

Antioxidant Activity, Total Phenol and Flavonoid Contents in Some Selected Medicinal Plants of Nepal

¹Sabina Parajuli, ¹Nirmala Tilija Pun, ¹Shraddha Parajuli, ¹Nirmala Jamarkattel-Pandit

¹School of Health and Allied Sciences, Pokhara University, Nepal

ABSTRACT

Different exogenous factors and normal metabolism of oxygen in human body lead to the generation of free radicals. Excess of such free radicals can cause oxidative damage resulting to different diseases. Antioxidants prevent the production of such highly reactive species; scavenge free radicals and aid in repairing from oxidative damage. Natural antioxidants from plant species are considered safer and bioactive and people are now more interested in finding out the treatment through the natural remedies. The aim of this study was to determine the antioxidant activity of some selected medicinal plants of Nepal and find out their total phenol and flavonoid contents. For the determination of antioxidant activity, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and ferric to ferrous reduction ability were performed. Folin Ciocalteu method was used to determine total phenols contents while aluminum chloride colorimetric method was used for determination of total flavonoids contents of selected plant samples. Among the studied plants, *Diplazium stoliczkae*, *Mimosa rubicaulis himalayana*, *Stephania japonica* and *Drynaria propinqua* showed strong free radical scavenging activity and reducing ferric to ferrous transformation power along with IC50 value almost similar to ascorbic acid. The extracts of these plants also showed higher contents of total phenol and flavonoids. These findings suggest that these plants could possess therapeutic effects arising from their antioxidant activity, in area such as various degenerative diseases and also give a scientific basis to the traditional uses of the investigated plants.

Key words: *Nepalese medicinal plants, Antioxidant, Phenol, Flavonoids, DPPH*

Corresponding address: *Nirmala Jamarkattel-Pandit, PhD, Associate Professor, School of Health and Allied Sciences, Pokhara University, Nepal, E-mail: nirmalajk@hotmail.com*

INTRODUCTION

Free radicals such as superoxide anions, hydrogen peroxide and hydroxyl radicals, which are highly reactive species, are generated due to different exogenous factors and normal metabolism of oxygen.¹ These molecules cause depletion of naturally prevailing antioxidants leading to oxidative stress along with change in gene expression, production of abnormal protein and biochemical damage of the cells and tissues, which results in the diseases like atherosclerosis, cancer, hypertension, diabetes mellitus, renal failure, liver diseases and early aging.^{2,3} Antioxidants prevent the production of such highly reactive oxygen species; scavenge free radicals; aids in repairing from oxidative damage and effective functioning of naturally prevailing antioxidants in body.⁴ They are added in dietary supplements as nutraceuticals, food additives to prevent food deterioration, medicine, food and cosmetics as preservatives.⁵

At present, most of the antioxidants are manufactured synthetically. Several synthetic antioxidants, e.g., butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylated hydroquinone (TBHQ) and gallic acid esters are commercially available. Such synthetic antioxidants are known to have potential side effects and toxicity when taken in vivo, hence, their use is being restricted nowadays and there is increase interest in finding out safer and bioactive natural antioxidants present in plant species.^{6,7} Phenols are secondary metabolites in plants and phenolic compounds isolated from plant species are known to have various biochemical activities such as antioxidant, antimutagenic, anticarcinogenic and also modify the gene expression.⁸

Likewise various flavonoids isolated from medicinal plants have been reported with antioxidative, anti-inflammatory, oestrogenic, antimicrobial, antiallergic, cardiovascular and cytotoxic antitumour activities.⁹

Plants are the universal resources of various medicines. Due to geographic and climatic diversity, the relatively small country Nepal [latitudes 26°22' and 30°27' north and longitudes 80°4' and 88°12' east] is rich in biological diversity. About 90% of Nepalese populations, residing in rural areas where modern health care facilities are lacking, are still using the medicinal plants for their primary health care.¹⁰ Despite widespread use of medicinal plants in Nepal, there are limited studies of these plants for their antioxidant activity. The main aim of this study was to determine the antioxidant activity of some selected medicinal plants of Nepal and to find out their total phenol and flavonoid contents. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and ferric to ferrous ion reduction ability was performed to determine antioxidant activity. Folin Ciocalteu and aluminum chloride colorimetric method were used to determine total phenol and flavonoid contents of the selected medicinal plants respectively.

MATERIAL AND METHODS

Chemicals and reagents

Gallic acid, quercetin and 2, 2-diphenyl-1 picrylhydrazyl (DPPH) radical were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Aluminum chloride, Ascorbic acid, Folin-Ciocalteu reagent and Trichloroacetic acid were

purchased from Qualigens Fine Chemicals (India). All chemicals and reagents used were of analytical grade.

Plant materials

Plant parts of *Stephania japonica*, *Amaranthus viridis*, *Mimosa rubicaulis himalayana*, *Schima wallichii*, *Aeschynanthus parviflorus*, *Diplazium stoliczkae*, *Drynaria propinqua*, *Maoutia puya*, *Colocasia esculenta*, *Pogostemon benghalensis*, *Berberis aristata* and *Bryophyllum pinnatum* were collected from central region of Nepal, Pokhara, Kaski (Table 1). All the plants were collected from the original sources. Crude samples of the collected plants were kept in the Pharmacognosy Laboratory of School of Health and Allied Sciences, Pokhara University, Lekhnath, Nepal.

Extraction

After collection of plants, they were washed with water and cut into pieces. They were allowed to dry in shades. After complete drying of these plant samples, they were ground into smaller pieces. Each plant sample of 50 g was mixed with 400 ml of methanol by maceration for 4 days (96 hours). The methanol solutions were then filtered and the filtrates were dried using rotatory evaporator under reduced pressure. The extract yields obtained from the methanol extraction are shown in Table 1. Stock solutions (10 mg/ml) of each plant extract were prepared by using methanol as solvent.

Determination of antioxidant activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical assay

The DPPH radical assay was performed according to the method of Kim et al., 2007¹¹ with some modifications. In brief, 2 ml of different extract solution (0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml) of each plant sample were mixed with 2 ml of DPPH solution (60 µM). The mixture was allowed to stand for 30 minutes to perform complete reaction. Then absorbance was measured at 517 nm using UV spectrophotometer. Radical scavenging activity of each sample was calculated by using following formula: Radical scavenging activity (%) = [(A0- AS)/ A0] × 100. Where, A0 = Absorbance of control and AS= Absorbance of sample. Control is the test solution without sample. IC50 is defined as the amount of antioxidant required to inhibit 50% of DPPH free radicals under the experimental conditions. Similar process was done with ascorbic acid solution of concentrations (0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml). Ascorbic acid was taken as standard solution.

Total reduction ability by Fe³⁺- Fe²⁺ transformation

The capacity of each plant extract to reduce the ferric-ferricyanide complex to the ferrous ferricyanide complex of Prussian blue was determined by the method of Oyaizu¹² with some modifications. In brief, 2.5 ml of different extract solutions (1 µg/ml, 10 µg/ml and 100 µg/ml) were mixed with 2.5 ml of phosphate buffer pH 6.6 and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 minutes. After incubation, 2.5 ml of 10 % trichloroacetic acid was added to mixture and was centrifuged for 10 minutes at 1000 x g. 2.5 ml of the upper layer of solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1%

ferric chloride. Then the absorbance was measured at 700 nm using UV spectrophotometer. Similar process was done with ascorbic acid solution of concentrations (1 µg/ml, 10 µg/ml and 100 µg/ml). Ascorbic acid was taken as standard solution. Increased absorbance of the reaction mixture indicates increased reduction capability.

Determination of Total Phenols

Total Phenols were determined by Folin Ciocalteu reagent² with some modifications. In brief, 200 µl of each extract solution (1 mg/ml) was mixed with 1800 µl of distilled water and 2 ml of Folin reagent. After standing for 3 minutes, 2 ml of 10% sodium carbonate was mixed and shaken. The mixture was allowed to stand for 1 hour and the absorbance was measured at 760 nm. The calibration curve was prepared using gallic acid as the standard of concentrations 50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L (Figure 4). Total phenol values were expressed as mg gallic acid equivalent per gram dry extract weight.

Determination of Total Flavonoids

Aluminum chloride colorimetric method was used for flavonoids determination¹³ with some modifications. In brief, 1 ml of each extract solution (1 mg/ml) was mixed with 4 ml of distilled water. Then, 300 µl of 5% sodium nitrite was added. After 5 minutes, 300 µl 20% aluminum chloride was added and allowed to stand for 6 minutes. Then after, 2 ml of 1M sodium hydroxide was added. The mixture was shaken and the absorbance was measured at 510 nm using UV spectrophotometer. The calibration curve was prepared using quercetin as the standard of concentrations 50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L (Figure 5). Total flavonoid values were expressed as mg quercetin equivalent per gram dry extract weight.

Statistical analysis

For each experiment, data are expressed as mean value ± standard deviation in triplicates.

RESULTS

DPPH Radical Scavenging Activity

In this study, the hydrogen atom or electron donation ability of each plant extract against DPPH free radical was measured from the bleaching of violet colored DPPH solution at 517 nm. DPPH radical scavenging activity of each sample at different concentrations is shown in figure 1. Sample *S. japonica*, *M. rubicaulis himalayana*, *D. stoliczkae*, *D. propinqua*, *M. puya* (root), *C. esculenta* and *B. aristata* showed concentration dependent DPPH free radical scavenging activity. The concentration of each plant extracts required to inhibit 50% of DPPH free radicals are shown in figure 2. Among the studied plant extracts, the extracts of *D. stoliczkae*, *M. rubicaulis himalayana*, *S. japonica*, *D. propinqua* showed higher scavenging activity even at 10 µg/ml and their IC50 value were close to that of standard ascorbic acid.

Total Reduction Ability by Fe³⁺- Fe²⁺ Transformation

The evaluation of the reducing power was done by the

reduction of hexaferricyanide [Fe (III)] to hexaferrocyanide [Fe (II)] and determined by measuring absorbance at 700 nm.¹⁴ The absorbance of the each extracts with different concentrations (1 - 100 µg/ml) is given in figure 3. It was found that the absorbance of extract *S. japonica*, *A. viridis*, *M. rubicaulis himalayana* and *D. propinqua* was increased in a concentration dependent manner which indicates that increase in absorbance of the reaction mixture increases reduction ability.

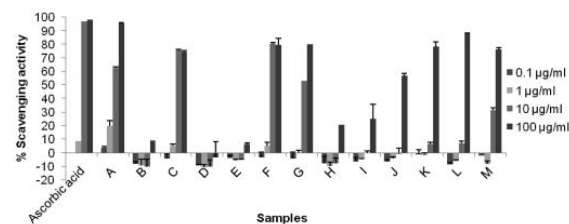


Figure 1: Graphical Representation of percentage scavenging of DPPH free radicals by extracts/ascorbic acid at 517 nm.

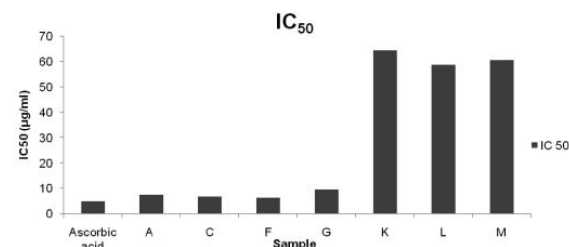


Figure 2: Graphical representation of IC₅₀ (µg/ml) values of plant extracts for free radical.

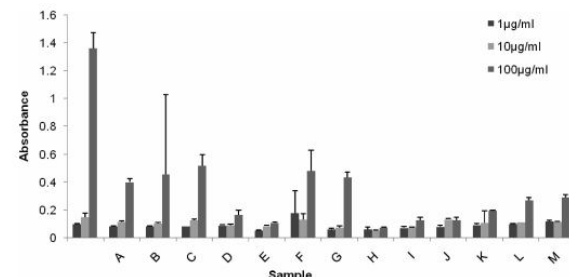


Figure 3: Graphical Representation of optical density of samples at different concentrations at 700 nm.

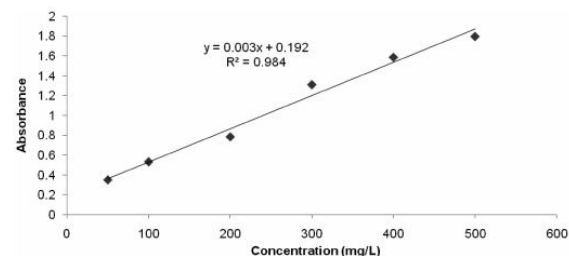


Figure 4: Calibration curve of gallic acid for total phenol calculation.

Total Phenol Determination

The quantitative determination of total phenol was carried out using Folin Ciocalteu reagent in terms of gallic acid equivalent. It involves the oxidation of phenols in alkaline solution by the yellow molybdotungstophosphoric

heteropolyanion reagent and colorimetric measurement of the resultant molybdotungstophosphate blue.¹⁵ This blue pigment has a maximum absorption depending on the qualitative and/or quantitative composition of phenol mixtures besides the pH of solutions, usually adding sodium carbonate.¹⁶ The calibration curve with gallic acid at different concentrations 50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L is shown in figure 4. Total phenol content (mg gallic acid equivalent per gram dry extract weight) of each plant extract is shown in table 2. The extracts which displayed the lowest and highest content of total phenols are the extract of *A. viridis* (24.9 ± 0.0 mg GAE/ g dry extract) and *D. stoliczkae* (440.6 ± 0.4 mg GAE/ g dry extract) respectively.

Table 1: List of plants selected for the study with their extraction yield value in methanol.

Scientific name	Common name	Sample	Parts used	% yield value
<i>Stephania japonica</i>	Batule pat	A	Leaves	9.70
<i>Amaranthus viridis</i>	Lude sag	B	Whole parts	5.22
<i>Mimosa rubicaulis himalayana</i>	Areli kada	C	Bark	8.62
<i>Schima wallichii</i>	Chilaune	D	Bark	2.22
<i>Aeschynanthus parviflorus</i>	Thirjo	E	Stem	6.66
<i>Diplazium stoliczkae</i>	Kalo niuro	F	Rhizomes	19.50
<i>Drynaria propinqua</i>	Kammari	G	Rhizomes	11.08
<i>Maoutia puya</i>	Puwa/bark	H	Barks	3.80
<i>Maoutia puya</i>	Puwa/root	I	Roots	3.44
<i>Colocasia esculenta</i>	Gava	J	Leaves	10.10
<i>Pogostemon benghalensis</i>	Rhudilo	K	Roots	4.42
<i>Berberis aristata</i>	Chutro	L	Stem	6.10
<i>Bryophyllum pinnatum</i>	Pattharchur	M	Leaves	9.76

% Yield = Weight of extract yield / Weight of crude sample taken × 100 %

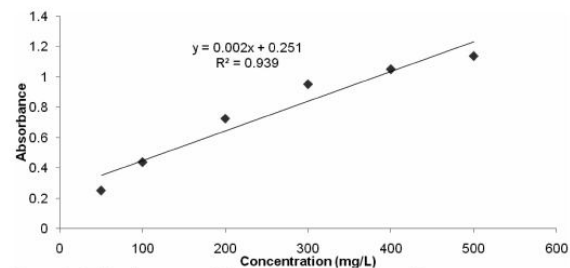


Figure 5: Calibration curve of Quercetin for Total flavonoid content.

Total Flavonoid Determination

The flavonoids in the presence of aluminum chloride have an intense yellow fluorescence which observed under UV spectrophotometer at 510 nm.¹³ The calibration curve with quercetin at different concentrations 50mg/L, 100 mg/L, 200

mg/L, 300 mg/L, 400 mg/L and 500 mg/L is shown in figure 5. Total flavonoid content (mg quercetin equivalent per gram dry extract weight) of each plant extract is shown in table 2. Among the studied samples, extract of *P. benghalensis* showed the lowest content of total flavonoids (34 ± 0.0 mg QE/g dry extract) and the extract of *D. stoliczkae* showed the highest content of total flavonoid (625.5 ± 0.4 mg QE/g dry extract) respectively.

Table 2: Total phenol and flavonoid contents in each sample

Sample	Total Phenol Content (mg GAE /g dry wt. of sample)	Total Flavonoid content (mg QE /g dry weight of sample)
A	189.2 ± 0.1	218.8 ± 0.2
B	24.9 ± 0.0	41.7 ± 0.1
C	375.9 ± 0.1	281.5 ± 0.1
D	39.3 ± 0.1	298.3 ± 0.2
E	45.6 ± 0.1	323.8 ± 0.1
F	440.6 ± 0.4	625.5 ± 0.4
G	173.9 ± 0.1	208.2 ± 0.1
H	25 ± 0.1	115.5 ± 0.1
I	43.6 ± 0.1	131.7 ± 0.4
J	47.3 ± 0.1	135.8 ± 0.0
K	56.7 ± 0.1	34.0 ± 0.0
L	80.2 ± 0.1	122.2 ± 0.4
M	112.8 ± 0.1	88.5 ± 0.1

DISCUSSION

In the present study, different plants were selected on the basis of their wide ethnomedicinal uses in Nepal and we determined the antioxidant activity, total phenol and flavonoid contents of selected medicinal plants.

The antioxidant activities of the prepared plant extracts were investigated by using two methods, DPPH radical scavenging assay and reduction ability of ferric to ferrous assay. DPPH scavenging assay has wide applications in determining radical scavenging activity of antioxidants.¹⁷ Extracts of *S. japonica*, *M. rubicaulis himalayana*, *D. stoliczkae* and *D. propinqua* showed significant radical scavenging activity with IC₅₀ value close to that of the standard, ascorbic acid. The ability of these extracts to scavenge DPPH free radicals suggests that they might be electron donors and react with free radicals to convert them to more stable products and terminate radical chain reactions.

These plants also showed potent reducing power in concentration dependent manner. This reflects their electron donating capacity to neutralize free radicals and form stable products.¹¹ In the study performed by Rahman and his coworkers, the methanolic extract of *S. japonica* showed moderate antioxidant activity in DPPH radical scavenging assay.¹⁸ In comparison to the results of Rahman, DPPH radical scavenging activity of *S. japonica* was better in our study.

In the comparative bioactivity studies on two *Mimosa* species performed by Genest et al., 2008, both *M. pudica* and *M. rubicaulis* showed prominent antioxidant activity.¹⁹ In our

study, *M. rubicaulis himalayana* showed potent antioxidant activity against DPPH free radical scavenging and Fe³⁺-Fe²⁺ reduction power. For *D. stoliczkae* and *D. propinqua*, we could find no studied reports regarding their antioxidant activities.

Plant phenols represent one of the major groups of compounds acting as primary antioxidants or free radical terminators.²⁰ Thus, it was reasonable to determine their total amount in the selected plant extracts. Among the studied plant extracts, there is variation in total phenol content ranging from *A. viridis* (24.9 ± 0.0 mg GAE/g dry extract) to *D. stoliczkae* (440.6 ± 0.4 mg GAE/g dry extract). Those plant extracts which showed better antioxidant activities in this study also demonstrated higher total phenol content which indicated that total antioxidative activity may correlate with total phenolic contents present in plant extract.^{21,22}

Flavonoids, as one of the most diverse and widespread group of natural compounds, are probably the most important natural phenols. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties.⁹ The antioxidant properties of flavonoid depend on their structure, particularly hydroxyl position in the molecule, and their ability as electron donor to a free radical.¹³ Among the studied plant extracts, there is variation in total flavonoid contents ranging from *A. viridis* (34.0 ± 0.0 mg QE/g dry extract) to *D. stoliczkae* (625.5 ± 0.4 mg QE/g dry extract). *S. japonica*, *B. aristata*, *D. stoliczkae*, *D. propinqua* and *M. rubicaulis himalayana* with better scavenging activity also showed higher (>100 mg QE/g dry extract wt.) flavonoid content. However, the extracts of *S. wallichii* and *A. parviflorus* contained high amount of flavonoid but could not show potent radical scavenging activity (Table 2, Figure 1).

With the increase in evidence that indigenous antioxidants may be useful for the management of disorders due to oxidative stress, there is also increase in interest in natural antioxidants present in herbs and medicinal plants for their biochemical functions.²³ Among the studied plants, the extracts of *S. japonica*, *D. stoliczkae*, *D. propinqua* and *M. rubicaulis himalayana* showed better antioxidant activities as well as contained higher amount of phenols and flavonoids. These assays indicate that these plant extracts could be significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidative activity are currently unclear. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in plant extracts. Furthermore, the in vivo activity of these extracts needs to be assessed prior to clinical use.

Those plant extracts which could not show potent antioxidant activities, might contain other phytochemicals that support those traditional medicines remain valuable source in the potential discovery of natural pharmaceuticals. Further work on isolation and identification of active compounds and their efficacy needs to be done.

CONCLUSION

The present study concludes that the extract of *Stephania japonica*, *Mimosa rubicaulis himalayana*, *Diplazium stoliczkae* and *Drynaria propinqua* inhibit the free radicals or scavenging of formed radicals. The high content of phenols and/or flavonoids may cause such inhibition of free radical or scavenging of formed radicals.

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