Preliminary Phytochemical Screening and Quantitative Analysis of Total Flavonoid and Phenolic Content of Some Medicinal Plants Found In Nepal

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ABSTRACT

A.vulgaris L. (common Mugwort), *Nyctanthes arbor-tristis*, *Utrica diociea* also called *Titepati*, *Parijata and Sisno* respectively in Nepali, are the species with great importance in the history of medicine and were called the "mother of herbs" in the middle ages. All of them were common herbaceous plants that exhibit high morphological and phytochemical variability depending on the location where they occur. The main objective of this research paper was to identify the different phytochemical constituents qualitatively and quantitatively. The aerial parts of the plants were shaded dried, grinded and soaked in the methanol solvent to prepare the extract. The extracts were, then, examined for the qualitative tests. Different phytochemical constituents (Flavonoids, Phenols, Alkaloids, Steroids, Tannins, Terpenoids, Coumarins, Quinones, and Glycosides) were identified from the methanolic extract of the leaves of plant. Similarly, quantitatively Total Flavonoid Content (TFC) and Total Phenolic Content (TPC) were calculated by using UV/Vis Spectrophotometer. As a result, this study highly supported the medicinal use of the plant species owing their different phytochemical constituents.

Keywords: Artemisia vulgaris, Phytochemical screening, Total flavonoid, Total Phenolic

Background of the Study

Nepal occupies only 0.1 percent of the global surface area. The landscape of Nepal is predominantly composed of hills and mountains covering 83 percent of the total land area of Nepal. However, the great variation in topography with altitudes ranging from 60 m to 8,848 m over a distance of 190 km has resulted in a great diversity of plant. In terms of species richness, Nepal is the 11th position in Asia and the 25th position in the world.

Plants are the most important sources of traditional medicines throughout the world and are the sources of many major pharmaceutical drugs. At present,

about 30,000 to 70,000 plant species are using medicinally across the world and 70 % of the world's rural people depending upon such plants for their primary health care (WHO, 2002). In Nepal, a total 1950 species of plants are found to be medicinal of which 1614 species are native and much more yet to be explored. Therefore, Nepal carries a great potential for the research on plant varieties, where in the world in the single landmass.

The natural product is a term commonly used in reference to a chemical substance found in nature that have distinctive pharmacological effects. This definition encompasses many compounds such as carbohydrates, proteins, lipids, and nucleic acids, all of which play an important and primary role in metabolic reactions.

There are two types of metabolic reactions taking place in living organism primary and secondary metabolism. All the chemical products involved in the Primary metabolism are the primary metabolites which are associated with essential cellular functions such as nutrition assimilation, energy production, growth and development. Secondary metabolite in contrast to the primary metabolite are the compound that have no known primary biochemical role in producing organism but useful for interaction with others, like chemical communication and chemical defense because of their ability to modulate biochemical and signal transduction pathways.

A crude extract from natural products contain a range of structurally diverse and novel chemical compounds. Chemical diversity in nature is based on biological diversity, so researchers travel around the world obtaining samples to analyze and evaluate drug discovery screens. This is widely accepted to be true when applied to drug discovery in olden times before the advent of high-throughput screening and the post genomic era. The clinical, pharmacological, and chemical studies of traditional medicines, which were derived predominantly from plants, were the basis of most early medicines such as aspirin, digitoxin, morphine, quinine, and pilocarpine.According to recent studies conducted by the World Health Organization (WHO), about 80 % of the world's population relies on traditional medicine. (D. Sasmal. *et al.* 2007).

Medicinal plants are the plants or their parts used for the health care. It is estimated that, plant materials are present or have provided the models for 50 % of the Western drugs because of their perceived effectiveness, minimal side effects and relatively low cost. Medicinal plants are important source for the production of new drugs due to their richness in bioactive compounds but are still largely unexplored. (Singh *et al.* 2001).

Data Analysis

Primary data were obtained from lab experiment; quantitative data for it was taken in triplicate with mean and standard deviation. Calculation was carried out by Ms-excel 2007 and data was explained by bar and line graph.

Experimental equipments. The equipments use in this research work were burettes, pipettes, micropipettes thermometer, Graduate pipette, beakers, conical flasks, test tubes, reagent bottles, incubator, vials, etc. and other are listed below with their manufacturer name.

Table 1: List of experiment equipment used

Equipments	Manufacturer
Electric Grinder	Mohit
Digital weighing Balance	GT-210
Automated Water bath	Clifton
Micropepettes	Erba BIHOT
UV/Vis Sphectrophotometer	ACZET Pvt. Ltd. (0210702206)
Cuvettes	ACZET Pvt. Ltd.

Chemicals. Most of the chemicals and solvents were used of laboratory grade which are listed below:

Table 1: List of Chemicals Used

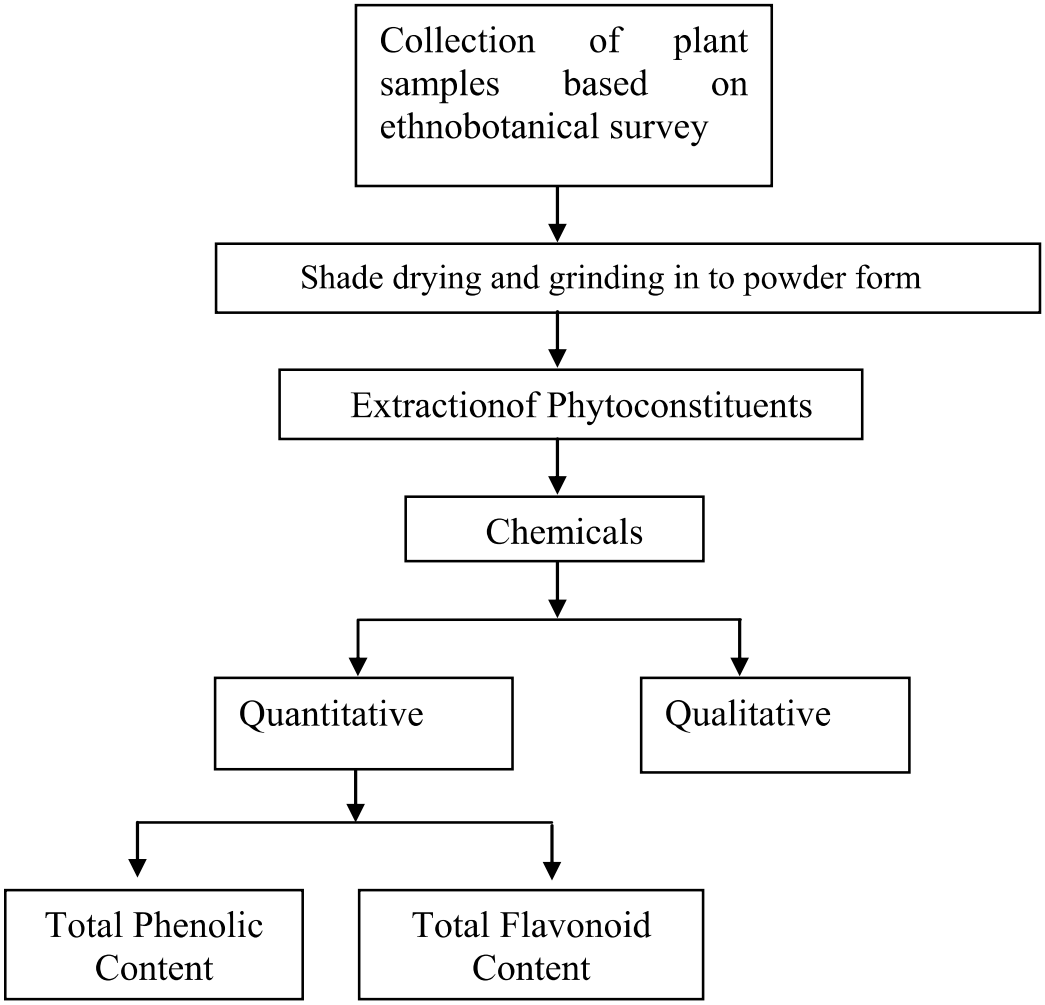
Chemicals	Manufacturer
Methanol	Wattman
Quercetin	Loba Chemie Pvt. Ltd.
Gallic Acid	Loba Chemie Pvt. Ltd.
Folin-Ciocalteu	Loba Chemie Pvt. Ltd.

Plant Material

Collection and identification of plant sample. The fresh leaves of *sample plant* were collected during November, 2021 from different district of Nepal. The collected leaves were identified by Prof. Dr. Ananta Singh Head of Department of Butwal Multiple Campus, Butwal.

Sample preparation. The collected fresh leaves of *sample plant* were washed with tap water to remove the contaminants. Then the leaves were shade dried. The shade dried leaves were grounded into powder form in electric grinder available in laboratory and the powdered sample were collected in clean plastic bag and were stored in cool and dry place until used for further experiment.

Flow chart. The flow chart to represent the methodology is given below:



Preparation of extract. The phytochemicals present in the powdered leaves were extracted by cold percolation method using methanol as a solvent. An amount of 100 g of powdered samples were weighed out by digital balance and kept in clean and dry conical flask. 250 ml methanol were added to flask and kept for 7 days with frequent shaking. After complete maceration the mixture were decanted and filtered with the help of cotton plug and solvent from thus obtained filtrate were removed by evaporation with the help of water bath maintaining temperature lower than the boiling point of the solvent (below 70^{o}C). The concentrated filtrate was kept in 100 ml beaker of known weight covered with aluminium foil containing many small holes to facilitate evaporation of solvent and left for drying. After complete drying semi solid methanolic extract of *sample plant* were obtained and were analysed for % yield. Similarly, a small portion of extract was separated in vials in order to perform phytochemical screening, biological screening and stored until use.

The percentage yield of the extract was calculated by using the following formula.

$$\% \text{ Yield} = \frac{\text{weight of the extract in g}}{\text{weight of the powdered sample taken}} \times 100\ \%$$

Preparation of different reagent

a) **Mayer's reagent:** Mercurric chloride 0.679 gm was weighed in a 50 ml volumetric flask and dissolved in distilled water. To this solution, 2.5 gm of Potassium Iodide was added. The scarlet red ppt was dissolved by shaking and volume was made upto the mark by adding distilled water.

b) **Dragendroff's reagent:** Bismuth Nitrate (4.00 gm) was dissolved in 5N Nitric acid (10 ml) to make solution A. Next potassium iodide (13.5 gm) was dissolved in distilled water (20 ml) to make solution B. These two solution was mixed together in a 50 ml Volumetric flask.

c) **Mollisch reagent:** α – Nepthol 5 gm was dissolved in 50 ml methanol to prepare the Molisch Reagent.

d) **Neutral $FeCl_3$ solution:** Ferric chloride crystals (1.0 gm) were dissolved in 100 ml distilled water. To this solution, sodium carbonate crystals were added little by little with stirring until the slight turbidity was persistent. Finally the mixture was filtered and the colourless filtrate was used as Neutral $FeCl_3$ solution.

Phytochemical Analysis

Qualitative phytochemical tests: The Methanolic extracts of the leaves of *Urtica* were used to screen for the presence of flavonoids, alkaloids, terpenoids, tannins, steroids, saponins and phenols. The screening tests for these major phytoconstituents were carried out using standard qualitative procedures as described by Trease and Evans (2002) and Adegoke *et al.* (2010).

Detection of flavonoids (Alkaline reagent test): Extracts (0.2 g) were treated with six drops of 2% sodium hydroxide solution. The formation of intense yellow colour, which developed into a colourless solution on addition of dilute acid, gave an indication of the presence of flavonoids in the extracts. (Adegoke *et al.*, 2010).

Detection of alkaloids (Mayer's test): Extracts (0.5 g) were dissolved in 3 ml of 2% dilute hydrochloric acid and the solution was divided into two test tubes and the following tests were performed.

a) **Mayer's Test:** Few drops of Mayer's reagent were added to first part, formation of pale yellow color indicates the presence of alkaloids.

b) **Dragendorff's Test (DDT):** Few drops of Dragendorff's reagent were added to the second part, formation of orange red ppt indicates the presence of alkaloids.

Detection of terpenoids (Salkowski's test): To 0.2 g of the extracts, 2 ml of chloroform ($CHCl_3$) was added followed by 3 ml of concentrated sulphuric acid carefully. The formation of reddish-brown precipitate gave an indication of the presence of terpenoids in the extracts (Trease and Evans, 2002).

Detection of tannins (Ferric chloride test): A mass of 0.2 g of the extracts were mixed with an equal volume of distilled water in a test tube and three drops of dilute ferric chloride was added. The formation of brownish blue or dark colour gave an indication of the presence of tannins in the extracts (Adegoke *et al.*, 2010).

Detection of steroids (Liebermann-Burchard's test): Extracts (0.5 g) were mixed with 2 ml of chloroform. 2 ml of concentrated sulphuric acid was then added to the mixture in a test tube. The appearance of red colour in the lower chloroform layer gave a positive result for steroids in the extracts (Adegoke *et al.*, 2010).

Detection of saponins (Foam test): To 0.2 g of the extracts, 6 ml of distilled water was added and shaken vigorously in a graduated cylinder for 15 min lengthwise. The formation of bubbles or persistent foam for 10 min gave an indication of the presence of saponins in the extracts (Trease and Evans, 2002).

Detection of phenols (Ferric chloride test): To 0.2 g of the extracts, 2 ml of 5% aqueous ferric chloride was added. The formation of dark green colour gave a positive result for phenols in the extracts (Adegoke *et al.*, 2010).

Test for quinones: To about 2 ml of extract 1 ml freshly prepared ferrous sulphate solution and few crystals of ammonium thiocynate (NH_4SCN) were added and the solution was treated with conc. H_2SO_4 drop by drop. The appearance of persistent deep red coloration indicates the presence of Quinones.

Test for gloycosides: About 0.5 g of extracts was dissolved in 2 ml of methanol amd divided into two parts ansd following test were performed.

Detection of coumarin: To about 1 ml of extract 1 ml of 10% of NaOH solution was added formation of yellow color indicates the presence of Coumarins.

Test for reducing sugar: To about 1 ml of extract 1 ml of distilled water was added followed by addition of 1 ml Fehling's Reagent (1:1 Mixing Fehling A and B). The mixture was warmed over a water bath for 30 min.The appearance of brown red ppt. indicates the presence of Reducing Sugar.

Test for proteins (Xanthoproteic test): In 2 ml of extract, 2 ml of conc. HNO_3 was added formation of orange yellow colour indicates the presence of proteins

Quantitative phytochemical determinations: The methanolic extracts were used in the quantification of detected phytochemicals. Quantitative assay was carried out for total flavonoids, phenols, saponins and alkaloids.

Determination of Total Flavonoid Content (TFC): The total flavonoids content of the crude extract was determined by aluminum chloride colorimetric method as described by Piyanete *et al.* (2009). Quercetin was used as standard and flavonoid content determined as quercetin equivalent. From the standard quercetin solution, the following concentrations (31.25, 62.5, 125, 250 and 500 µg/ml) were prepared in methanol. 100 µl of each of the quercetin dilution was mixed with 500 µl of distilled water and then with 100 µl of 5% sodium nitrate and allowed to stand for 6 min. Then 150 µl of 10% aluminum chloride solution was added and allowed to stand for 5 min after which 200 µl solution of 1 M sodium hydroxide was added sequentially.

The absorbance of this reaction mixture was measured at 510 nm using UV/Vis Spectrophotometer. The same procedure was repeated for methanolic extracts of the leaves of *sample plant* All measurements were performed in triplicate for each analysis. The total flavonoids content was determined from the linear equation of a standard curve prepared with quercetin and expressed as mg/g quercetin equivalent (QE) of dry extract.

Determination of Total Phenol Content (TPC): The total phenols content of *sample plant* extracts was determined using Folin- Ciocalteu reagent following a slightly modified method of Ainsworth and Gillespie (2007). S. Adusei *et al.* Heliyon 5 (2019). Gallic acid was used as a reference standard for plotting the calibration curve. A volume of 0.5 ml aliquot of 31.25, 62.5, 125, 250 and 500 µg/ml Gallic acid solutions were mixed with 2 ml of Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 4 ml of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min with intermittent shaking for colour development.

The absorbance of the resulting blue colour was measured at 765 nm using UV/Vis Spectrophotometer. The same procedure was repeated for both the aqueous and the methanolic extracts of the leaves of *sample plant*. All measurements were performed in triplicate for each analysis. The total phenols content was determined from the linear equation of a standard curve prepared with Gallic acid and expressed as mg/g Gallic acid equivalent (GAE) of dry extract.

Calculation of percentage yield

Dry weight of sample = 96.9 g
Dry weight of extract = 10.123 g
Percentage yield = 10.44 %

Thus, the percentage yield of methanolic extract of N. *arbotristis* was found to be 10.44

Preliminary phytochemical screening test

Table 3: Preliminary phytochemical screening test

S.N	Test	Result
1.	Alkaloids	+++
2.	Terpenoids	++
3.	Coumarins	++
4.	Flavonoids	+++
5.	Quinones	+
6.	Glycosides	-
7.	Polyphenol	+
8.	Reducing sugar	+
9.	Saponins	-
10.	Tannins	+
11.	Steroids	++
12.	Proteins	+
13.	Phenolic compounds	+

Table 4: Preliminary phytochemical screening test of *artemesia vulgaris*

S.N	**Test**	**Result**
1.	Alkaloids	+++
2.	Terpenoids	-
3.	Coumarins	+
4.	Flavonoids	+
5.	Quinones	++
6.	Glycosides	++
7.	Polyphenol	+++
8.	Reducing sugar	-
9.	Saponins	+
10.	Tannins	++
11.	Steroids	++
12.	Proteins	++
13.	Phenolic compounds	+

Table 5: Preliminary phytochemical screening test of *utrica dioica*

S.N	Test	Result
1.	Alkaloids	+++
2.	Terpenoids	-
3.	Coumarins	+
4.	Flavonoids	+
5.	Quinones	++
6.	Glycosides	++
7.	Polyphenol	+++
8.	Reducing sugar	-
9.	Saponins	+
10.	Tannins	++
11.	Steroids	++
12.	Proteins	++
13.	Phenolic compounds	+

Where, +++: Significantly present, ++: Moderately present, +: Weakly prese

Total Phenolic Content

Phenols and phenolics are very important phytoconstituents with free radical scavenging ability due to their hydroxyl groups, but the antioxidant effects do not necessarily always correlate with the presence of large quantities of phenolics and related compounds. Methanolic extract of *sample plant* leaves were subjected for the determination of total phenolic content which was done by Folin-Ciocalteu colorimetric method using Gallic acid as a standard. The absorbance was measured with the help of UV- Spectrophotometer at a wavelength of 760 nm for 31.25, 62.5, 125, 250, 500µg/ml concentration. The absorbance of the resulting blue colour was measured at 765 nm using UV/vis Spectrophotometer. The same procedure was repeated for both the aqueous and the methanolic extracts of the leaves of N. *arbotristis*. All measurements were performed in triplicate for each analysis. The total phenols content was determined from the linear equation of a standard curve prepared with Gallic acid and expressed as mg/g Gallic acid equivalent (GAE) of dry extract.

Table 6: Total Phenolic content of *Nyctanthes arbortristis*

Concentration (ppm)	Absorbance (Y)	Total Phenolic Content (TPC) mgGAE/gm
500	2.583	533
250	1.733	617
125	0.858	625
62.5	0.746	624
31.25	0.169	638

Table 7: Total phenolic content *A. vulgaris*

Concentration (ppm)	Absorbance (Y)	Total Phenolic Content (TPC) mg GAE/gm
500	0.584	273.6
250	0.311	274.3
125	0.175	276.6
62.5	0.096	297.8
31.25	0.244	370.9

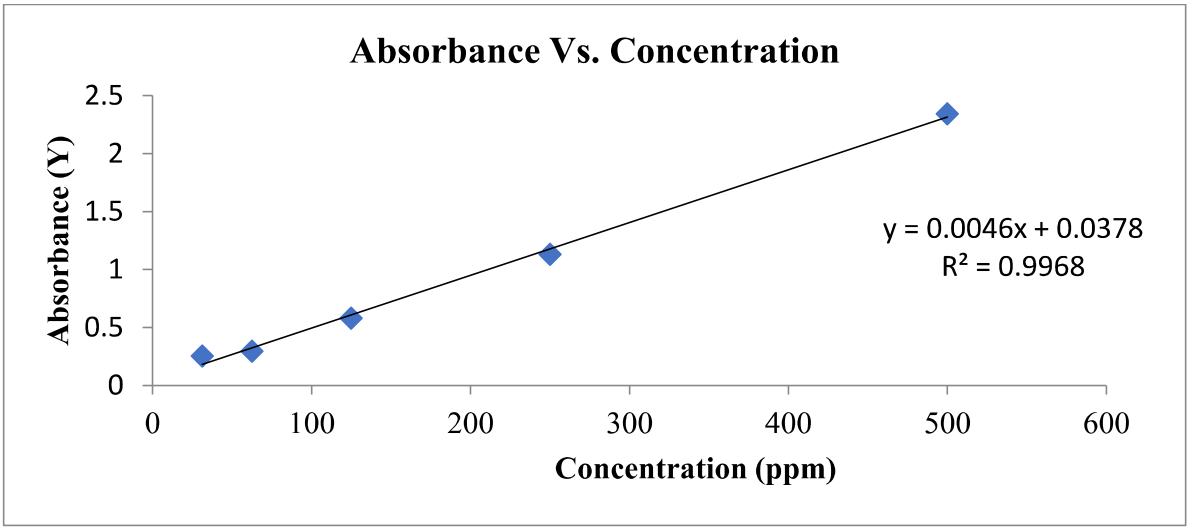
Table 8: Total phenolic content *U. Dioicea*

Concentration (ppm)	Absorbance (Y)	Total Phenolic Content (TPC) mg GAE/gm
500	0.584	380
250	0.311	383
125	0.175	420
62.5	0.096	483
31.25	0.244	487

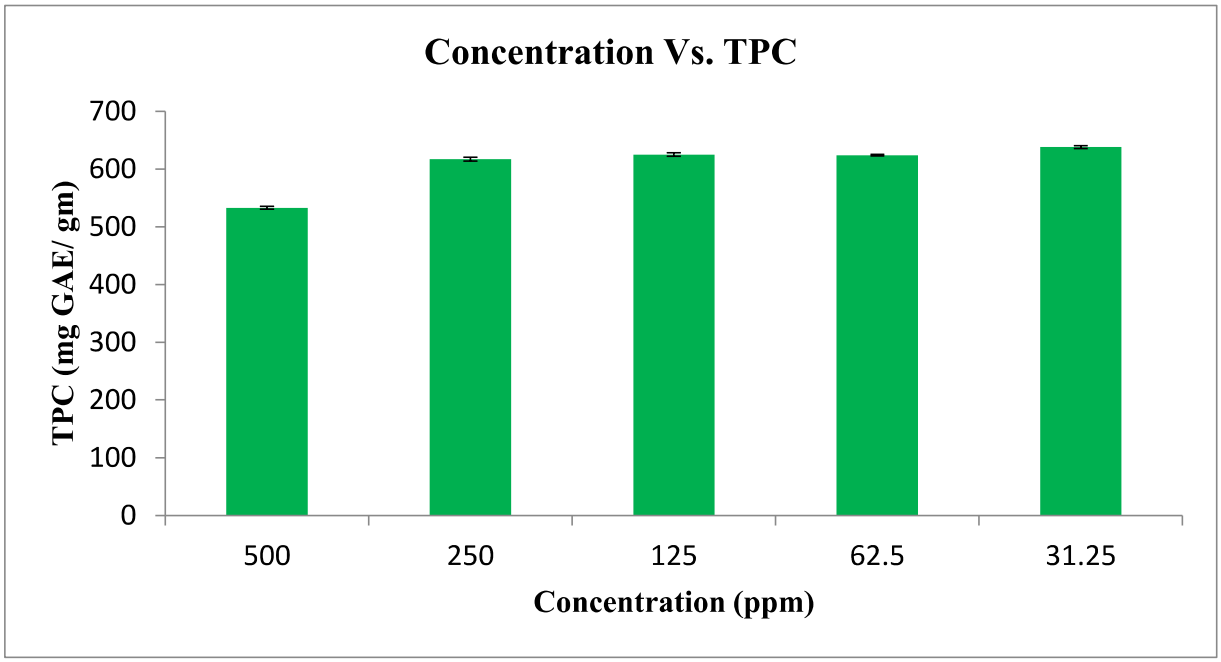
Callibration Curve for Standard Gallic Acid

Table 8: Absorbance of gallic acid at different concentration

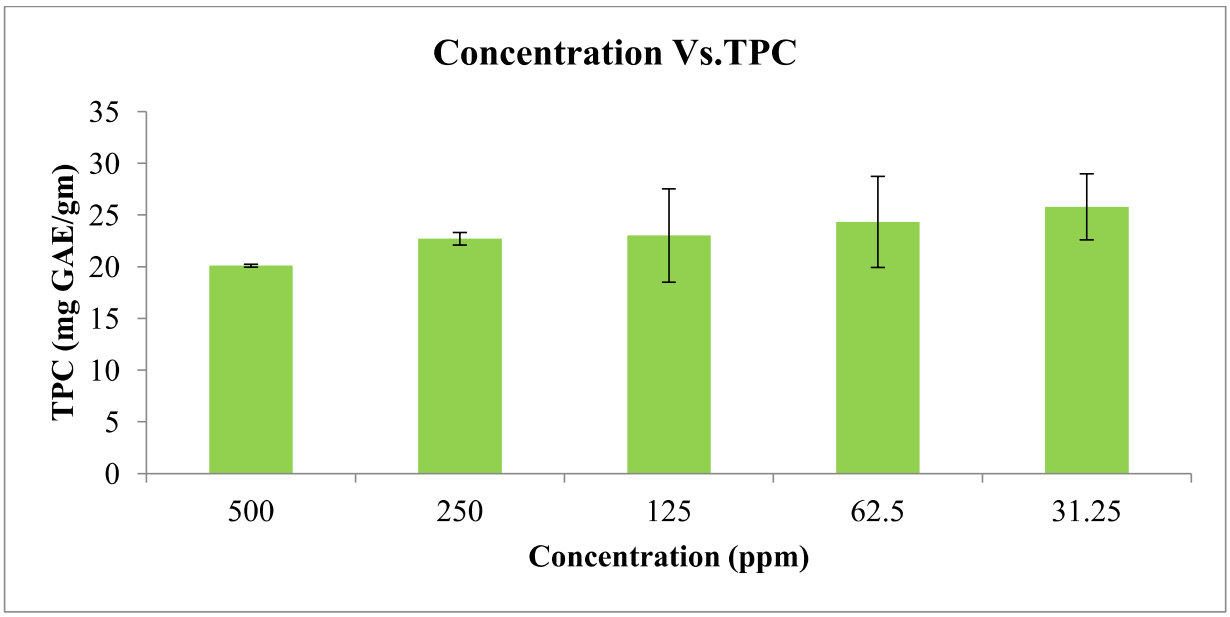
Concentration of Gallic acid (ppm)	Absorbance (Y)
500	2.343
250	1.131
125	0.579
62.5	0.294
31.25	0.253



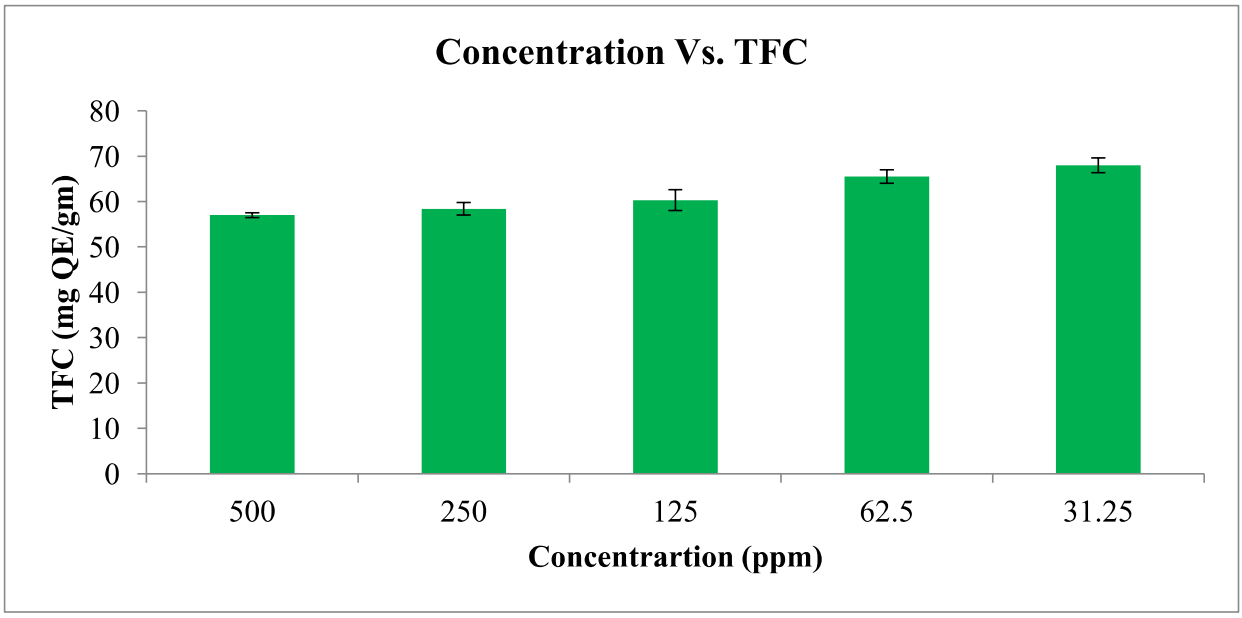
Calibration curve for standard Gallic acid



Bar Graph with Standard error of Phenolic content of *Nyctanthes*



Bar Graph with Standard error of Phenolic content of A. *vulgaris*



Bar Graph with Standard error of Phenolic content of U. *dioicea*

Total Flavonoid Content

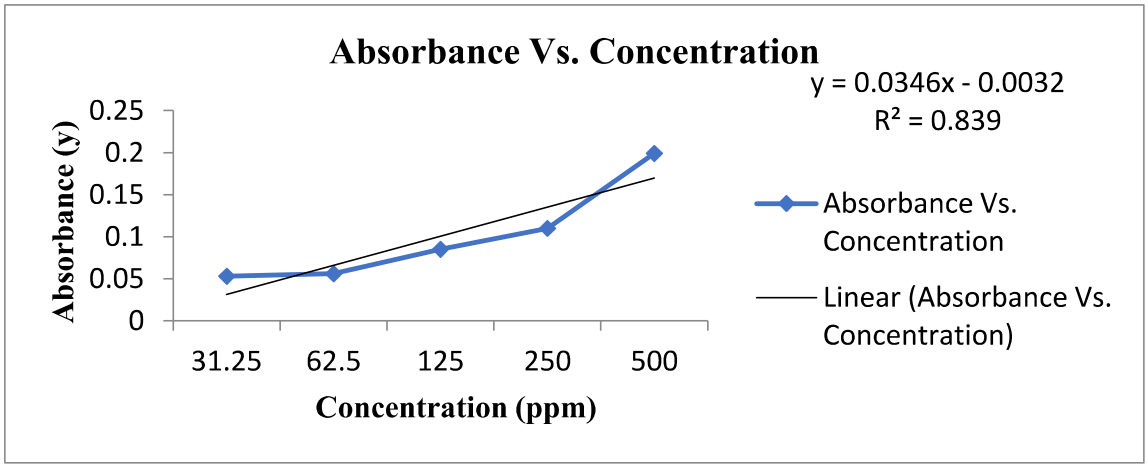
The total flavonoid content of the crude methanolic extract of leaves of *sample plant* was determined using the aluminum chloride method reported by Pekal *et al.* The absorbance was read at 510 nm. Quercetin was used as the standard. Extracts were analyzed in triplicates..

The total flavonoid content of *sample plant* was calculated from the slope (y = 0.034x) obtained from above curve. The absorbance of *sample plant* at different concentration was used to evaluate the total flavonoid content which were tabulated.

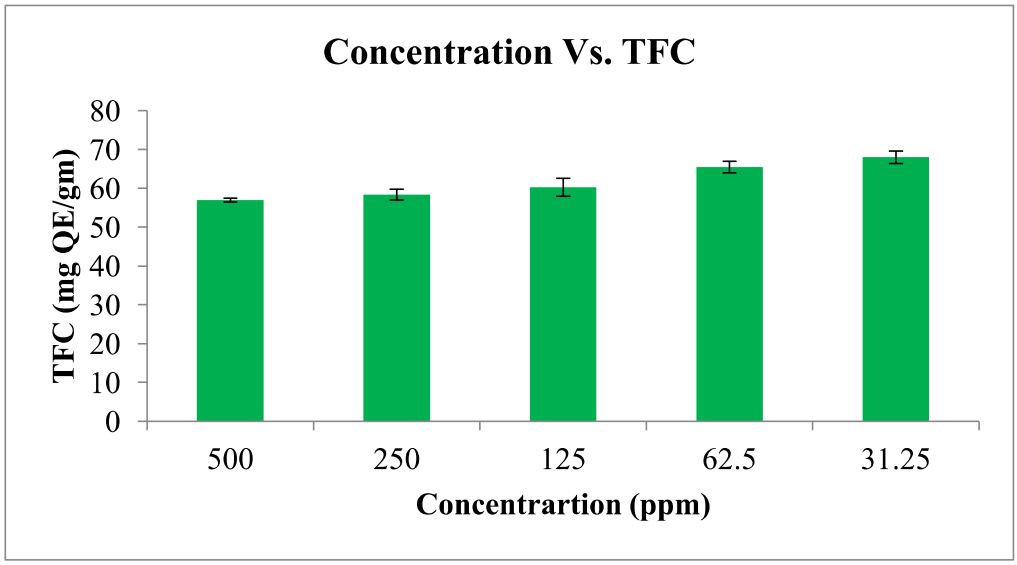
Callibration Curve for Standard Quercetin

Table 8: Absorbance of quercetin at different concentration

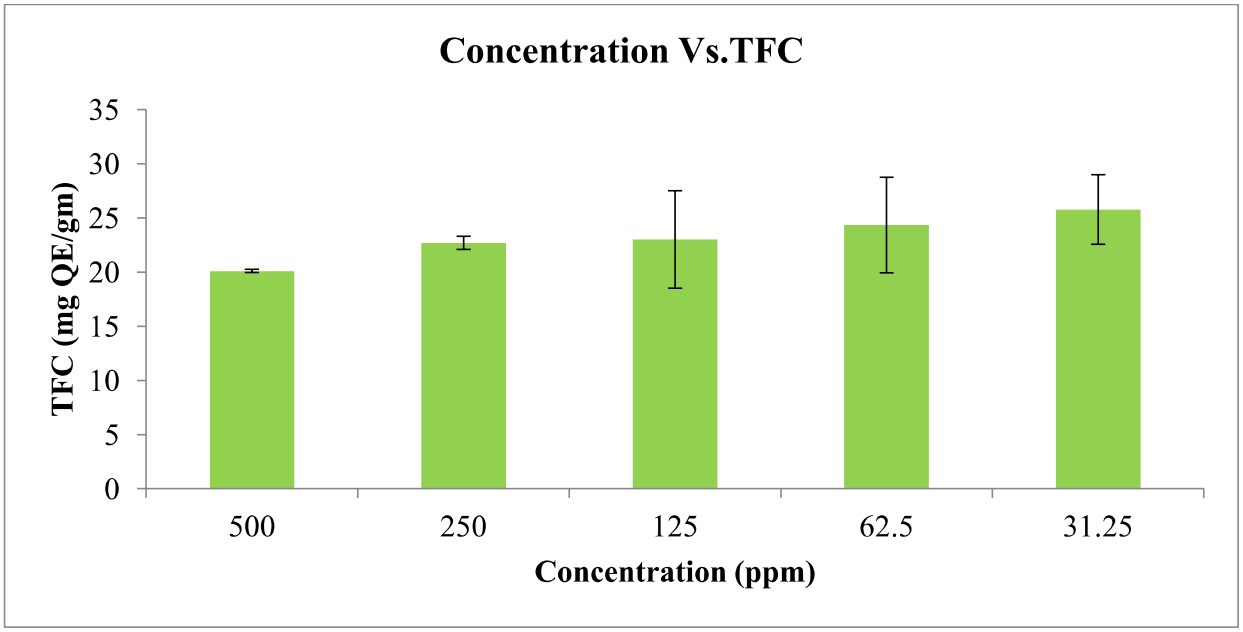
Concentration of Standard Quercetin(ppm)	Absorbance (Y)
500	0199
250	0.110
125	0.085
62.5	0.056
31.25	0.053



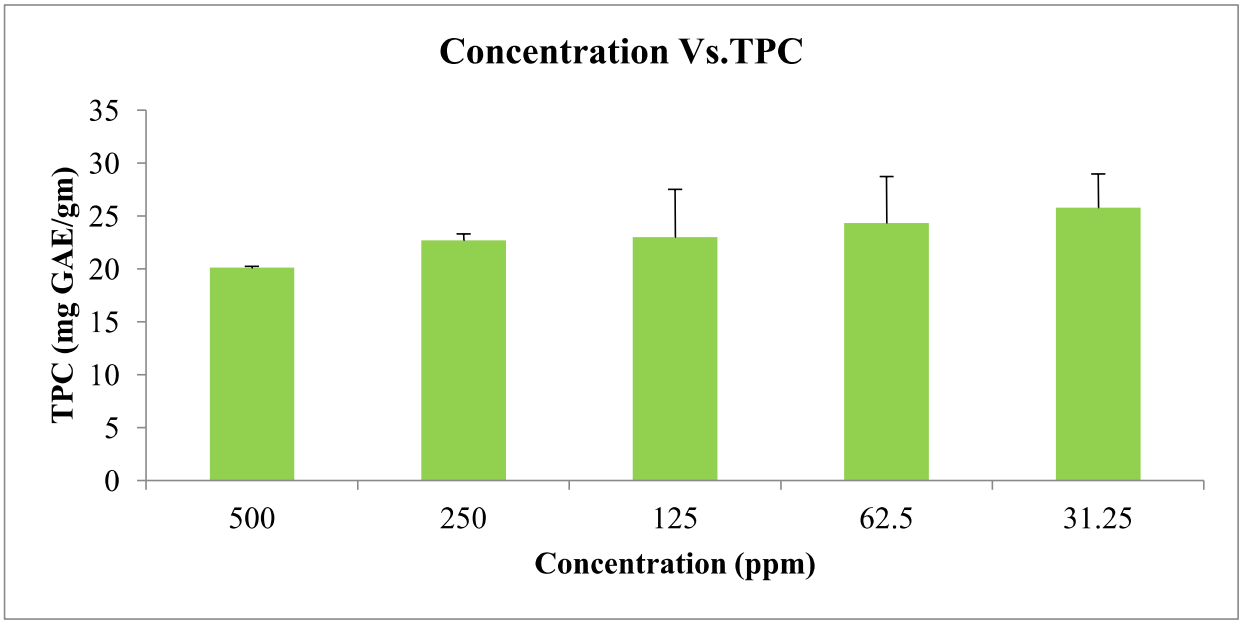
Calibration curve for standard Quercetin



Bar Graph with Standard error of Flavonoid content of *Nyctanthes*



Bar Graph with Standard error of Flavonoid content of *A. vulgaris*



Bar Graph with Standard error of Flavonoid content of *U. dioicea*

Calculations

For Total Phenolic Content (TPC). The total phenolic content in the extract was calculated using the formula

$$T = CV / m \quad \text{..... (1)}$$

Where,

T = Total content of the phenolic compounds (mg/g) in Gallic acid equivalent (GAE)

C = Concentration of Gallic acid established from the calibration curve in mg/ml

V = Volume of extract (ml) m = Weight of the plant extract in mg

Statistical analysis. Data were recorded as a mean of three determinations of absorbance for each concentration, from which linear correlation coefficient (R^2) value was calculated. The regression equation is given as:

$Y = mx + c$..... (2)

Where, y = Absorbance of the extract

m = Slope from the calibration curve

x = Concentration of the extract

c = Intercept

Using this regression equation, concentration of the extract was calculated. Thus, with the calculated value of concentration of the extract, the total phenolic content was calculated from the equation (1). The results of phenolics are expressed in terms of Gallic acid mg/g extract.

For Total Flavonoids Content (TFC). The total flavonoid content of the extract was calculated by using following formula

$T = CV/m$ ..... (3)

Where,

T = Total content of flavonoid compounds in mg/g, in Quercetin equivalent (QE)

C = Concentration of Quercetin established from calibration curve in mg/ml

V = Volume of extract in ml

m = the weight of plant extract

Statistical analysis. Linear correlation coefficient (R^2) value was calculated from the data recorded as a mean of three determinations of absorbance for each concentration. The regression equation is given as

$Y = mx + c$ ..... (4)

Where,

Y = Absorbance of extract

m = Slope from the calibration curve

x = Concentration of extract

c = Intercept

Concentration of extracts was calculated using this regression equation. Thus, the flavonoid content was calculated by the equation (3) from the calculated value of concentration of extract.

Conclusion

The phytochemical screening of methanol extracts of leaves of *sample plants* revealed the presence of alkaloids, flavanoids, tannins, polyphenols, steroids, glycosides, terpenoids, Coumarins, and Quinones whereas the test for saponins and Reducing Sugar showed negative result.

The total phenolic content present in methanolic extract of *Nyctanthes arbortristis*, A.*vulgaris and Urtica diociea* was found to 638 ± 2.6 mg, 370.9 ± 6.45 and 496 ± 3.2 Gallic acid equivalent per gram (mg GAE/gm) respectively. The amount of total flavonoid content present in methanolic extract of *Nyctanthes arbortristis*, A.*vulgaris and Urtica diociea* calculated in this study was 68 ± 1.6, 25.78 ± 3.2 and 78 ± 2.6 mg Quercetin equivalent per gram (mg QE/mg).

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