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EXPERIMENTAL STUDY

Antioxidant activity and phenol and flavonoid contents of eight medicinal plants from Western Nepal

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Abstract

OBJECTIVE: The purpose of this study was to evaluate the antioxidant activity, phenolic and flavonoids contents of selected medicinal plants form the Western region of Nepal.

METHODS: The antioxidant activity of selected medicinal plants were determined by using 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity; total ferric ion reducing ability through spectroscopic analysis. The content of total phenols was determined using Folin-Ciocalteu reagent, whereas Aluminum chloride colorimetric method was used for flavonoid determination.

RESULTS: Extracts of Syzygium Operculatus (87%), Astilbe Rivularis (83%) and Mallotus Philippnensis (88%) showed significant free radical scavenging activity with effective concentration (EC_{50}) close to that of ascorbic acid. Syzygium Operculatus (96%), Astilbe Rivularis (97%) and Mallotus Philippnensis (97%) had potent reducing power in concentration dependent fashion. Those plant extract with higher free radical scavenging and ferric reducing effect also showed the greater content of both phenols and flavonoids, suggesting the correlation between polyphenolic content and antioxidant activity. Those plant extracts which showed better antioxidant activity assays, also demonstrated higher total phenol and flavonoid contents. These three plants showed the presence of higher amount of phenols and flavonoids.

CONCLUSION: This study may provide the scientific basis for the traditional use of those plants and may provide valuable idea for further research.

Key words: Plants, medicinal; Antioxidants; 2, 2-diphenyl-1-picrylhydrazyl; Phenol; Flavonoids

INTRODUCTION

Oxidative stress occurs when the balance between the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) amount of antioxidants is destroyed in living cell, which cause the damage to cell components such as proteins, lipids and nucleic acids and eventually leads to cell death.[1] ROS such as hydrogen peroxide and hypochlorous acid and free radicals such as hydroxyl radical and superoxide anion are produced as normal products of cellular metabolism. ROS are produced endogenously by mitochondria during respiratory chain reaction and exogenously by various sources such as ultraviolet radiations, pollutants, inflammation, etc.[2] Rapid production of free radicals can lead to oxidative damage to biomolecules and may

cause disorders such as cancer, diabetes, inflammatory disease, asthma, cardiovascular diseases, neurodegenerative diseases, and premature aging.[3] ROS and RNS are the main source of free radicals, and they lead to serious damage causing neurodegeneration in the pathogenesis of neurological disorders such as Alzheimer's disease, Parkinson's disease and strokes.[4-6] Antioxidants provide protection from damage caused by uncontrolled production of ROS and concomitant lipid peroxidation, protein damage and deoxyribonucleic acid strand breakage.[7] An ideal antioxidant must be readily absorbed, quench free radicals and chelate metal redox at physiologically relative levels.[8] Human body has complex antioxidant defense system that includes the antioxidant enzymes: superoxide dismutase, glutathione 2 peroxidase and catalase and non-enzymatic antioxidants such as glutathione,[9] Vitamin E and C, thiol antioxidants (glutathione, thioredoxin and lipoic acid), melatonin, carotenoids and other compounds.[8] Currently available synthetic antioxidants, like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone and gallic acid esters have been reported to cause several side effects.[10,11] Therefore, investigations have been focused on naturally occurring substances. Plant phytochemicals not only to counteract free radical induced oxidative stress but also overcome the side effects of synthetic antioxidants.

Herbal medicine represents one of the most important fields of traditional medicine all over the world and the uses of herbal remedies for various medical conditions have been popularly growing. There is increasing trend in correlating phytochemical constituents of plants with its pharmacological activities.[12] Recent studies have reported that natural antioxidants obtained from medicinal plants protect from toxic and harmful effects of free radicals and have wide range of pharmacological effects, including antimicrobial, antimutagenic, antiallergic, antioxidant free radical scavenging activity and anticarcinogenic effects.[13-15] Natural antioxidants, from medicinal or edible plants, have recently received much attention as promising agents for reducing the risk of oxidative stress-induced neurological diseases.[16,17] Ethnomedicinal literature contains a large number of plants having polyphenols as active compounds that can be used against diseases in which ROS and free radical play important role.[7] The importance of natural phenolic compounds from plants materials is also raising interest due to their redox properties which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. In addition, they have metal chelating properties as well.[18,19] Polyphenolic compounds are secondary plant metabolites found in numerous plant species and they are reported to have multiple functions to counteract the free radicals and they also inhibit different types of oxidizing enzymes.[1]

The aim of the present study is to evaluate antioxidant activity and to determine the content of phenol and flavonoid of selected traditional medicinal plants from Western Nepal. We chose eight different medicinal plants, namely Premna coryembosa, Premna Mucronata, Mallotus Philippnensis, Trichosanthus Bracteata, Maharanga Bicolor, Hibiscus Rosa-sinensis, Astilbe Rivularis, and Syzygium Operculatus for the study purpose. These plants were used in traditional systems of medicine in Nepal from ancient period of time in order to cure different human ailments such as wound healing, gout, diabetes, arthritis, fever, inflammatory disease, immunomodulation, respiratory disease, vaginal problems, antimicrobial, etc.[20] In spite of the abundance use in the traditional and ethnomedicinal systems, scientific studies to explore the pharmacological studies of these plants have not been carried out yet. To evaluate antioxidant activity, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and ferric reduction assay were performed.

MATERIALS AND METHODS

Collection of plant material

Plants were collected from different regions of Kaski district of Western Nepal from July 2012 to November 2012. All plants were collected from original sources after proper identification from Botanist Dr. Radheshyam Kayastha and through the literature comparison. Herbarium and Crude sample specimen of the collected plants were preserved in the Pharmacognosy Laboratory of School of Health and Allied Sciences, Pokhara University, Nepal. The herbarium specimen number of each plant have been presented in Table 1.

Extraction

Selected plants were properly dried and grinded to make the powder. The powder were then extracted using methanol (1 : 10) in reflux condensation at 50℃ for 3 h. Extracts obtained were filtered and concentrated in rotary evaporator. After complete dryness of extracts, the yield value is calculated and stored for the further experiment.

Free Radical Scavenging Activity

DPPH free radical scavenging activity: DPPH free radical scavenging activity was determined spectrophotometrically as described by Cheel *et al*,[21] with some modification. Ascorbic acid was used as positive control. In brief, 2 mL of different extract solution (1, 10 and 100 μg/mL) of each plant and ascorbic acid sample were mixed with 2 mL of DPPH solution (60 μM). The mixture was allowed to stand for 30 min to perform complete reaction. Finally, the absorbance (Abs) of each plant samples was measured at 517 nm by using UV spectrophotometer. Free radical scavenging activity of each sample was calculated by using following formula:

Total Fe^{3+} - Fe^{2+} Reduction Ability Determination: the ferric reducing power of each extracts was determined as described by Zou *et al*,[22] with slight modifications. Briefly, 1.0 mL of different concentration of samples was mixed with 2.5 mL of a 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of a 1% (w/v) solution of potassium ferricyanide. The mixture was incubated in a water bath at 50℃ for 20 min. Afterward 2.5 mL of a 10% (w/v) trichloroacetic acid solution was added, and the mixture was then centrifuged at 3000 rpm for 10 min. 2.5 mL of supernatant was combined with 2.5 mL of distilled water and 0.5 mL 0.1% ferric chloride solution, and the absorbance was measured spectrophotometrically at 700 nm.

Total phenol content determination

The amount of total phenols in the herb extracts was determined using Folin-Ciocalteu reagent according to the method of Slinkard and Singleton,[23] with some modifications. Gallic acid was used as a positive control. Samples (200 µL, three replicates) were introduced into test cuvettes, and then 1.0 mL of Folin-Ciocalteu's reagent and 0.8 mL of sodium carbonate (7.5%) were added. The absorbance of all samples was measured at 765 nm using the UV spectrophotometer after incubating at 30℃ for 1.5 h. Results were expressed as milligrams of gallic acid equivalent (GAE) per gram dry extract.

Total Flavonoid content determination

Aluminum chloride colorimetric method was used for flavonoid determination,[24] with some modifications. In brief, 1 mL of sample solution was mixed with 4 mL of distilled water. 300 µL of sodium nitrite was added. After 5 min, 300 µL aluminum chlorides was added and allowed to stand for 6 min. Then, 2 mL of sodium hydroxide was added and the mixture was shaken to mix well. The absorbance was measured at 510 nm using UV spectrophotometer.

Statistical analysis

All results are presented as mean ± standard deviation ($\bar{x} \pm s$). Significant differences between experimental groups were determined by using one way ANOVA followed by the Tukey's Post Hoc test using GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA). $P<0.05$ is considered as statistically significant level.

RESULTS

Plant parameters and yield value

Table 1 shows the parameters of the selected medicinal plants and yield value after extractions. The vernacular names of plants are presented in Nepalese languages. Selected medicinal plants were collected from western Nepal, especially in Kaski district Gandaki zone.

DPPH free radical scavenging activity

DPPH free radical scavenging activities of selected medicinal plants are presented in Figure 1. All plants showed the dose dependent antioxidant activity. Among the studied samples, Astilbe Rivularis, Mallotus Philippenensis and Syzygium Operculatus plant extract showed the significant antioxidant activity with the EC_{50} values of 4.05, 3.17, 3.07 µg/mL respectively. Other plants, Premna Coryembosa, Premna Mucronata, Trichosanthus Bracteata, Maharanga Bicolor, and Hibiscus Rosa-sinensis showed relatively weaker antioxidant activity with higher EC_{50} values (data are not shown). The EC_{50} value of Ascorbic acid was found to be 2.31 µg/mL.

Total Reduction Ability by Fe^{3+}- Fe^{2+} Transformation

The evaluation of the reducing power is based on the reduction of hexaferricyanide to hexaferrocyanide.[25] The absorbance of the extracts with concentrations 1, 10 and 100 µg/mL for each sample is given in the Figure 2. While analyzing the result of reducing activity in methanol extract, it was found that the absorbance of all samples were increased in a concentration dependent manner whereas the absorbance of the positive control, ascorbic acid was set as the standard for the further calculations. The ferric reduction ability of the selected medicinal plants are presented in the Figure 2.

Table 1 Plant parameters and yield values

Serial No.	Scientific name (herbarium No.)	Vernacular name	Family	Parts used	Yield value (%)
1	Premna Coryembosa (PH-316)	Seto gindari	Verbenaceae	Bark	12.87
2	Premna Mucronata (PH-317)	Kalo gindari	Verbenaceae	Bark	16.75
3	Mallotus Philippnensis (PH-318)	Sindure	Euphorbiaceae	Bark	16.95
4	Trichosanthus Bracteata (PH-319)	Indrayani	Euphorbiaceae	Stem	13.90
5	Maharanga Bicolor (PH-320)	Maharangi	Euphorbiaceae	Root	10.60
6	Hibiscus Rosa-sinensis (PH-321)	Ghantiphul	Malvaceae	Leaves	19.32
7	Astilbe Rivularis (PH-322)	Thulo okhati	Saxifragaceae	Bark	20.58
8	Syzygium Operculatus (PH-323)	Kyamuno	Myrtaceae	Bark	19.44

Note: %Yield=weight of extract/weight of crude sample taken×100%.

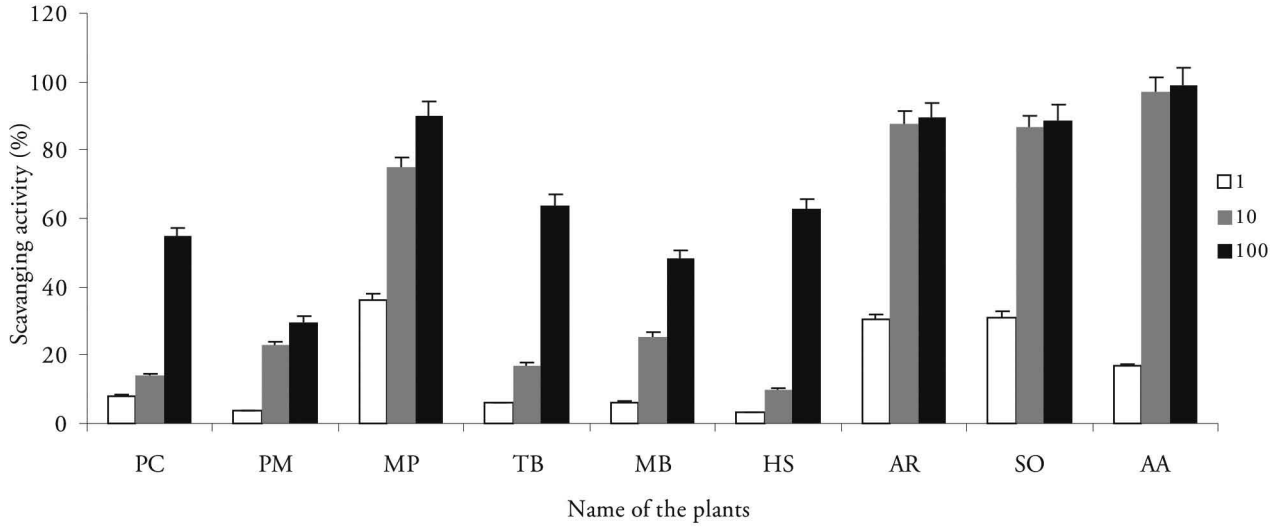

Figure 1 DPPH free radical scavenging activity of selected medicinal plants
PC: Premna Coryembosa; PM: Premna Mucronata; MP: Mallotus Philippnensis; TB: Trichosanthus Bracteata; MB: Maharanga Bicolor; HS: Hibiscus rosa-sinensis; AR: Astilbe Rivularis; SO: Syzygium Operculatus; AA: ascorbic acid. The concentration of each plant extracts and standards are in μg/mL. Data expressed as mean value±standard deviation (*n*=3) and the concentration of the extract is provided in terms of μg/mL.

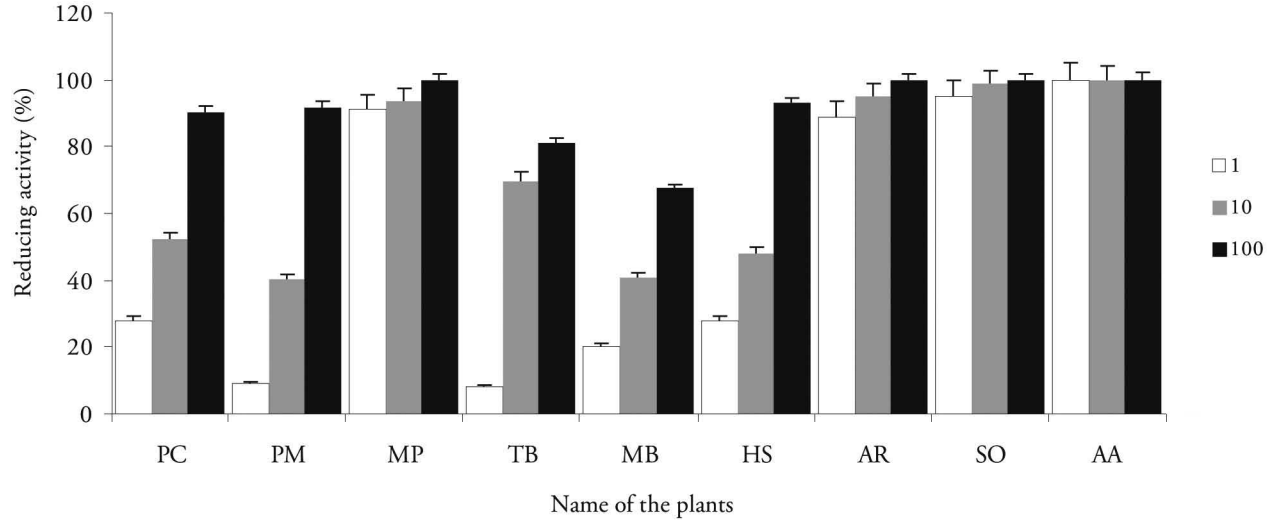

Figure 2 Ferric ion reducing ability of selected medicinal plants
PC: Premna Coryembosa; PM: Premna Mucronata; MP: Mallotus Philippnensis; TB: Trichosanthus Bracteata; MB: Maharanga Bicolor; HS: Hibiscus rosa-sinensis; AR: Astilbe Rivularis; SO: Syzygium Operculatus; AA: ascorbic acid. The concentration of each plant extracts and standards are in μg/mL. Data expressed as mean value±standard deviation (*n*=3) and the concentration of the extract is provided in terms of μg/mL.

Total phenol determination

The quantitative determination of total phenol was carried out using Folin Ciocalteu reagent in terms of gallic acid equivalent. Total phenol content is expressed as mg gallic acid equivalent per gram dry extract weight (Table 2). There is variation in total phenol content ranging from Mallotus Philippnensis [(284.12± 0.60) mg GAE/g dry extract wt.] to Premna Mucronata [(28.04±0.10) mg GAE/g dry extract wt.].

Total flavonoid determination

Quantitative total flavonoid determination is performed by precipitating the extracts with aluminum chloride in an alkalinized medium. The flavonoid in the presence of aluminum chloride have an intense yellow fluorescence when observed UV spectrophotometer.[26] Total flavonoid content were expressed as mg quercetin equivalent (QE) per gram dry extract weight (Table 2). Among the studied plant extracts, there is variation in total flavonoid content ranging from Mallotus Philippnensis [(879.48±24.75) mg QE/g dry extract wt.)] to Premna Mucronata [(27.89±14.40) mg QE/g dry extract wt.]. Astilbe Rivularis, Hibiscus Rosa-sinensis, Maharanga Bicolor, Syzygium Operculatus and Trichosanthus Bracteata also showed higher (> 100 mg QE/g dry extract wt.) flavonoid content.

DISCUSSION

This study was performed to determine antioxidant activity, total phenol and flavonoid contents of some selected medicinal plants from different regions of Western Nepal. Total eight plants were selected on the basis

Table 2 Total phenol and flavonoid content of selected medicinal plants (*n*=3)

Plant sample	Total phenol content (mg GAE /g dry extract wt.)	Total flavonoid content（mg QE/g dry extract wt.）
Premna Coryembosa	56.01±0.10	75.12±9.74
Premna Mucronata	28.04±0.10	27.89±14.40
Mallotus Philippnensis	284.12±0.60	879.48±24.75
Trichosanthus Bracteata	63.20±0.10	657.26±18.01
Maharanga Bicolor	400.14±0.30	130.27±19.09
Hibiscus Rosa-Sinensis	49.13±0.30	715.59±7.24
Astilbe Rivularis	183.11±0.50	857.26±10.38
Syzygium Operculatus	155.21±0.60	134.25±8.78

Notes: data expressed as mean value±standard deviation. GAE: galic acid equivalent; QE: quercetin equivalent; wt.: weight.

of their wide ethnomedicinal uses with limited scientific studies.

Antioxidant capacity of the methanolic extract of each plant was examined using DPPH radical scavenging assay and ferric reduction assay, because evaluation of antioxidant properties of plants cannot be carried out accurately by single universal method. The DPPH free radical scavenging assay is a simple, acceptable and most widely used method to evaluate antioxidant activity of plant extracts. In the presence of an antioxidant, DPPH radical form a stable molecules by gaining one more electron or hydrogen atom from the antioxidant and the UV absorbance decreases which signifies the scavenging activity of natural products as well as synthetic compounds.[27] Extracts of Syzygium Operculatus, Astilbe Rivularis and Mallotus Philippnensis showed potent radical scavenging activity with EC50 close to that of ascorbic acid. Lower EC_{50} value indicates higher antioxidant activity.[11] These three plants extract showed the similar antioxidant potency to that of ascorbic acid and remaining plant species showed the dose dependent free radical scavenging activity, both in DPPH free radical scavenging and ferrus reduction potency. The ability of these extracts to scavenge DPPH 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.

Reducing power is used to measure the reductive ability of antioxidant, and it is evaluated by the transformation of Fe (Ⅲ) to Fe (Ⅱ) in the presence of the sample extracts.[28] The reduction ability by ferric to ferrous transformation assay is convenient to perform and the reaction is reproducible and linearly related to the molar concentration of the antioxidant.[29] In this study, the reducing power of Syzygium Operculatus, Astilbe Rivularis and Mallotus Philippnensis were found to increase in concentration dependent manner. The outcome of the reducing reaction is to terminate the radical chain reactions.

Phenols are very important plant constituents. There is a linear relationship between total phenol and antioxidant activity of plant species, because of the scavenging ability of their phenolic hydroxyl groups.[30] Phenolic compounds are also reported to be effective hydrogen donors, making them very good antioxidants.[31] 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 Mallotus Philippnensis [(284.12 ± 0.60) mg GAE/g dry extract wt.] to Premna Mucronata [(28.04± 0.10) mg GAE/g dry extract wt.]. Those plant extracts which showed better activity in antioxidant activity assays such as Mallotus Philippnensis, Astilbe Rivulars and Syzygium Operculatus also demonstrated higher total phenol content, which indicates that there is correlation between total phenol content and radical scavenging activity.

The antioxidant activity of medicinal plants could be attributed to its flavonoid content. Flavonoid act as scavengers of various oxidizing species i.e. superoxide anion, hydroxyl radical or peroxy radicals, they also act as quenchers of singlet oxygen.[32] Among the studied plant extracts, there was variation in total flavonoid content ranging from Mallotus Philippnensis [(879.48± 24.75) mg QE/g dry extract wt.)] to Premna Mucronata [(27.89 ± 14.40) mg QE/g dry extract wt.].Astilbe Rivularis, Hibiscus Rosa-sinensis, Maharanga Bicolor, Syzygium Operculatus and Trichosanthus Bracteata also showed higher (>100 mg QE/g dry extract wt.) flavonoid content. Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. The pharmacological activities of flavonoids were closely related to their functional group. In addition, there may be some interference rising from other chemical components present in the extract. Moreover, flavonoids may exert their cell structure protection through a variety of mechanisms; one of their potent effects may be through their ability to increase levels of glutathione, a powerful antioxidant, as suggested by various research studies.[33]

On the basis of the results of these antioxidant activity assays, phenolic and flavonoid content determination,

Astilbe Rivularis and Mallotus Philippnensis showed potent free radical scavenging activity and high content of phenol and flavonoid. Whereas, Premna Mucronata and Premna Corymbosa extracts showed lower antioxidant activity and low content of phenol and flavonoid. Among the selected plant samples, the comparative antioxidant study with similar methods has been found for Hibiscus Rosa-sinensis. In the study performed by Rahman, the methanolic extract of Hibiscus Rosa-sinensis showed moderate antioxidant activity in DPPH radical scavenging assay [34] which is as found to be similar in our study as well.

In this study, *in vitro* antioxidant assays indicate that plant extracts showing higher antioxidant activity and higher phenol and flavonoid contents could be significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses and its related disorders. Out of selected medicinal plants used for this study, few of them showed potent antioxidant activity but there is limited scientific evidence on those plants. So, further study is necessary to prove their potency. Further investigation is also needed to isolate and identify the components responsible for the antioxidative activity which are currently unclear. Furthermore, the *in vivo* activity of these extracts needs to be assessed prior to their clinical uses.

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