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PHYTOCHEMICAL SCREENING, EVALUATION OF ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND FLAVONOID CONTENT OF SELECTED NEPALESE MEDICINAL PLANTS

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ABSTRACT

This research work aimed to analyze the phytochemicals, total phenolic, flavonoid contents, and antioxidant properties of four selected medicinal plants of Gulmi and Rupandehi districts using in vitro studies. They were Dioscorea bulbifera from Gulmi, the other three Aegle marmelos, Nyctanthes arbortristis, Urtica dioica from Rupandehi. Methanolic extract of these plants was analyzed. The DPPH radical scavenging assay was used to measure the antioxidant activity, the Folin-Ciocalteu method to measure total phenolic content, and the aluminum chloride colorimetric method to measure total flavonoid content. Results revealed that plant extract contained phytochemicals such as flavonoids, alkaloids, polyphenols, terpenoids, saponins, quinonnes, etc. The Dioscorea bulbifera showed the highest % of radical scavenging activity up to 82.31±2.43µg/ml with an IC_{50} value of 51.102±6.424µg/ml followed by Nyctanthes arbortristis and the lowest in Urtica dioica with IC_{50} value 70.716±1.55 µg/ml, 179.103±3.580 respectively. Dioscorea bulbifera revealed the highest TPC and TFC of 136.667±5.08 mgGAE/g and 33.41±1.720 mgQE/g followed by Nyctanthes arbortristis and lowest in Urtica dioica. These parameters were analyzed from the period of 15 September 2021 to 20 October, 2021.

Keywords: Antioxidant, 2,2-Diphenyl-1-picrylhydrazyl, Flavonoids, Phenolic content, Phytochemicals.

INTRODUCTION

Due to its distinctive geographical variances, Nepal is home to an enormous variety of medicinal plants. Nepal is located in the portion of Central Himalayas and has contributed about 10% of medicinal plants of the expected 7000 species of flowering plants(Muhammad et al., 2011). The Gulmi and Rupandehi districts of Nepal are rich in biodiversity (GC et al., 2019). Medicinal plants are potent sources of medicine to treat various diseases. Despite the widespread use of medicinal plants in Nepal, there are limited studies on phytoconstituents and their antioxidant activity (Manandhar et al., 2002). Synthetic drugs are used to cure various diseases but they are expensive & if used in the long run, they show harmful side effects. Hence, drug development from natural products is a promising field. So, it is necessary to identify them, analyze and explore the antioxidant flavonoid and phenolic contents in the natural resources of Nepal. Hence, the present study mainly focused on four traditionally used medicinal plants found in Gulmi and Rupandehi districts Oxidative stress causes the human body to develop chronic illnesses like diabetes, heart disease, and cancer (Rezaeizadeh et al., 2011). According to Naziroglu et al. (2004), oxidative stress damages the body's ability to maintain a balance between the production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and the quantity of antioxidants. This finally results in cell death.

The primary sources of free radicals that cause major diseases including Alzheimer's disease, Parkinson's disease, and strokes are reactive nitrogen species (RNS) and ROS (Willcox et al., 2004). Smooth muscle relaxation, neuronal signaling, the prevention of platelet aggregation, and other physiological processes are all powerfully mediated by nitric oxide (NO), a powerful mediator. Superoxide and NO radicals play a role in host defense, but their excessive synthesis is the cause of inflammatory illnesses (Halliwell et al., 1989). Antioxidants slow down or stop the oxidative processes that lead to oxidative damage (Shyur et al., 2005). Antioxidants should be consumed properly to prevent diseases and reduce health issues. In the present context, many antioxidants are manufactured synthetically such as tertiary butylated hydroxyquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT). These artificial antioxidants have several harmful side effects. A significant number of antioxidants are found in natural products, primarily in meals, which reduce oxidative harm (Elmastaş et al., 2006). Plants contain

phytochemicals such as flavonoids, phenolic diterpenes, phenolic acid, and tannins that are beneficial sources of natural antioxidants (Lee et al., 2004). Plant extracts, particularly flavonoids (polyphenols), are potent antioxidants (Bernards et al., 2010). Flavonoids are potent free radicals and the most oxidizing molecular scavengers (Shrestha et al., 2022). According to Kharel et al., (2020), S. pinnata extract had the highest percentage of radical scavenging activity, reaching 87.94±1.88, with an IC_{50} of 17.51±1.27 g/mL, followed by B. variegata, while Urtica dioica had the lowest percentage (Kharel et al., 2020). According to Kharel et al., hydro-alcoholic extract of Urtica dioica demonstrated effective antioxidant activity with an IC50 value of 88.33 ± 2.88 µg/ml. The presence of phenolic compounds suggests that the presence of ferulic acid may be the cause of the antioxidant activity (Khare et al., 2012). Because of the presence of glycosides and phenolic compounds, extracts of Nyctanthes arbor-tristis have a variety of pharmacological effects, including hepatoprotective, antileishmanial, antipyretic, antihistaminic, antimalarial, antibacterial, anti-inflammatory, antioxidant, antiviral, and antifungal effects (Venkataraman et al., 2019). Similarly, Dioscorea bulbifera fruit powder's scavenging activity in vacuum drying ranged from 65.36% to 81.33% at concentrations of 200 g/ml to 1000 g/ml. which was the highest radical scavenging activity so an effective method to protect the antioxidant properties of Dioscorea bulbifera fruit powders is vacuum drying(Wijewardana et al., 2016). By Charoensiddhi et.al., (2008) the antioxidant activities in aegle marmelos fruit by DPPH free radical scavenging and FRAP assays resulted in 6.21 µg dw/ µg DPPH and 102.74 µM trolox equivalent (TE)/ g dw, respectively. Similarly, n-butanol and ethyl acetate fraction of Justicia adhatoda extract exhibited high antioxidant activity with a minimum IC_{50} value of < 105.33 µg/ml (Kaur et al., 2015). According to Padhan et.al., (2020) the total phenolic, flavonoid, and total antioxidant capacity of the samples, Dioscorea species ranged from 2.19 to 9.62 mg g−1 dry weight, 0.62–0.85 mg g−1 dry weight, and 1.63–5.59%, respectively

The IC_{50} values were found 77.9–1164, µg ml−1 for DPPH, scavenging activity (Padhan et al., 2020). However, there is little attempt has been carried out for the systematic screening and search of the bio-active components from traditionally used Nepalese medicinal plants for new natural drug discovery.

The present study was to analyze secondary metabolites, total phenolic, flavonoid contents, and antioxidant activity of four different medicinal plants.

MATERIALS AND METHODS

Collecting and identifying plant samples

Four medicinal plants' various components were collected from Rupandehi and Gulmi Districts (Dioscorea bulbifera from Gulmi, other three from Rupandehi). These plants were collected from15 March 2021 to 25 April 2021 and were identified by Botanist Ananta Gopal Singh Ph.D., Department of Botany, Butwal Multiple Campus.

Table 1: List of selected medicinal plants

Name of Plants	Local Name	Utilized parts	Family
Aegle marmelos	bel	Raw fruits	*Rutaceae*
Dioscorea bulbifera	Vyakur	Tubers, bulbils	*Dioscoreaceae*
Nyctanthes arbor-tristis	Parijat	Leaves	*Oleaceae*
Urtica dioica	Sisnu	Leaf	*Urticaceae*

Drying and extract preparation

The raw fruits and leaves of the plant were collected locally and processed. The collected plant's parts were washed and dried at the shade ambient temperature. They were mechanically ground into a powder after being cut into pieces. and stored in a clean plastic bag at a low temperature. The extract was prepared by the Cold percolation method. Dried powdered parts (150 gm) were mixed with 400 ml methanol in separate clean and dry conical flasks. The flask was properly sealed, and extraction was carried out periodically for 72 hours while being frequently shaken and filtered. A rotary evaporator was used to concentrate the filtrate. Different plant species' solid methanolic extracts were obtained and kept in a refrigerator at 4°C until analyzed (Kharel et al., 2020).

Chemicals and reagents used

All chemicals used were of analytical grade. Methanol, NH_4SCN, $FeSO_4$, NH4OH, Distilled water, $NaNO_2$, $AlCl_3$, Aluminium chloride, hydrochloric acid, ascorbic acid (LOBA Chemie Pvt. ltd), Fehling's solution, alpha-Naphthol, $FeCl_3$, $NaHCO_3$, $Bi(NO_3)_3$, KI, $HgCl_2$, Picric acid, sodium carbonate, disodium hydrogen phosphate, sodium chloride, sodium hydroxide, sodium nitroprusside, dimethyl sulphoxide, etc. The Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Gallic acid, and Quercetin were purchased

from Qualigens Fine Chemicals, India. Meyer's reagent, Dragendorff's reagent, Molisch's reagent, and other reagents were made into laboratory-grade formulations.

Apparatus used

Mechanical grinder, digital balance, hot air oven, cuvettes, burettes, pipettes, micropipettes, thermometer, condensers, beakers, conical flasks, test tubes, vial tubes, round bottom flasks, Rotary evaporator, UV-visible spectrophotometer (EI machinery Scanning Visible Spectrophotometer 2306, Product ID:201605028).

Phytochemical analysis

The technique utilized followed the phytochemical screening protocol provided by Ciulei I et al. (2013). There were found to be numerous secondary metabolites (bioactive substances) in plants. Utilizing several particular reagents, the analysis was carried out utilizing the color reaction. The qualitative findings are shown as (+) for the existence of phytochemicals, (++) for their substantial presence, and (-) for their lack.

The phytochemical screening was carried out to test for Alkaloids (Meyer’s test, Dragendorff’s test), Coumarins, Flavonoids, Quinones, Polyphenols, Glycosides, Reducing sugar, Saponins, Tannins, and carbohydrates (Molish’s test) respectively.

Table 2: Phytochemical analysis of various plant samples' methanolic extracts

Phytochemicals	A.M	D.B	N.A	U.D
Alkaloids	+	+	+	+
Terpenoids	+	+	+	+
Coumarins	+	+	+	+
Flavonoids	+	+ +	+	-
Quinones	+	+	+	+
Polyphenols	+	+	+	-
Glycosides	+	+ +	+	-
Reducing sugar	+ +	+ +	-	-
Saponins	+	+ +	+	-
Tannins	+	-	+	-
Carbohydrates	+ +	+ +	-	+

Note: A.M=Aegle marmelos, D.B=Dioscorea bulbifera, N.A=Nyctanthes arbortristis, U.D=Urtica dioica, (-) for absence & (+) for presence.

Estimation of antioxidant activity (DPPH radical scavenging assay)

The DPPH radical scavenging assay was conducted following the protocol provided by Paulsamy et al., (2012). For each plant sample, 2 ml of extract at various concentrations (10 g/ml to 100 g/ml) were mixed with 2 ml of DPPH solution (60 µM). To ensure a full reaction, the mixture was let to stand in the dark for 30 minutes. Finally, a UV spectrophotometer was used to assess each plant sample's absorbance at 517 nm. Using the following formula, the radical scavenging activity of each sample was determined.:

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

A_1 is the absorbance of the sample extract, while A_0 is the absorbance of the control. The test solution without a sample is the control. The standard was ascorbic acid. The same process was applied when using an ascorbic acid solution with concentrations ranging from 10µg/ml to 100 µg/ml. Each sample's antioxidant activity was quantified using its IC_{50} value. The amount of sample needed to effectively scavenge 50% of the DPPH free radicals is known as the 50% inhibitory concentration which is IC_{50} value. By graphing the extract concentration versus the corresponding scavenging action, the inhibition curve was used to determine the IC_{50} values (Giri et al., 2022).

Determination of total phenolic content

With a few adjustments, following protocol given by Khan et al., (2018) the total phenolic content was determined (Shackelford et al., 2009). The Folin-Ciocalteu method was used to determine it, with gallic acid serving as the standard. 5 ml of 10% Folin-Ciocalteu reagent were combined with 1 ml of crude extract. It was allowed to stand for 5 minutes before being well mixed with 4 ml of 7% (w/v) sodium carbonate. After 40 minutes of incubation, the mixture's absorbance at 760 nm was measured. Every experiment was run in triplicate. Gallic acid was used as the standard in the creation of the calibration curve. The calibration curve was used to quantify the total phenolic content (TPC), and the results were represented as mg GAE/gram dry weight of the extract.

The calculation was performed using the formula TPC=CV/M, where C=conc of gallic acid received from the calibration curve in mg/ml, V=volume of extract in ml, M=weight of plant extract in mg, and R^2 the linear correlation coefficient was derived from the gallic acid calibration curve. The concentration of each extract was determined using

the regression equation. Consequently, the TPC was determined using the calculated value of each extract's concentration (Shrestha et al., 2022).

Estimation of total flavonoid content

With a little adjustment, protocol given by Bag et al., (2015), the flavonoid concentration was determined using the aluminum chloride colorimetric method. Quick addition of 0.3 ml of 5% sodium nitrite was made after combining 1 ml of each extract solution with 4 ml of distilled water. After waiting for five minutes, added 0.3 ml of 10% aluminum chloride and let it stand for six minutes. After shaking the mixture well and adding 2 ml of 1 M sodium hydroxide and 2.4 ml of distilled water to make the volume 10 ml, the absorbance at 510 nm was measured using the UV spectrophotometer. Quercetin was used as the standard in the preparation of the calibration curve (Shrestha et al., 2022). The calibration curve was used to determine the total flavonoid content, which was then represented as mg of quercetin equivalent (QE) per gram of dry extract weight

Statistical analysis

There were three copies of each experiment run. The mean and ±standard deviation (SD) was used to express the results. The IC_{50} values and all the statistical analysis were carried out through Microsoft Excel 2016.

RESULTS AND DISCUSSION

Yield value of extract

The methanolic solvent was used to extract the chosen plant sample(s). The yield percentage of methanolic extract of plant samples is given in Table 2.

Table 3: Extract yield percentage of plant samples in the methanolic solvent

Name of Plants	Extract Yield %
Aegle marmelos	12.42%
Dioscorea bulbifera	6.75%
Nyctanthes arbor-tristis	10.28%
Urtica dioica	11.42%

Estimation of antioxidant activity (Variation of DPPH radical scavenging activity)

Figure 1 displays the percentage of DPPH radical scavenging activity in methanol at various concentrations, whereas Table 3 displays the DPPH radical scavenging activity represented as an IC_{50} value. The highest DPPH radical scavenging activity with a minimum IC_{50} value was found in Dioscorea bulbifera of 51.102µg/ml and followed by Nyctanthes arbor-tristis, IC_{50} value of 70.7166 µg/ml whereas Urtica dioica with IC_{50} value of 179.5361 µg/ml possessed very low DPPH radical scavenging activity regarding ascorbic acid standard (IC_{50} value 55.40 µg/ml). This assay is a straightforward and frequently used method to assess the antioxidant potency of plant extracts. The visible quenching of the stable purple-colored DPPH radical to the yellow-colored DPPH represents antioxidant action.

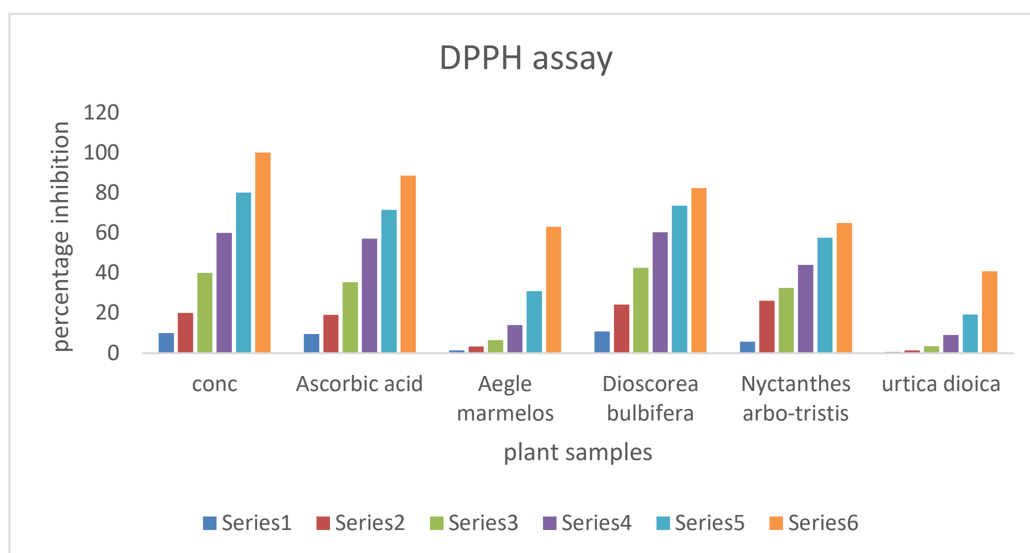

Figure 1: Percentage Results of ascorbic acid-related scavenging of DPPH free radicals by various plant extracts are expressed as the mean± standard deviation (n=3) at concentrations of 10, 20, 40, 60, 80, and 100 µg/ml.

Table 4: Ascorbic acid and methanolic extract of plant samples' ability to scavenge DPPH radicals as measured by their IC_{50} values

S.N	Name of plant extracts/reference	IC_{50} (µg/mL), mean±SD
1.	Ascorbic acid (reference)	55.40±0.89
2.	Aegle marmelos	99.872±1.27
4.	Dioscorea bulbifera	51.102±6.42
5.	Nyctanthes arbotristis	70.506±1.55
6.	Urtica dioica	179.103±3.58

Note: A.M: Aegle marmelos, D.B:Dioscorea bulbifera, N.A: Nyctanthes arbotristis, U.D:Urtica dioica, SD: Standard deviation, IC_{50}: Half maximal inhibitory concentration

Variation of Total phenolic content

By applying the Folin-Ciocalteu method and gallic acid as a reference, the total phenolic content of methanolic extracts was calculated. At 760 nm, the highest absorption was noted. Beer's Law was validated at 760 nm by a gallic acid solution with a concentration of 10–100 µg/ml and a regression coefficient R^2 of 0.9698. (Figure 2). With the aid of a calibration curve using gallic acid as the reference, the total phenolic content was determined and expressed as mg GAE/g dry extract weight. The total phenolic content was found highest in Dioscorea bulbifera (136.667±5.08 mg GAE/g dry extract weight) and was estimated to be lowest in Urtica dioica (24.36±1.33 mg GAE/g dry weight).

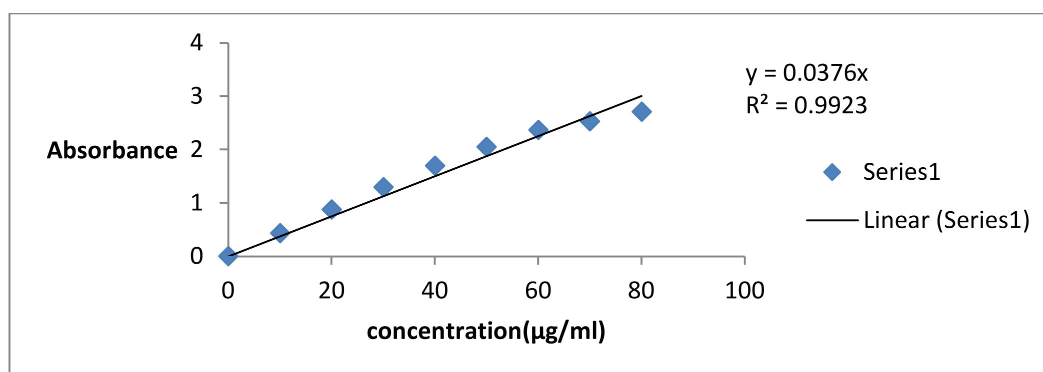

Figure 2: Calibration curve for gallic acid.

Table 5: Total phenolic content of methanolic extract of plant samples

Name of plants	Absorbance			TPC (mg GAE/g)			Mean±SD
	A1	A2	A3	C1	C2	C3	
A.M	1.645	1.646	1.624	88.919	88.973	87.78	88.559±.6.715
D.B	2.567	2.421	2.597	138.757	130.865	140.378	136.667±5.08
N.A	1.846	1.664	1.915	99.7831	89.8459	103.514	97.647±7.01
U.D	0.473	0.424	0.455	25.5676	22.9189	24.5946	24.36±1.33

Note: GAE: Gallic acid equivalent, SD: Standard deviation, A.M: Aegle marmelos, D.B: Dioscorea bulbifera, N.A: Nyctanthes arbo-tristis,U.D:Urtica dioica, TPC: Total phenolic content

Variation of Total flavonoid content

Using quercetin as a reference, the total flavonoid content of methanolic extracts was determined using the aluminum chloride colorimetric technique following protocol Bag et al., 2015. Beer's Law was followed by the quercetin solution of concentration(10-100µg/ml) at 510 nm, with a regression coefficient R^2 of 0.9729. (Figure 3). When expressed as mg QE/g dry extract weight, the total flavonoid content was found to be highest in Dioscorea bulbifera (33.41±1.720 mgQE/g) and lowest in Urtica dioica (10.838±5.231).

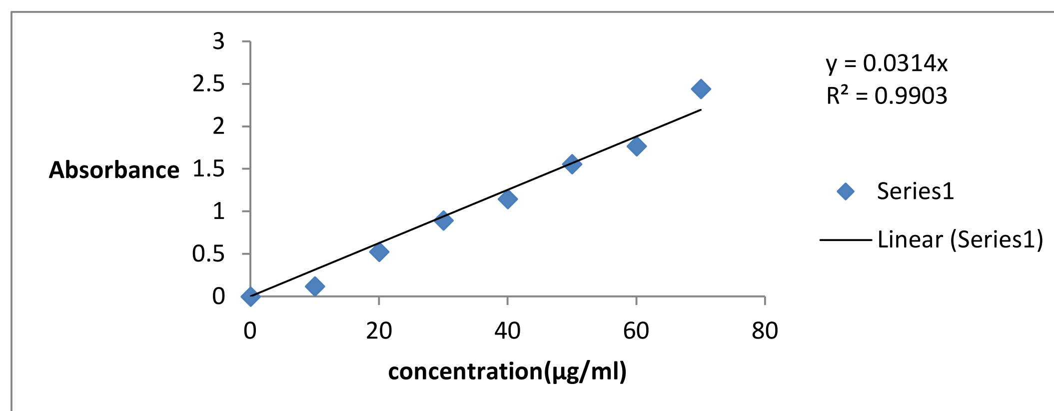

Figure 3: Quercetin calibration curve

Table 6: Total flavonoid content of different plant extracts

Plants name	Absorbance			TFC (mg QE/g)			Mean±SD
	A1	A2	A3	C1	C2	C3	
A.M	0.172	0.171	0.168	11.0968	11.0323	10.838	10.989±0.1343
D.B	0.497	0.509	0.548	32.064	32.838	35.354	33.41±1.720
N.A	0.2	0.323	0.223.	17.612	20.838	14.387	17.61±3.225
U.D	0.172	0.171	0.168	11.0468	11.032	10.438	10.838±5.231

Note: A.M:Aegle marmelos, D.B: Dioscorea bulbifera,N.A:Nyctanthes arbotristis,U.D:Urtica dioica, QE: Quercetin equivalent, SD: Standard deviation, TFC: Total flavonoid content (n=3)

CONCLUSION AND IMPLICATION

The DPPH radical scavenging activities and subsequent IC_{50} values of the selected plants' methanolic extracts revealed various degrees of antioxidant capability, with Dioscorea bulbifera exhibiting the highest percent scavenging activity and N. arbor-tristis the second-highest. The normal ascorbic acid has an IC_{50} value of 55.40±0.89 g/ml, but D. bulbifera exhibits the maximum percentage of scavenging at 51.102±6.42 g/ml. Their increased antioxidant capacity may be attributable to the presence of chemically active components like phenol, flavonoid, tannin, terpene, etc. Additionally, D. bulbifera had the greatest TPC and TFC levels, followed by N. arbo-tristis and A. marmelos, and U. dioica had the lowest levels. Consequently, these plants may serve as potential sources of free radical scavengers. Even though several medicinal plants demonstrated considerable antioxidant properties, they cannot be used directly as pharmaceuticals. It is necessary to do additional, in-depth phytochemical and pharmacological research. Further research is required to determine whether these plants are a potential source of natural medicines.

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