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Estimation of Total Phenolic and Flavonoid Contents in Some Medicinal Plants and Their Antioxidant Activities

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Abstract

Quercetin was isolated from ethyl acetate fraction of a methanol extract of the outer scale of onion by repeated sephadex LH-20 column chromatography. Methanol, 50% aqueous methanol and 70% aqueous acetone extracts of different parts of four medicinal plants, *Ficus benghalensis, Elaeocarpus sphaericus, Ipomea carnea* and *Azeratum conyzoides* were prepared and screened for total phenolic and flavonoid contents and free radical scavenging activity. The total phenolics contents were measured spectrophotometrically by using Folin-Ciocalteu reagent and total flavonoids content by using aluminum chloride colorimetric method. Free radical scavenging activity was evaluated using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay. All the investigated medicinal plant extracts contained high amount of phenolics. The highest amount was detected in 70% aqueous acetone extract of *E. sphaericus* (298.8±9.03 mg GAE/g extract) and lowest amount in 50% aqueous methanol extract of *F. benghalensis* (6.7±0.73 mg GAE/g extract). The highest amount of flavonoid was found in methanol extract (78.2±2.72 mg quercetin/g extract) and the lowest amount was detected in 50% aqueous methanol extract (2.1±0.25 mg quercetin/g extract) of *F. benghalensis*. DPPH free radical scavenging assay was carried out only in 70% acetone extracts. The highest IC_{50} value was observed for *E. sphaericus* (34.0 µg/ml). A strong linear correlation between total phenolic content and antioxidant activity was found indicating that the major antioxidants are phenolics.

Key words: antioxidant activity, gallic acid, medicinal plants, quercetin, total flavonoids phenolics

Introduction

Reactive oxygen and/or nitrogen species such as superoxide anion, hydrogen peroxide, hydroxyl radical and peroxy-nitrite free radicals are constantly produced as the normal products of cellular metabolism. When the cellular concentration of oxidant species is increased to an extent that overcome the endogenous antioxidant defense system, oxidative stress occurs, leading to lipid, protein and DNA damage which is considered a main factor contributing to carcinogenesis and evolution of cancer, and may cause many disorders such as diabetes, asthma, inflammation, premature aging, cardiovascular and neurodegenerative diseases (Ames *et al.* 1993, Khan *et al.* 1995). Due to the ability of natural phenolic compounds to scavenge and reduce the production of free radicals and act as transition metal chelators, they can exert a major chemopreventive activity (Kampa *et al.* 2007). Quercetin and other phenols (Alia *et al.* 2006, Aherne & O'Brine 1999, Johnson & Loo 2000) were shown to exert protective effects against cellular oxidative damage in different human cell lines. In addition, phenolic compounds possess anti-angiogenesis effects (Mojzis *et al.* 2008), which is an important aspect in the inhibition of tumor growth, invasion and metastasis.

Medicinal plants contain a wide variety of phytochemicals such as phenolics, flavonoids, chlorophyll derivatives, tocopherols, carotenoids and ascorbic acid as natural antioxidants. However, recently phenolics have been considered powerful antioxidants *in vitro* and proved to be more potent than vitamin C and E (Rice-Evans *et al.* 1995, Rice-

Evans *et al.* 1996, Scalbert *et al*. 2005). These natural antioxidants act by scavenging free radical species or by inhibiting the generation of reactive species during the course of normal cell metabolism, thus preventing damage to lipids, proteins and nucleic acids, and eventual cellular damage and death (Cotelle *et al.* 2001). Plant phenolics appear to exhibit different anti-carcinogenic mechanisms including inactivating potentially carcinogenic reactive electrophiles and scavenging reactive oxygen species that may initiate tumor production (Heinonen *et al.* 1998, Ames *et al.* 1993). Thus, phytochemicals responsible for such antioxidant activity should be isolated and used for the prevention and treatment of free redical related disorders.

Nepal is a natural store house of medicinal plants. Approximately 70 to 80% of the population of Nepal depends on traditional medicines. Indigenous people residing in different belts depend on local plants and plant products to meet their daily requirements for food, fodder and medicines. Local herbs and other plant resources found in rural area are the principle source of medicines for treating diseases since time immemorial. Despite widespread use of medicinal plants, little is known about their chemical composition while quantitative data on phenolics content and the antioxidant potential are missing which is the subject of investigation. In our previous study, we have quantified the total flavonoids content in different parts of *Camellia* plants, and total phenolics content in different medicinal herbs and also assessed the selected extracts for their antioxidant activity using DPPH free radical (Acharya *et al*. 2013, Gewali *et al*. 2013).

The aim of the present study is to evaluate the total phenolics and flavonoids contents in different extracts prepared from different parts of medicinal plants and compare the radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. In addition, the relationship between the DPPH radical scavenging activity and total phenolic content of some extracts are also established.

Methodology

Chromatographic materials and chemicals

Thin layer chromatography foils (precoated Silica gel 60 GF_{254}, 0.2 mm) were purchased from Merck, Darmstadt, Germany. Sephadex LH-20 was purchased from Pharmacia Biotech, Uppasala, Sweden. DPPH was purchased from Sigma Chemical Company, USA. Gallic acid was purchased from Merck, Darmstadt, Germany. Authentic quercetin was provided by Prof. S. M Tuladhar, Research Centre for Applied Science and Technology, RECAST, Tribhuvan University. Aluminum chloride reagent and Folin Ciocalteu reagent were purchased from SD fine-chemicals. All other chemicals were of analytical grade.

Plant materials

Plant materials were collected from Kirtipur, Kathmandu, Nepal on February 2013. They were authenticated by comparision with the voucher specimens deposited at the National Herbarium at Godwari. The name of plants, their local names and collected parts are given in Table 1.

Table 1. Name of plants, local names and collected parts

Name of Plants	Local Names	Collected Parts
Ageratum conizoides Linn.	Raawanne	Whole plant
Allium cepa Linn.	Pyaaj	Outer scales
Elaeocarpus sphaericus (Gaertn.) K. Schum	Rudraksha	Leaves
Ficus benghalensis Linn.	Bar	Leaves
Ipomea carnea Jacq	Beshram	Leaves

Extraction of dry scales of onion and isolation of quercetin

Finely crushed onion scale (200 g) was extracted with methanol (1 liter) for twelve hours in a soxhlet extractor. The methanol extract was concentrated under reduced pressure in a rotavapour to get viscous liquid (51.4 g) which was suspended in 100 ml distilled water and then extracted with hexane (200 ml x 4) in a separatory funnel. The aqueous layer was again extracted with ethyl acetate (200 ml x 5). The ethyl acetate layer was collected and evaporated in a rotavapour under reduced pressure and 6 g crude extract was chromatographed on a sephadex LH- 20 column filled with 97 g sephadex and eluted with methanol. Sub-fractions were collected and monitored by TLC on silica gel GF_{254} using solvent system ethyl acetate - methanol – water, EMW (100 : 10 : 5). The sub-fractions were pooled into four major fractions on the basis of TLC character. The quercetin accumulated fraction (2.1 g) was further chromatographed on a sephadex LH- 20 column filled with 97 g sephadex and eluted with 75% aqueous methanol. Sub-fractions were collected and monitored by TLC and pooled into five major

fractions. The fraction containing quercetin was concentrated and purified by recrystallization with hot water to get 700 mg of yellow powder.

Preparation of extracts for the determination of total phenolics and flavonoids

An amount of 20 g each of the dried and powdered plant materials was extracted with methanol (200 ml) in a soxhlet extraction apparatus for 10 hours. The residue was extracted with 50% aqueous methanol under reflux for six hours. Similarly, 20 g each dried and powdered sample was percolated with 70% acetone and subjected to ultrasound-assisted extraction for 15 minutes. The extracts were filtered and the solvent was evaporated in a rotary evaporator under reduced pressure to get respective extracts.

Determination of total phenolic content in different extracts

Preparation of standard

The total phenolic content in plant extracts was determined by using Folin-Ciocalteu colourimetric method based on oxidation-reduction reaction (Waterhouse 2002). Various concentrations of gallic acid solutions in methanol (100, 75, 50, 25 and 10 µg/ml) were prepared. In a 20 ml test tube, 1 ml gallic acid of each concentration was added and to that 5 ml Folin-Ciocalteu reagent (10%) and 4 ml 7% Na_2CO_3 were added to get a total volume of 10 ml. The blue coloured mixture was shaken well and incubated for 30 minutes at 40 ^{0}C in a water bath. Then, the absorbance was measured at 760 nm against blank. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve.

Preparation of sample

Various concentrations of the extracts (200, 100, 50 and 25 µg/ml) were prepared. Following the procedure described for standard, absorbance for each concentration of the extract was recorded. Total phenolic content of the extracts was expressed as mg gallic acid equivalents (GAE) per gram dry extract (mg/g). Total phenolic content in all samples were calculated using the formula: C = cV/m where, C = total phenolic content mg GAE/g dry extract, c = concentration of gallic acid obtained from calibration curve in mg/ml, V = volume of extract in ml, m = mass of extract in gram.

Determination of total flavonoid content in different extracts

Preparation of standard

The total flavonoid content was determined by aluminum chloride colorimetric assay (Zhishen *et al.* 1999). Various concentrations of standard quercetin (2.0, 1.0, 0.5 and 0.25 mg/ml) were prepared. An aliquot of 1 ml quercetin of each concentration in methanol was added to a 10 ml volumetric flask containing 4 ml double distilled water. At the zero time, 0.3 ml 5% sodium nitrite was added, after 5 min, 0.3 ml of 10% $AlCl_3$ was added and at 6 min, 2 ml of 1 M sodium hydroxide was added to the mixture. Immediately, the total volume of the mixture was made up to 10 ml by the addition of 2.4 ml double distilled water and mixed thoroughly. Absorbance of the pink color mixture was determined at 510 nm versus a blank containing all reagents except quercetin. The average absorbance values obtained at different concentrations of quercetin were used to plot the calibration curve.

Preparation of sample

Various concentrations of the extracts (2.0, 1.0, 0.5 and 0.25 mg/ml) were prepared. Following the procedure described for standard, absorbance for each concentration of extract was recorded. Total flavonoid content of the extracts was expressed as mg quercetin equivalents (QE) per gram of dry extract (mg/g). Total flavonoid content is calculated by using the formula: C = cV/m where, C = total flavonoid content mg QE/g dry extract, c = concentration of quercetin obtained from calibration curve in mg/ml, V = volume of extract in ml, m = mass of extract in gram.

Statistical analysis

All the experiments were carried out in triplicates and data reported are mean ± standard deviation. Calculation of linear correlation coefficient and correlation analysis were carried out using MS Office Excel 2007. The linear regression equation for a straight line is, y = mx + c where, y = absorbance of extract, m = slope of the calibration curve, x = concentration of extract, c = intercept. Using this regression equation, concentrations of extracts were calculated. From the calculated values of concentration of each extract, the total phenolics and flavonoid content were calculated.

Determination of antioxidant activitiy using 2,2-diphenyl-1-picrylhydrazyl free radical

Antioxidant activity of the selected extracts was assessed using DPPH free radical (Brand-William *et al.* 1995). DPPH solution (0.1 mM) was prepared by dissolving 3.9 mg of DPPH in 100 ml methanol and stirred overnight at 4° C. Thus, prepared purple colored DPPH free radical solution was stored at -20° C for further use.

Three different concentrations (5, 10 and 15 µg/ml) of methanolic solutions of each extract were prepared by the serial dilution of the stock solution of the respective extract. To each 0.5 ml extract solution, 2.5 ml 0.1 mM methanolic DPPH solution was added. A control was prepared by mixing 0.5 ml distilled water and 2.5 ml 0.1 mM methanolic DPPH solution. These samples were shaken well and kept in dark for 30 min at room temperature. The absorbance was measured at 517 nm against the blank solution consisting 2.5 ml MeOH and 0.5 ml distilled water. The radical scavenging activity was expressed as the radical scavenging percentage using the equation where; A_S = absorbance of sample solution, A_b = absorbance of blank and A_C = absorbance of control.

$$\% \ scavenging \quad = \left[\frac{(As - A_b)}{Ac} \right] x100$$

IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the graph of radical scavenging activity against the concentration of extracts. Statistically, the correlation between antioxidant activity and total phenolic and flavonoid content was determined by plotting IC_{50} (µg/ml) against total phenolics and total flavonoids (mg/g).

Results and Discussion

Isolation and characterization of quercetin from onion scale

Finely powdered onion scale was extracted with methanol and the solvent was evaporated to get viscous liquid which was then extracted with ethyl acetate. The ethyl acetate soluble portion was subjected to repeated chromatography on sephadex LH- 20 column and quercetin was isolated. which was purified by recrystallization with hot water. On thin layer chromatogram, it appeared as light yellow spot, turned into dark yellow spot on exposure to air, brown colored spot under UV 254 nm, bright yellow under UV 366 nm, dark yellow when exposed to ammonia vapour. Thin layer chromatography (R_f 0.79 in butanol-acetic acid-water 4:1:5; 0.88 in chloroform-acetic acid-water 10:9:1; 0.87 in ethylacetate-methanol-water 100:10:5; 0.45 in toluene-ethylacetate-formic acid 10:8:1) and the melting point (315-317 °C) of the isolated quercetin is in good agreement with the authentic quercetin. The UV spectrum of the isolated and authentic quercetin showed absorption bands at 230 and 358 nm. The IR spectrum of the isolated and authentic quercetin has a broad band around 3600-2400 cm^{-1} region corresponding to the superimposed aliphatic and aromatic C-H and phenolic O-H stretching. Other stretchings were comparable with IR spectra of authentic quercetin.

Amount of different extracts obtained under different extraction conditions

For the determination of total phenolics and total flavonoids in plant extracts, different extraction approaches such as soxhlet using methanol, reflux using 50% aqueous methanol and ultrasound assisted using 70% acetone were applied. The yield of the extract varied according to the extraction method and plant samples used. The results are shown in Table 2. Among the plants included in this study, both the highest and the lowest amount of extracts were obtained with methanol (7.74 g) and 50% aqueous methanol (0.31 g) extract of *E. sphericus* respectively.

Table 2. Amount of extracts (gram) from 20 g of plant materials

Name of Plant	Methanol Extract	50% Methanol Extract	70% Acetone Extract
F. benghalensis	4.95	0.63	5.45
E. sphaericus	7.74	0.31	6.74
I. carnea	2.17	0.43	3.41
A. conizoides	2.08	0.71	2.10

Total phenolic content in different plant extracts

The total phenolic content in plant extract was determined by using Folin-Ciocalteu (F-C) colourimetric method. Gallic acid was used as a standard compound. The absorbance values obtained at different concentrations of gallic acid was used for the construction of calibration curve (Fig 1). F-C method is based on the transfer of electrons in alkaline medium

from phenolic compounds to phosphomolybedic/ phosphotungstic acid complexes to form blue coloured complexes, $(PMoW_{11}O_{40})^{-4}$ that are determined spectrophotometrically at 760 nm. Total phenolic content of the extracts was calculated from the regression equation of calibration curve (y = 0.013x, R^2 = 0.999) and expressed as mg gallic acid equivalents (GAE) per gram of extract (mg/g). The results are presented in Table 2.

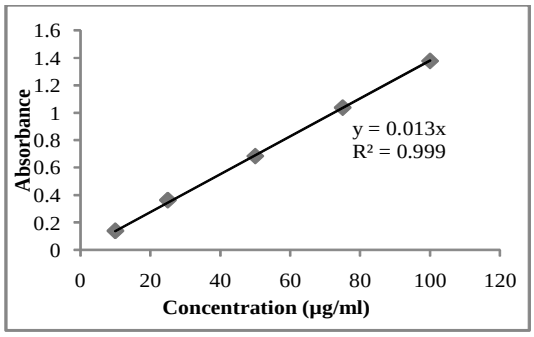

Fig. 1. Callibration curve for standard gallic acid

Total flavonoid content in different plant extracts

Total flavonoid content in the extracts was determined by reaction with sodium nitrite followed by the development of colored flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which was monitored spectrophotometrically at maximum wavelength of 510 nm. Quercetin was used as a standard compound. The absorbance values obtained at different concentrations of quercetin was used for the construction of calibration curve (Fig 2). Total Flavonoids content of the extracts was calculated from the regression equation of calibration curve (y = 0.004x; R^2 = 0.995) and expressed as mg quercetin equivalent (QE) per gram extract (mg/g). The results are presented in Table 3.

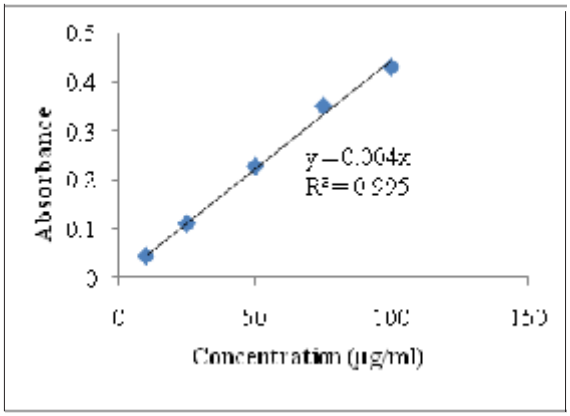

Fig. 2. Callibration curve of standard quercetin

Table 3. Total phenolic, TP and flavonoid, TF content in different extracts and their ratio

Name of Plant	Methanol mg GAE/g	Methanol mg QE/g	Ratio TF/TP	50% Methanol mg GAE/g	50% Methanol mg QE/g	Ratio TF/TP	70% Acetone mg GAE/g	70% Acetone mg QE/g	Ratio TF/TP
A. conizoides	18.2±1.81	12.7±2.24	0.697	26.0±1.17	2.5±0.10	0.096	12.1±0.23	9.62±0.47	0.795
E. sphaericus	247.6±3.91	61.9±2.83	0.250	30.6±0.76	7.7±0.73	0.251	298.7±9.03	44.1±4.21	0.147
F. benghalensis	212.4±3.46	78.2±2.71	0.368	6.7±0.73	2.1±0.25	0.313	131.3±1.42	78.0±2.44	0.594
I. carnea	47.0±3.93	19.2±1.62	0.408	60.4±0.86	11.3±1.28	0.187	34.4±1.74	10.0±0.00	0.290

Total phenolic content in methanol and 70% acetone extracts were relatively high when compared with 50% aqueous methanol extract in *F. benghalensis* and *E. sphaericus*. However, in the case of *I. carnea* and *A. conizoides*, total phenolic content in 50% aqueous methanol extracts were relatively high when compared with methanol and 70% aqueous acetone extracts. *F. benghalensis* and *E. sphericus* contained higher amount of phenolic compounds than *I. carnea* and *A. conizoides* in methanol and 70% aqueous acetone extracts. The highest amount of phenolic was detected in 70% aqueous acetone extract of *E. sphericus* (298.7±9.03 mg GAE/g) and lowest amount in 50% aqueous methanol extract of *F. benghalensis* (6.7±0.73 mg GAE/g).

In most cases, it was found that total flavonoid content in methanol and 70% aqueous acetone extracts was relatively high when compared with 50% aqueous methanol extract. In all cases total flavonoid content in methanol extract was found to be higher than 70% aqueous acetone extract and 50% aqueous methanol

extract. The highest total flavonoid content was found in methanol extract (78.1±2.71 mg QE/g) and the lowest was found in 50% aqueous methanol extract (2.1±0.25 mg QE/g) of *F. benghalensis*. As an exception, in *I. carnea*, the lowest flavonoid content was found in 70% aqueous acetone extract (10.0±0.00 mg QE/g) and the highest was found in methanol extract (19.2±1.62 mg QE/g).

The ratios of total flavonoid to total phenolic content were found to be different in different extracts. In the case of methanol extract of *A. conizoides*, the greatest ratio (0.69) was observed whereas in *E. sphaericus*, the lowest ratio (0.25) was observed which indicated that 69% of total phenolics in *A. conizoides* are flavonoids and only 25% of total phenolics in *E. sphaericus* are flavonoids. Similarly, in 70% acetone extract, 79% of total phenolics are flavonoids in *A. conizoides* and only 15% of total phenolics are flavonoids in *E. sphaericus*. However, in the case of 50% aqueous methanol, the highest ratio (0.31) was observed in *F. benghalensis* and the lowest (0.09) was observed in *A. conizoides* which indicated that only 31% and 9% are flavonoids in the case of *F. benghalensis* and *A. conizoides* respectively.

Determination of antioxidant activity

DPPH assay was carried out for 70% acetone extracts of medicinal plants. The absorbance values were measured at wavelength 517 nm for different concentrations of extracts and the control. These values were used to calculate the percentage inhibitions of DPPH radicals against the samples. The IC_{50} values of various extracts were calculated from the percentage inhibitions at various concentrations are given in Table 4. The calculated percentage of inhibition showed that all the four extracts have shown antioxidant activity. The extract with the highest phenolic content showed the greatest radical scavenging activity.

Table 4. IC_{50} values of various extracts

Plant	IC_{50} (µg/mL)	TPC (mg GAE/g)
A. conizoides	312.000	12.192
E. sphaericus	34.000	298.769
F. Benghalensis	135.000	131.326
I. carnea	238.000	34.442

Correlation between DPPH radical scavenging activity (IC_{50}) and total phenolic content

The correlation between antioxidant activity and total phenolic content had been determined by plotting IC_{50} (µg/ml) against total phenolic content (mg GAE/g). The relation between total phenolic contents and free radical scavenging activity, FRSA of the samples is shown in Fig. 3.

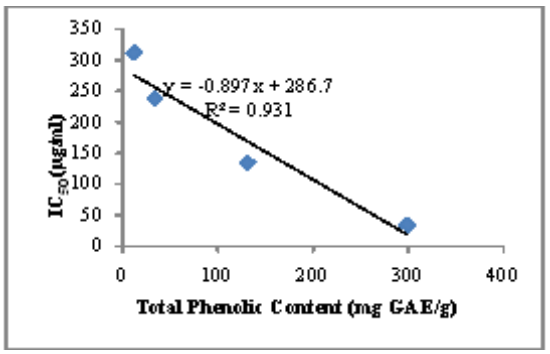

Fig. 3. Correlation between IC_{50} value and total phenolic content

A direct correlation between radical scavenging activity (IC_{50}) and TPC of the samples was demonstrated by linear regression analysis. The relationship between radical scavenging activity (Y) and total phenolic content (X) revealed coefficient of correlation (R^2) of 0.931. It can be stated that scavenging effects of extracts is not limited to phenolic compounds but other antioxidant secondary metabolites in the extracts such as volatile oils, carotenoids and vitamins may also play significant role (Javanmardi *et al.* 2003).

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References

Acharya, P. P., G. R. Gewali and M. Rajbhandari. 2013. Isolation of catechin from *Acacia catechu* Willdenow, estimation of total flavonoid content in *Camellia*

sinensis Kuntze and *Camellia sinensis* Kuntze var. *assamica* collected from different geographical region and their antioxidant activity. *Scientific World* **11:** 32-36

Aherne, S.A. and N. M. O'Brien. 1999. Protection by the flavonoids myricetin, quercetin, and rutin against hydrogen peroxide-induced DNA damage in Caco-2 and Hep G2 cells. *Nutrition and Cancer* **34:** 160-166.

Alia, M., R. Mateos, S. Ramos, E. Lecumberri, L. Bravo and L. Goya. 2006. Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2). *European Journal of Nutrition* **45:** 19-28.

Ames, B. N., M. K. Shigenaga and T. M. Hagen. 1993. Oxidants, antioxidants and the degenerative diseases of aging. *Proceeding of National Academy of Science U.S.A.* **90** (17) **:** 7915-7922.

Brand-William, W., M. E. Cuvelier and C. Berset. 1995. Use of a free radical method to evaluate antioxidant activity *Lebensmittel-wissenschaft und Technologie* **28 (1):** 25-30.

Cotelle, N. 2001. Role of flavonoids in oxidative stress. *Current Topics in Medicinal Chemistry* **1:** 569-590.

Gewali, G. R., P. P. Acharya and M. Rajbhandari. 2013. Isolation of gallic acid and estimation of total phenolic content in some medicinal plant and their antioxidant activity**.** *Nepal Journal of Science and Technology* **14:** 95-102

Heinonen, M. I., A. S. Meyer and E. N. Frankel. 1998. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. *Journal of Agriculture and Food Chemistry* **46:** 4107-4112.

Javanmardi J., C. Stushnoff, E. Locke and J. M. Vivanco. 2003. Antioxidant activity and total phenolic content of Iranian *Ocimum* accessions. *Food Chemistry* **83:** 547-550.

Johnson, M.K. and G. Loo. 2000. Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA. *Mutation Research* **459:** 211-218.

Kampa, M., A. P. Nifli, G. Notas and E. Castanas. 2007. Polyphenols and cancer cell growth. *Reviews of Physiology, Biochemistry and Pharmacology* **159:** 79-113.

Khan, A.U. and T. Wilson. 1995. Reactive oxygen species as cellular messengers. *Chemistry and Biology* **2:** 437-445

Mojzis, J., L. Varinska, G. Mojzisova, I. Kostova and L. Mirossay. 2008. Antiangiogenic effects of flavonoids and chalcones. *Pharmacological Research* **57:** 259-265.

Rice-Evans, C.A., N. J. Miller, P. G. Bolwell, P. M. Bramley and J. B. Pridham. 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research* **22:** 375-383.

Rice-Evans, C.A., N. J. Miller and G. Paganga. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine* **20:** 933-956.

Scalbert, A., C. Manach, C. Morand, C. Remesy and L. Jimenez. 2005. Dietary polyphenols and the prevention of diseases. *Critical Review of Food Science and Nutrition* **45:** 287-306.

Waterhouse, A. 2002. Determination of total phenolics. In: *Current protocols in food analytical chemistry* (Ed Wrolstad, R. E). John Wiley and Sons, New York, Units I1.1.1-I1.1.8.

Zhishen, J., T. Mengcheng and W. Jianming. 1999. The determination of ûavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry* **64:** 555-559.