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Isolation of Gallic Acid and Estimation of Total Phenolic Content in Some Medicinal Plants and Their Antioxidant Activity

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Abstract

Gallic acid was isolated from ethylacetate soluble portion of the methanol extract of fruit pulp of *Terminalia chebula* and characterized by comparing the melting point, R_f values, UV and IR spectra with authentic gallic acid. Quantitative determination of phenolics from different extracts of medicinal herbs such as *Adhatoda vasica* Nees, *Bergenia ciliata* (Haw) Sternb, *Phyllanthus emblica* Linnaeus, *Terminalia bellirica* (Gaerth) Roxb, *Terminalia chebula* Retzius and *Vitex negundo* Linnaeus which are commonly used as home herbal remedies for the primary health care was carried out using Folin-Ciocalteu colorimetric method. Gallic acid was used as the standard for the estimation of phenolics. All the investigated plant extracts contained high amount of phenolics but the highest amount was detected in 70% acetone extract of *B. ciliata* (357.08 mg GAE/g sample) and the lowest amount was detected in 50% aqueous methanol extract of *T. bellirica* (108.69 mg GAE/g sample). The antioxidant activity of selected extracts was determined by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay. The extract having the highest phenolic content showed the lowest IC_{50} demonstrating the positive correlation between radical scavenging activity and total phenolic content.

Key words: antioxidant activity, gallic acid, medicinal herbs, total phenolic content

Introduction

Natural products, especially plants are the sources of different classes of phytochemicals. Phenolics are aromatic compounds bearing one or more hydroxyl substituents. Plant phenolics include phenolic acids, flavonoids, tannins, stillbenes and lignins. Phenolic acids are divided into derivatives of benzoic acid such as gallic acid and derivative of caeffic acid such as coumaric, caeffic and ferulic acids. Both flavonoids and phenolic acids are the most common polyphenols in our diet and distributed widely in fruits, vegetables, cereals and beverages.

In recent years, much attention has been paid by nutritionist on the dietary polyphenols due to their potent antioxidative effects and their credible effects in the prevention of various oxidative stress associated diseases. Oxidation process is one of the most important ways for producing free radicals in food and even in living systems. Free radicals cause many human diseases like cancer, Alzheimer's, cardiac, kidney and liver diseases, fibrosis, atherosclerosis, arthritis, neurodegenerative disorders and aging (Halliwell & Gutteridge 1990). The preventive effects of plant polyphenols and their use in treating diseases are deduced from the epidemiological data as well as *in vitro* and *in vivo* studies (Art & Hollamn 2005).

Many research studies have demonstrated that medicinal plants, fruits, and vegetables contain various phytochemicals with antioxidant activity, which are responsible for their beneficial health effects (Scalbert *et al*. 2005). In addition to vitamin C, E and carotenoids, polyphenols have shown strong antioxidant capacity (Rica-Evan *et al*. 1995). Due to their natural origin, the antioxidants obtained from plants are of greater benefit in comparison to synthetic ones such as butylatedhydroxy anisole (BHA) and butylatedhydroxy toluene (BHT).

Nepal is rich in medicinal plants and we consume several types of these plants and plant products as herbal medicine for primary health care. These plants could be the potential source of natural antioxidants. However, no quantitative information are available on the polyphenol content and antioxidant effect of Nepalese herbs. In our previous study, we have quantified the total flavonoid content in different parts of *Camellia* plants and assessed their antioxidant activity (Acharya *et al*. 2013). The present study was conducted to quantify the total phenolic content in different medicinal plants using gallic acid as the standard. The antioxidant activity of selected extracts were determined using DPPH free radical scavenging assay. The correlation between total phenolic content and antioxidant activity was established. This will help to find new sources of safe and inexpensive natural antioxidants to use them in food or nutraceutical and pharmaceutical preparations to replace synthetic antioxidants.

Methodology

Chromatographic materials and chemicals

TLC foils (precoated) Silica gel 60 GF_{254}, 0.2 mm and TLC foils (precoated) Cellulose F, 0.2 mm, were purchased from Merck, Darmstadt, Germany. Sephadex LH-20 was purchased from Pharmacia Biotech, Uppasala, Sweden. Gallic acid was purchased from Merck, Germany and DPPH from Sigma Chemical Company, USA. Folin-Ciocalteu reagent was purchased from SD Fine Chemical Limited, Mumbai. All other chemicals were of analytical grade.

Plant materials

The dried fruits of *T. chebula* were purchased from the local market of Kathmandu. Other plants materials were collected from Gulmi district at an altitude between 1200-1600 m in March 2012. The plants were authenticated by Prof. R. P. Chaudary, Central Department of Botany, Tribhuvan University. Voucher specimens were deposited at the Research Centre for Applied Science and Technology, Tribhuvan University, Kathmandu, Nepal.

Extraction of *T. chebula* fruits and isolation of gallic acid

The flesh of dried fruits of *T. chebula* were ground to a fine powder (100 g) and extracted with 250 ml methanol in a Soxhlet extractor for 15 hours. The extract was dried under reduced pressure at a Rotavapour to get viscous mass (36 g) which was suspended in 100 ml distilled water and then extracted with hexane (300 ml) followed by ethyl acetate (300 ml) in a separatory funnel. The solvent was evaporated in a rotavapour under reduced pressure to get hexane (5 g) and ethylacetate (9 g) extracts.

The ethyl acetate extract (9 g) was chromatographed on a Sephadex LH-20 column eluting with methanol. Altogether five major fractions were collected after monitoring by TLC for the presence of gallic acid (R_f 0.44) in solvent systems, toluene-ethylacetate-formic acid (6:6:1). The fraction F-4 (200 mg) containing gallic acid was further purified by preparative thin layer chromatography in the solvent system toluene-ethylacetate-formic acid (6:6:1). An amount of 20 mg of sample F-4 was loaded on the home made preparative thin layer chromatographic plate (Silicagel GF_{254}, 20 x 20 cm, 1 mm thickness). The band corresponding to gallic acid was scratched off. Silica gel was washed with methanol and the solvent was evaporated to get white crystals of gallic acid (3 mg). It was finally purified by re-crystallization with hot water.

Preparation of extracts for the determination of total phenolics

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 6 hours. Similarly, 20 g of each 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.

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 (10, 25, 50 and 75 µg/ml) were prepared. In a 20 ml test tube, 1 ml gallic acid of each concentration was added and to that 5 mL of Folin-Ciocalteu reagent (10%) and 4 mL of 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 (25, 50, 100 and 200 µg/mL) were prepared. Following the procedure described for standard, absorbance for each concentration of extract was recorded. Total phenolics content of the extracts was expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g). The total phenolic contents in all samples was calculated the using the formula: C = c V/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.

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 content was calculated.

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

Antioxidant activity of the selected extracts was assayed using DPPH free radical (Brand-Williams *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 ^{o}C. Thus prepared purple colored DPPH free radical solution was stored at -20 ^{o}C for further use.

Three different concentrations (5, 10 and 15 µg/ml) of methanolic solutions of each extracts were prepared by the serial dilution of the stock solution (10 mg/ml) of the respective extract. To each 0.5 ml extract solution, 2.5 ml of 0.1 mM DPPH solution was added. A control was prepared by mixing 0.5 ml distilled water and 2.5 ml 0.1 mM DPPH solution. These samples were shaken well and kept in dark for 30 minutes 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

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

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 content was determined by plotting IC_{50} (µg/ml) against TPC (mg/g).

Results and Discussion

Isolation and characterization of gallic acid from *T. chebula*

Column chromatograph of the ethyl acetate phase of the methanol extract of *T. chebula* over Sephadex LH 20 followed by preparative thin layer chromatography on silicagel GF_{254} yielded gallic acid, which was purified by recrystallization with hot water. On TLC it appeared as dark coloured spot under UV 254 nm, blue colour with ferric chloride reagent and folin reagent with R_f value 0.4 in ethyl acetate-benzene (9:11) and 0.44 in toluene-ethyl acetate-formic acid (6:6:1). The melting point was found to be 248-250 ^{o}C. Thin layer chromatography behavior and the melting point of the isolated gallic acid was in good agreement with the authentic gallic acid. The UV spectrum of the isolated and authentic gallic acid showed absorption bands at 220 and 270 nm. The IR spectrum showed the band at 3495, 1666, 1612, 1319, 3278, 1535, 1419 cm^{-1}. On the basis of all these facts, the compound isolated from *T. chebula* was confirmed as gallic acid.

Amount of different extracts obtained by different extraction methods

For the determination of total phenolics in plant extracts, different extraction approaches such as

soxhlet using methanol, reflux using 50% aqueous methanol and ultra sound assisted using 70% acetone were applied. Generally methanol is used for the extraction of low molecular weight polyphenols, 50% aqueous methanol for the extraction of glycosides while 70% aqueous acetone is used for the extraction of higher molecular weight tannin components. Conventional long extraction time and high temperature increase the chance of oxidation of phenolics so ultrasound assisted extraction (UAE) technique was applied. The mechanism involves the propagation of the acoustic waves in KHz range that produce physical, chemical and mechanical effect causing disruption of biological membrane to facilitate the release of extractable compounds (Loborde *et al*. 1998). The different extraction approaches provided different extracts in various amounts. The prepared extracts were used for the determination of total phenolics. The names of plants and the respective parts used for extraction and the amount of different extracts obtained per 20 g of dry plant materials under different extraction conditions are summarised in Table 1.

Table 1. Amount of different extracts obtained from 20 g of dried plant materials under different extraction conditions

Name of plants	Parts used	Local name	Methanol extract (g)	50% Methanol extract (g)	70% Acetone extract (g)
Terminalia bellirica (Gaerth) Roxb	Fruit	Baro	2.0	5.0	3.5
Terminalia chebula Retzius	Fruit	Harro	3.5	5.0	3.0
Phyllanthus emblica Linnaeus	Fruit	Amala	1.5	-	1.5
Bergenia ciliate (Haw) Sternb	Rhizome	Pashanveda	5.5	2.5	3.0
Adhatoda vasica Nees	Leaves	Kalovashak	1.5	1.0	1.5
Vitex negundo Linnaeus	Leaves	Simali	2.0	2.0	3.0

Construction of calibration curve and calculation of total phenolic content in different plant extracts

Total phenolic content in different extracts were determined by Folin-Ciocalteu (F-C) method using gallic acid as the standard. 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 ($Y = 0.014x$; $R^2 = 0.999$) and expressed as mg gallic acid equivalents (GE) per gram of sample in dry weight (mg/g). The results are presented in Table 2.

Table 2. Total phenolic content in different extracts

Plant	Methanol (mg/g) GAE	50% Methanol (mg/g) GAE	70% Acetone (mg/g) GAE
T. chebula	237.827 ± 10.130	318.361 ± 10.370	238.865 ± 11.173
T. bellirica	149.690 ± 6.088	108.692 ± 9.914	186.288 ± 10.618
P. emblica	197.371 ± 4.244	-	250.420 ± 11.540
B. ciliata	304.00 ± 18.180	117.428 ± 9.316	357.079 ± 11.946
A. vasica	109.512 ± 9.589	111.2 ± 8.806	186.495 ± 10.570
V. negundo	151.618 ± 6.3886	150.98 ± 9.979	226.518 ± 11.0412

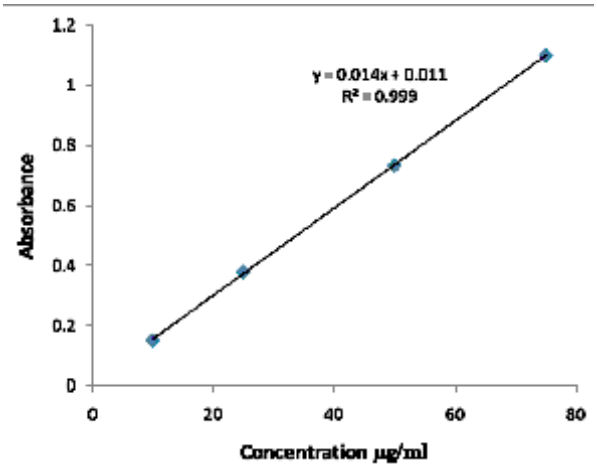

Fig 1. Calibration curve for standard gallic acid

In the case of *B. ciliate,* the highest amount of phenolic was detected in 70% acetone extract (357.08 mg GAE/ g sample) and in methanol extract (304.0 mg GAE/g sample) than in 50% aqueous methanol extract (117.43 mg GAE/g sample). In the case of *T. chebula* the highest amount of phenolics was detected in 50% aqueous methanol extract (318.36 mg GAE/g sample) whereas the methanol and 70% acetone extracts contain the same amount of phenolics. Again, in the case of *P. emblica* (250.42 mg GAE/g), *V. nigundo* (226.51mg GAE/g sample), *T. bellirica* (186.28 mg GAE/g sample) and *A. vasica* (187.49 mg GAE/g sample) high amount of phenolics were detected in 70% acetone extracts than in methanol and 50% aqueous methanol extracts. The lowest amount of phenolics were detected in 50% aqueous methanol extract of *T. bellirica* (108.69 mg GAE/g sample). Comparing the extraction methods, 70% acetone is found to be the best solvent for the extraction of phenolics.

It is well known that the genus *Bergenia* is a high polyphenol, specially tannin containing plant (Rajbhandari & Schoepke 2011). Previous phytochemical investigation of the rhizomes of *B. ligulata* led to the isolation of polyphenols like bergenin, 11-*O*-*p*-hydroxybenzoylbergenin, 6-*O*-galloylarbutin and epiafzelechin (Rajbhandari *et al*. 2011).This could be the reason for the presence of high amount of phenolic in 70% acetone extract. On the other hand, *T. chebula,* one of the constituents of Ayurvedic formulation, triphala is a rich source of hydrolysable tannins, both gallotannins and ellagitannins and traditionally used to treat heart and spleen diseases. It was reported that 33% of total phyto-constituents of the fruits of *T. chebula* are hydrolysable tannins that are responsible for various pharmacological activity. These tannins contain phenolic carboxylic acid like gallic acid, ellagic acid, chebulic acid and gallotannins such as 1,6-di-*O*-galloyl-*â*-D-glucose; 3,4,6-tri-*O*-galloyl-*â*-D-glucose; 2,3,4,6-tetra-*O*-galloyl-*â*-D-glucose; 1,2,3,4,6-penta-*O*-galloyl-*â*-D-glucose. Ellagitannin such as punaclagin, casurarinin, corilagin and terchebulin and others such as chebulanin, neochebulinic acid, chebulagic acid and chebulinic acid are also reported (Chattopadhaya & Bhattacharya 2007). Therefore, highest amount of phenolics were detected in 50% methanol extract. Similarly, *P. emblica* is reported to contains phenolic compounds such as tannins, phyllaemblic compounds, gallic acid, ellagic acid, 1 *O*-galloyl *â* D glucose, 3, 6 di *O* galloyl D glucose, chebulinic acid, chebulagic acid, corilagin together with flavonoids, and vitamin C (Zhang *et al*. 2003). This could be the reason for the presence of high amount of phenolics in 70% acetone extracts. In *V. negundo* flavonoids and lignins were reported (Chandramu *et al*. 2003; Maurya *et al*. 2007) and it could be reason for high phenolic content in 70% acetone extracts. It is well known that *A. vasica* is a alkaloid containing plant but very few reports about polyphenol content are available (Kathale 2013) and in our finding high level of phenolic was detected in 70 % acetone extract. *T. bellirica* is reported to contain 20% tannin, both condensed and hydrolysable; ethyl gallate, galloyl glucose, gallic acid, belliric acid and chebulic acid (Bele *et al*. 2010) were the main constituents. But the total phenolic content is found to be relatively low in all three extracts. Traditionally it is used to terat various ailments and also one of the constituents of triphala.

DPPH radical scavenging assay

The DPPH assay is based on the capability of an antioxidant to donate a hydrogen radical or an electron to DPPH radical, which is stable free radical with deep violet color. When an odd electron becomes paired in the presence of free radical scavenger of antioxidant agent, DPPH radicals get reduced to corresponding hydrazine, DPPH-H form (Piaxao *et al*. 2007) and the solution gets decolorized from its initial deep violet to light yellow colour. The degree of fall in the absorbance is measured spectrophotometrically and is proportional to the concentration of the antioxidant.

Few extracts, 70% acetone extract *B. ciliata* having highest phenolic content, methanol extract of *T. chebula* having medium phenolic content and 50% methanol extract of *T. bellirica* having the lowest phenolic content were selected for their free radical scavenging capacities using DPPH free radicals. The absorbance values were measured at wavelength 517 nm for different concentration of extracts and the control. These values are used to calculate the percentage inhibitions of DPPHÿ radicals against the samples. The IC_{50} values of various extracts were calculated from the percentage inhibitions at various concentrations. The extract containing high amount of phenolic showed high radical scavenging activity. The results are shown in Table 3.

Table 3. Total phenolic content and IC_{50} values

Plant /Extracts	Total Phenolic (mg/g)GAE	IC_{50} µg/ml
T. chebula/ Methanol extract	237.827	109.78
T. bellirica/ 50% Methanol Extract	108.692	213.11
B. ciliata/ 70% Acetone Extract	357.796	21.11

The correlation between antioxidant activity and TPC (Fig. 2) had been determined by plotting IC_{50} (µg/ml) against TPC (mg/g). A direct correlation between radical scavenging activity (IC_{50}) and TFC of the samples was observed which indicated that the phenolic compounds are responsible for DPPH free radical scavenging of the extracts.

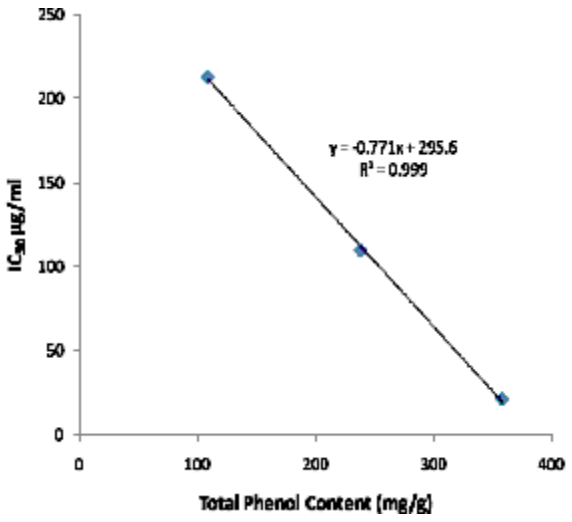

Fig. 2. correlation between DPPH radical scavenging activity and total phenolic

The results of the investigation indicated that the plants *T. chebula, T. bellirica, P. emblica, B. ciliate, A. vasica and V. negundo* that are often used as home herbal remedies are the rich source of high value polyphenol compounds. They are also the most commonly used components of Ayurvedic formulations. The consumption of these plants may have beneficial implications in human health such as in the treatment and prevention of cancer, cardiovascular disease and other pathologies by delaying or inhibiting the oxidation of lipids or other macromolecules and inhibiting the initiation or propagation of oxidative chain reactions. In addition, the easy availability of these plants make them promising sources of natural antioxidants and other bioactive compounds in food and pharmaceutical industries.

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