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Research Article

Comparative Phytochemical investigation and Antioxidant study of *Narcissus tazetta*, *Nymphaea mexicana* zucc. And *Indigofera heterantha*.

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ABSTRACT

The study was intended to investigate the presence of phytoconstituents in the bulbs of *Narcissus tazetta*, flowers of the plant *Nymphaea mexicana* and roots of *Indigofera heterantha*. The present study provides evidence that methanolic extracts of *Narcissus tazetta*, *Nymphaea mexicana* and *Indigofera heterantha* contain important compounds with marked bioactivity. Maximum phenolic content was observed in *Nymphaea mexicana* and maximum flavonoid content was observed in extract of *Indigofera heterantha*.

Keywords: Phytoconstituents, Antioxidant, *Nymphaea mexicana*, *Narcissus tazetta*, *Indigofera heterantha*

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INTRODUCTION

Antioxidants neutralize the free radicals resulting from various biochemical processes in body¹. A highly refined and complex antioxidant protection system has therefore been developed by human beings to counteract the damage from these free radicals, with both exogenous as well as endogenous mechanisms constituting this system. These include Nutrient-derived antioxidants like ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids, and other low molecular weight compounds such as glutathione and lipoic acid, Antioxidant enzymes, e.g. superoxide dismutase, glutathione peroxidase, and glutathione reductase, which catalyze free radical quenching reactions, Metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions, that are capable of catalyzing oxidative reactions and numerous other antioxidant phytonutrients present in a wide variety of plant foods. Plants have provided themselves as a potential source of Antioxidants. The plant constituents like phenolics and flavonoids are recognized free radical scavengers. Synthetic antioxidants have been suspected to cause negative health effects. Therefore, their use has been put under scanner and ways are being devised to substitute them with naturally occurring antioxidants².

Narcissus tazetta is a flowering plant belonging to family Amaryllidaceae and is reported to possess anti-malarial³, anti-inflammatory⁴, anti-cholinesterase⁵, anti-viral properties⁶.

Nymphaea mexicana is a flowering aquatic plant belonging to Nymphaeaceae, *Nymphaea* species are reported to possess anti-inflammatory and anti-diabetic properties⁷, plants of *Nymphaea* species are recognized to be larvicidal⁸, sedative⁹, anti-microbial^{8,10}, aphrodisiac¹¹, , anti-hyperlipidemic¹² etc.

Indigofera heterantha belonging to family fabaceae is reported to help stomachache and Diarrhoea¹³, It is used in renal disorders¹⁴ and is used as Anti-Diabetic and in Toothache¹⁵

Plants (herbal medicines) have remained an effective and diverse source of drugs since prehistoric times¹⁶. They have been used to treat various ailments since times immemorial¹⁷. The knowledge of Materia Medica for pharmacotherapy for years has been utilized and Herbal drugs have earned significance and popularity over the years for their efficacies and cost effectiveness¹⁸.

MATERIAL AND METHOD

Plant material:

Narcissus tazetta was collected from Kashmir University Botanical Garden. The curator of Center for Biodiversity & Taxonomy (CBT) identified the plant vides voucher no. 2608-(KASH) Herbarium, CBT. The plant was cleared of dirt and debris. Bulbs were obtained without adventitious roots. The bulbs were then shade dried.

Nymphaea mexicana flowers were collected from Nigeen lake. The said flowers were identified by curator CBT vide voucher no. 2609 –(KASH) Herbarium, CBT. Cleaned flowers were then shade dried.

Indigofera heterantha was obtained from Drang, Tangmarg. The plant was identified by curator CBT vide voucher no. 2607-(KASH) Herbarium. The plant roots were cleaned, and then shade dried.

Extraction procedure:

All the extracts of plants were made by cold maceration technique using methanol. 250 g each of *N. tazetta* bulbs, *N. mexicana* flowers and *I. heterantha* roots were macerated separately in three maceration bottles using methanol as solvent of extraction in the ratio 1:3. After repeated washings finally, the obtained liquid crude extract was subjected to drying to obtain dry extract. Dessicator was used for final drying.

Qualitative Phytochemical tests:

A stock concentration of 1 % (W/ V) of each extract was prepared by using methanol as solvent. These extracts were tested for the presence of active phytochemicals viz: tannins, alkaloids, phytosterols, triterpenoids, flavonoids, cardiac glycosides, anthraquinone glycosides, saponins, carbohydrates, proteins, amino acids, fixed oils & fats following standard methods^{19,20}:

1. Alkaloids

5 mg of extract were dissolved in 5 ml of distilled water. 2M HCL was further added until an acid reaction occurred and filtered. The filtrate was tested for the presence of alkaloids by following tests.

- a. **Dragendorff's Test:** To 2 ml of the filtrate, added 1 ml of Dragendorff's reagent along the side of the test tube. Formation of orange or orange reddish brown precipitate indicated presence of Alkaloids.
- b. **Hager's Test:** A drop or two of Hager's reagent were added to 1 ml of test solution. The formation of yellow precipitate indicated the presence of alkaloids.
- c. **Wagner Test:** Two drops of Wagner's reagent were added to 1ml of the test solution along the side of the test tube. The formation of yellow or brown precipitate confirmed the presence of alkaloids.

2. Tannins

- a. **Ferric Chloride Test:** To 2 ml of the test solution added a few drops of 5% ferric chloride solution. Formation of blue color indicated the presence of hydrolysable tannins.
- b. **Gelatin Test:** Added five drops of 1% gelatin containing 10% sodium chloride to 1 ml of the test solution. Formation of white precipitates confirmed presence of tannins.

3. Phytosterols

Liebermann-Burchard's Test: 2 mg extract was dissolved in 2 ml of acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulfuric acid was added along the sides of the test tube. A brown ring formation at the junction and the turning of the upper layer to dark green color confirmed the test positive for the presence of phytosterols.

4. Triterpenoids

Salkowski Test: 2 mg of dry extract was shaken with 1 ml of chloroform and few drops of concentrated sulfuric acid

were added along the sides of the test tube. A red brown color formed at the interface indicated the presence of triterpenoids.

5. Flavonoids

- a. **Shinoda test:** 5 drops of concentrated hydrochloric acid and few magnesium turnings were added drop wise to 1 ml test solution. A pink, scarlet, crimson red or occasionally green to blue color appeared after few minutes. This confirmed the presence of flavonoids.
- b. **Alkaline reagent test:** Addition of 5 drops of 5% sodium hydroxide to 1 ml of the test solution resulted an increase in the intensity of the yellow color which became colorless on addition of few drops of 2 M hydrochloric acid which indicated the presence of flavonoids.
- c. **Lead Acetate Test:** To 1ml of the test solution added few drops of 10% lead acetate, formation of yellow precipitate confirmed the presence of flavonoids.

6. Saponins

- a. **Foam Test:** 5 ml of the test solution in a test tube was shaken well for five minutes. Formation of stable foam confirmed the presence of saponins.
- b. **Olive Oil Test:** Few drops of olive oil were added to 2ml of the test solution and shaken well. The formation of a soluble emulsion confirmed the presence of saponins.

7. Cardiac Glycosides

Keller -Killiani Test

Added 0.4 ml of glacial acetic acid and a few drops of 5% ferric chloride solution to a little of dry extract. Further 0.5 ml of concentrated sulfuric acid was added along the sides of the test tube carefully. The formation of blue color in acetic acid layer confirmed the test.

Anthraquinone Glycosides

Hydroxyanthraquinone Test

Few drops of 10% potassium hydroxide were added to 1 ml of extract. Red color formation confirmed the test positive for anthraquinone glycosides.

8. Test for carbohydrates

- a. **Molisch's Test:** Few drops of 1 % naphthol were added to 1 ml of test solution and 2-3 ml concentrated sulfuric acid were added along the sides of test tube. The reddish violet or purple ring formed at the junction of two liquids confirmed the test.
- b. **Barfoed's Test:** 2 ml of the test solution and 2ml of reagent were mixed & kept a in boiling water bath for 1 min. Red precipitate formed indicates the presence of monosaccharides.
- c. **Seliwanoffs Test:** 3 ml of Seliwanoffs reagent was added to 1 ml of the test sample and heated on a water bath for one minute. The formation of rose red color confirmed the test positive for carbohydrates.

9. Test for Proteins

- a. **Biuret test:** 5 drops of 1% copper sulphate solution and 2 ml of 10% NaOH were added to 2 ml of the test solution. Mixed thoroughly, formation of purple or violet color confirmed presence of proteins.

10. Test for Amino Acids

- Millon's Test:** Added 5 drops of millon's reagent to 1 ml of test solution and heated on a water bath for 10 min, cooled and added 1% sodium nitrite solution. Appearance of red color confirmed the test positive for amino acids.
- Xanthoproteic Test:** The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.
- Ninhydrin Test:** To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

Total Phenolic Content estimation²¹

Total Phenolic content estimation Principal: The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method

Preparation of Standard: 50 mg Gallic acid was dissolved in 50 ml methanol, various aliquots of 25- 125µg/ml was prepared in methanol.

Preparation of Extract: 10 mg of dried extract dissolved in 10 ml methanol and filter. 200 µL (1mg/ml) of this extract was for the estimation of phenols.

Procedure: Briefly, 200 µL of extract (1 mg/mL) were made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin-Ciocalteu reagent for 3 min, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was allowed to stand further for 60 min in the dark, and absorbance was measured at 650 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

Total flavonoids content estimation²²:

Principal: Determination of total flavonoids content was based on aluminum chloride method .

Preparation of standard: 50 mg rutin was dissolved in 50 ml methanol, and various aliquots of 25- 125µg/ml were prepared in methanol.

Preparation of extract: 10 mg of extract dissolved in 10 ml methanol and filter. Three (1mg/ml) of this extract was for the estimation of flavonoid. 3 ml of extract or standard and allowed to stand for 15 min at room temperature; absorbance was measured at 510nm.

In-Vitro free radical scavenging activity (2, 2-diphenyl-1-picrylhydrazyl - DPPH)²³:

When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color.

The free radical scavenging capacity or electron donating ability of different extracts was determined using DPPH. DPPH solution (0.1 mM) was freshly prepared in 95% ethanol. Different concentrations (200 µg /ml – 600 µg /ml) of methanolic extracts were prepared. 0.1 ml of the various concentrations of above mentioned extracts were mixed with 1.0 ml of DPPH solution.

The reaction mixture was incubated in dark for 30 min. The absorbance was measured at 517 nm. Ascorbic acid was used as a standard (1mg/ml). The scavenging capacity of ascorbic acid with distilled water was also measured. Control was prepared in the same way except for the replacement of the test sample with corresponding extraction solvent. All the tests were performed in triplicate.

The scavenging ability of the plant extracts and ascorbic acid was calculated using this equation:

$$DPPH\text{Scavenging activity (\%)} = \frac{[(Abs. C - Abs. S)]}{(Abs. C)} \times 100$$

Where, Abs. C is the absorbance of DPPH + methanol;

Abs. S is the absorbance of DPPH radical + sample (i.e. extract or standard).

RESULTS AND DISCUSSION

Yield of Plant extracts:

After successful cold extraction the extract was subjected to drying using water bath and dessicator was used for final drying. The yields of extracts from all of the plants are enlisted in Table 1.

Table 1: Comparative yield of Plant extracts

Test Plant	Mass Taken (grams)	Extract Yield (grams)
<i>Narcissus tazetta</i>	250	13
<i>Nymphaea mexicana</i>	250	26
<i>Indigofera heterantha</i>	250	32

A portion of these extracts was subjected to various phytochemical tests for presence of different phytoconstituents. The outcome of the qualitative tests is enlisted in Table 2.

Table 2: Qualitative phytochemical tests

S.No	Phytochemical Tests	Extract		
		<i>N. tazetta</i>	<i>N.mexicana</i>	<i>I.heterantha</i>
1	Test for Alkaloids			
	Drangendroffs test	+	+	+
	Hagers test	+	+	+
	Wagners test	+	+	+
2	Test for triterpenoids			
	Salkowski test	+	+	+
3	Test for phytosterols			
	Libermann Buchard's test	-	+	-
4	Flavonoids			
	Shinoda test	+	+	+
	Alkaline reagent test	+	+	+
	Lead acetate test	+	+	+
5	Test for saponins			
	Foam test	+	-	+
	Olive oil stain test	-	-	+
6	Test for Glycosides			
	Keller killani test	+	+	+
	Test for anthraquinone glycosides	+	+	+
7	Test for proteins			
	Biuret test	+	-	-
8	Test for Amino acids			
	Millons test	+	-	-
	Ninhydrin test	-	+	-
	Xanthoproteic test	+	+	+
9	Test for carbohydrates			
	Molishs test	+	+	+
	Barfoeds test	+	+	+
	Seliwanoffs test	+	+	-
10	Tannins	+	+	+

All the three plant extracts exhibited presence of various phytoconstituents like carbohydrates, flavonoids, glycosides, tannins, terpenoids, alkaloids, phenols etc.

The content of total phenolic content (TPC) was expressed as mg/g of gallic acid equivalent of dry extract sample using the equation obtained from the calibration curve:

$y=0.009x+0.0559$ $R^2 = 0.9818$, where, x is the Gallic acid equivalent (GAE) and Y is the absorbance.

Total flavonoids content was calculated as rutin equivalent (mg/g) using the equation based on the calibration curve: $y=0.0099x+0.0499$ $R^2 = 0.9281$, where X is the rutin equivalent (RuE) and Y is the absorbance. The results are given in Table 3.

Table 3: Total phenolics and flavonoids content

Test plant	Total phytochemical extract mg/g	
	Total Phenolics (GAE)	Total Flavonoids (RUE)
<i>Narcissus tazetta</i>	407 ± 0.05	27 ± 0.04
<i>Nymphaea mexicana</i>	424 ± 0.04	66 ± 0.03
<i>Indigofera heterantha</i>	342 ± 0.09	89 ± 0.06

Table 4: DPPH scavenging Assay

Substrate	DPPH % inhibition (0.1 mM DPPH)		
	200 µg/ml	400 µg/ml	600 µg/ml
<i>Narcissus tazetta</i>	21.3 ± 0.5	42.2 ± 2.3	62.1 ± 0.43
<i>Nymphaea Mexicana</i>	41.9 ± 1.5	65.9 ± 2.2	71.6 ± 0.1
<i>Indigofera heterantha</i>	48.5 ± 0.1	66.4±0.2	79.2 ± 0.1
Ascorbic Acid	64.5 ± 0.6	73.6 ± 0.3	91.9 ± 0.3

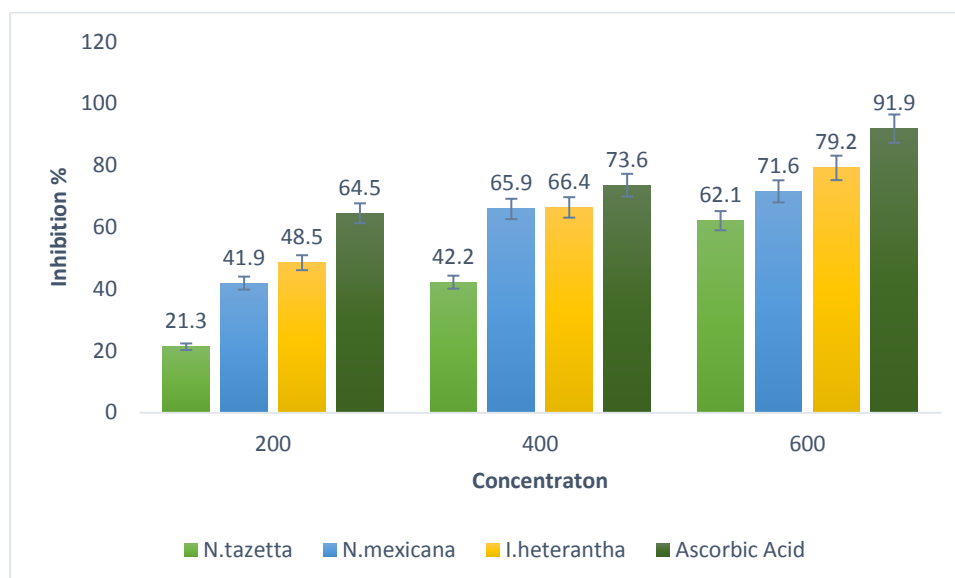


Figure 1: Bar Graph of *in vitro* free radical scavenging activity

DPPH scavenging assay is mostly accepted *in vitro* assay protocol to evaluate antioxidant potential of substances. With increase in concentration decrease in absorbance was observed. IC₅₀ value for standard was found to be 131.1 µg/ml, *Nymphaea mexicana* as 296.09 µg/ml, *Narcissus tazetta* as 479.73 µg/ml and *Indigofera heterantha* as 208.60 µg/ml. thus the antioxidant activity of all extracts was lesser than standard.

Disruption of balance between antioxidants and ROS due to either exhaustion of antioxidants or building up of ROS causes oxidative stress. Detoxification of reactive oxygen species is essential to the survival of all aerobic life forms. Thus, exploring newer sources of antioxidants is gaining importance increasingly. Antioxidants protect against neurological, cardiovascular, and various other metabolic disorders.

CONCLUSION

All the extracts have shown presence of various phytoconstituents and all these plant constituents can be positively exploited to be used as treatment strategies against different diseases. Carbohydrates, alkaloids, glycosides, flavonoids etc. were found in the extracts. Total phenolic contents of *Narcissus tazetta* bulb, *Nymphaea mexicana* flower and *Indigofera heterantha* roots were respectively 15, 6 and 4 times than corresponding flavonoid contents indicating an average of 6.5 times more phenolics than flavonoids in test plant extracts. In fact, mean yield of phenolics did not significantly differ between *Narcissus tazetta* bulbs and *Nymphaea mexicana* flowers while both exhibited significantly more phenolic content than that found in *Indigofera heterantha* roots.

The mean flavonoid content of *Indigofera heterantha* roots was not different from that of *Nymphaea mexicana* flowers while that of *Narcissus tazetta* was significantly lower than those of other two plant extracts. In this study this is evident that all the extracts are potent source of bioactive molecules.

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