreased in following order NNDE > NNFE > NNFA > NNFE > NNDA > NNDE, and was observed that fresh flowers had higher reduced glutathione content than dry flowers except ethanol extracts. Vit-C content was expressed as ascorbic acid equivalents per gm. The total Vit C content of the extracts of *N. nouchali* dry and fresh flowers ranged from 23.75±5.3 to 1±0.28 mg Asc AE/gm, and decreased in following order NNDM > NNDE> NNDA> NNFM > NNFE > NNFA. The methanol extracts of the *N. nouchali* dry flowers had higher Vit C content than other extracts

Antioxidant potential

Total antioxidant capacity

The total antioxidant capacity of the *N. nouchali* 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 NNDM showed high antioxidant capacity followed by NNDE, NNDA, NNFM, NNFE and NNFA 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 [44, 45].

Iron(III) to Iron(II) reducing activity

The reducing ability of a compound generally depends on the presence of reductants[46], which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom[47]. The presence of deductants in *N. nouchali* 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. The reducing power of methanol extract of *N. nouchali* dry flowers was very potent compared to other extracts. reducing power of *N. nouchali* flowers ranging from 32.625±3.3 to 17±1.37 mg Asc AE/gm, and decreased in following order NNDM > NNDA > NNDE > NNFM > NNFA > NNFE.

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[48]. 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. nouchali* 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. nouchali* flowers exhibit slight high inhibition than ethanol and aqueous extracts ranged from 62.95 to 53.25% inhibition and also seen that fresh and dry flowers do not show much significant difference in inhibit-ion of H_2O_2. All tested species can inhibit the presence and production of 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[49].

Table 3: Antioxidant capacity of *Nymphea nouchali (NN)* flowers

	Flowers	Extracts	TAA (AscAE)	% of Inhibition		Fe^{2+}-Fe^{3+} (AscAE)
				H_2O_2 Scavenging activity	Lipid peroxidation	
		Aqueous (A)	100.5±6.36	54.73	71.42±5.07	28.75±1.2
	Dry (D)	Methanol (M)	145.83±5.89	62.95	66.66±7.92	32.62±3.3
NN		Ethanol (E)	114.55±2.89	58.6	91.66±4.3	23.25±2.4
		Aqueous (A)	22.8±0.02	53.25	67.85±5.08	22.5±1.41
	Fresh (F)	Methanol (M)	24.165±5.89	61.428	49.995±2.56	23.86±3.6
		Ethanol (E)	22.495±1.18	58.47	83.33±11.78	17±1.37
		Aqueous (A)	63.665±5.18	51.096	78.56±7.09	51.12±1.9
Positive controls		Asc.A(100µg/ml)		92.94	96.42	

Lipid peroxidation assay

It is known that oxidation of poly unsaturated fatty acids in biological membranes often lead to the formation and propagation of lipid radicals, uptake of oxygen, and rearrangement of the double bonds in unsaturated lipids and even destruction of membrane lipids. Many of these biochemical activities can lead to the production of breakdown products that are highly toxic to most mammalian cell types[50]. Effects on the inhibition of lipid peroxidation by methanol, ethanol and aqueous extracts of *N. nouchali* flowers were tested by using liver cell homogenates as a lipid source. A drastic increase in the capability of inhibiting lipid peroxidation was detected in ethanol extracts compared to methanol and aqueous extracts this might be due to high presence of glutathione in ethanol extracts of tested flower as indicated earlier, glutathione and glutathione peroxidase play an important role in inhibiting lipid peroxidation in biological medium[40]. Lipid peroxidation inhibition by *N. noucahli* flowers was ranged from 91.66±4.3% to 49.995±2.56% and inhibition of lipid peroxidation was less in fresh flowers compared to dry flowers. All tested species can inhibit lipid peroxidation but lesser when compared to reference standards ascorbic acid and BHT.

***N. nouchali* dry flowers** ***N. nouchali* fresh flowers**

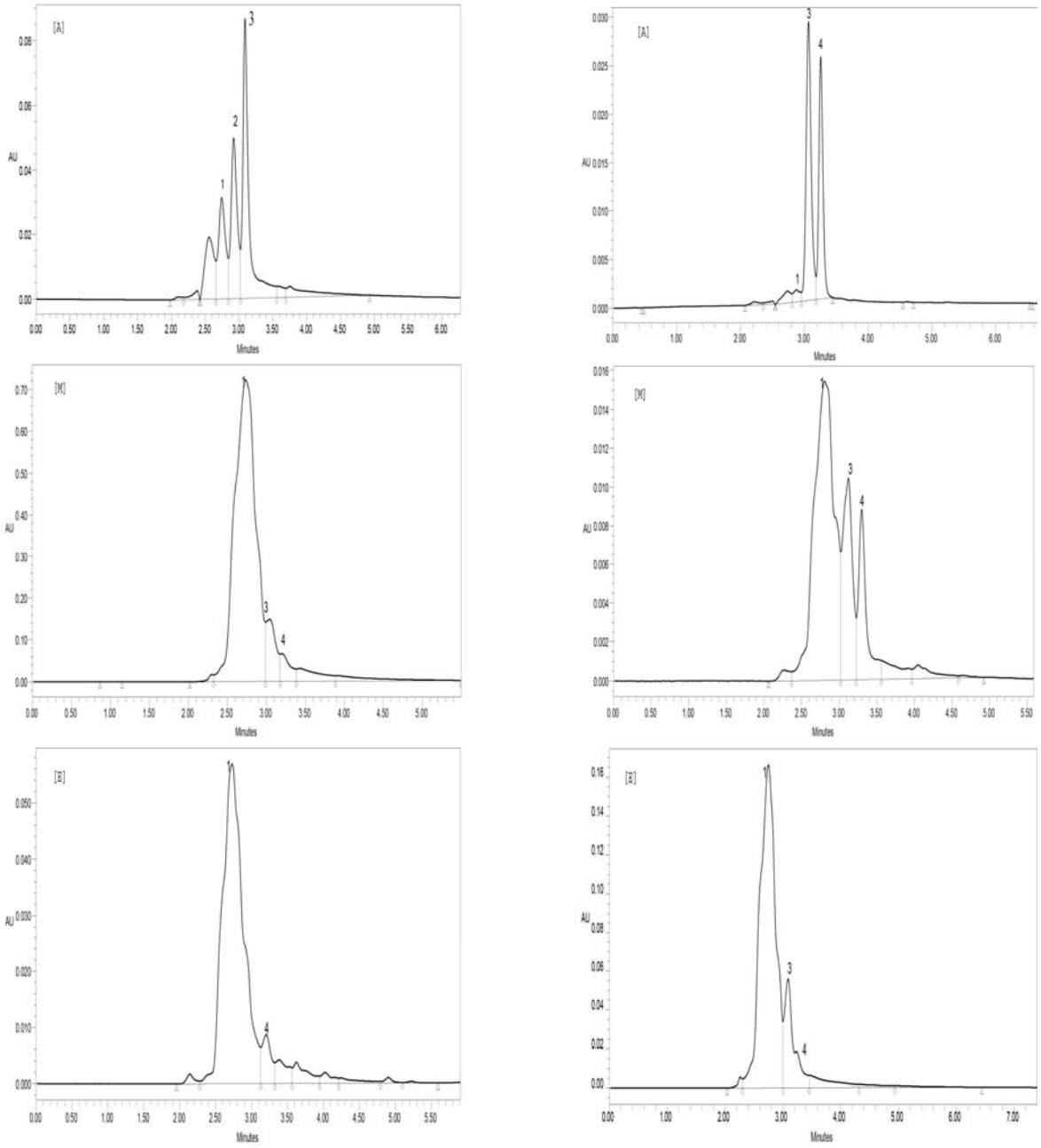

Fig. 1: HPLC profiles in dry and fresh flowers of *N. nouchali* analyzed: aqueous (A), methanol (M), ethanol (E). Peaks: 1-catechin, 2-quercetin, 3-caffeic acid, 4-*p*-coumaric acid.

HPLC analysis

Qualitative analysis of *N. nouchali* 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 caffeic acid was present in methanol extracts and catechin and *p*-coumaric acid in ethanol extracts and catechin, quercetin and caffeic acid in aqueous extracts of *N. nouchali* dry flowers. In *N. nouchali* fresh flowers, HPLC chromatogram showed the presence of catechin in methanol extracts and catechin, quercetin and *p*-coumaric acid were present in 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.

DISCUSSION

The relation between diseases and free radicals has been proved by many studies. UV light, radiation, smoking, alcohol consumption, stress and high cholesterol consumption can increase the process of cell oxidation[51]. This study aimed to establish a platform for *in vitro* evaluation of antioxidant capacity of herbal plants.

From the results, it was observed that methanol extracts contains more phenolic compounds than ethanol and aqueous extracts, why because methanol is less polar than water, due its low polarity nature it can release the cell wall bound polyphenols from cells[44] and also it can neutralize the activity of polyphenol oxidase which degrades the polyphenols in plants[52].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[53]. Other studies have also indicated that the anti-oxidative properties of polyphenols in edible plants and plant products may help prevent diseases[54]. 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 cardiovascular diseases[55]. 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 acid and tannins possess diverse biological properties such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities. These biological properties might be to due their antioxidant activities[56]. 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. nouchali* 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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