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 ACTIVITIES

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Abstract: Catechin, a flavanol was isolated from the ethylacetate soluble portion of the water extract of red heartwood of *Acacia catechu* Willd. The isolated catechin was characterized by comparing melting point, R_f values, UV and IR spectra with authentic catechin. Quantitative determination of flavonoids in different parts of *Camellia sinensis* Kuntz and *Camellia sinensis* Kuntz var. *assamica* under different extraction conditions, collected from Ilam, Jhapa and Pachthar was carried out using aluminum chloride colorimetric method. Catechin was used as the standard for the calibration of flavanoids. The greatest total flavonoid content was revealed in the methanol extract of the stem collected from Ilam (192.18 mg catechin/g sample) and the lowest was determined in 50% methanol extract of old leaves collected from Jhapa (19.21 mg catechin/g sample). The antioxidant activity of the selected tea extracts was determined by DPPH assay. The extract having the highest flavonoid content showed the lowest IC_{50} demonstrating the positive correlation between radical scavenging activity (IC_{50}) and total flavonoid content. The flavonoids contributed 65.8 % to free DPPH radical scavenging of the extracts. The result indicated that the *Camellia* plant is the rich source of high value polyphenol compounds as natural antioxidants to use in preventive medicines and food industry.

Kewwords: Acacia catechu; Catechin; Camellia species; Total flavonoid content; Antioxidant activity;Correlation.

INTRODUCTION

Polyphenols are the class of chemical compounds synthesized by fruits, vegetables, teas, cocoa and other plants that possess certain health benefits. They are an integral part of human diet and responsible for overall organoletpic properties of plant foods. Plant polyphenols have drawn increasing attention due to their potent antioxidant properties and their marked effects in prevention of various oxidative stress associated diseases such as cardiovascular, cancer and neurodegenerative diseases [1-4].

Catechin, a flavanol is a polyphenolic compound found in tea, coca and several fruits. The terminal three leaves of the Camellia plant is used for the production of green, oolong and black tea. The green tea contains 30 to 50% polyphenols[5] mainly epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate on the dry weight basis[6,7]. They constitute 90% of the total flavonoids[8] and a cup of green tea contains about 150 to 210 mg of polyphenols[9]. In black tea, catechins are converted to theaflavines[6,10]. Many researches have demonstrated that the phytochemicals present in tea have beneficial effect as they act as a free radical scavenger. They have shown strong antioxidant activity like vitamin C, E and carotenoids[11], anti-inflamatory [12], cholesterol lowering[13], antiviral and antibacterial activities[14,15].

Although processed teas were well investigated for their polyphenol content and antioxidant activities no

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information is available on the polyphenol content and antioxidant effect of Nepalese Camellia plants. Hence the present study was conducted to quantify the total flavonoid content in different parts and different extracts of Nepalese Camellia plants collected from different geographical regions using catechin as a standard, determination of antioxidant activity of selected extracts using DPPH free radical scavenging assay and to find out correlation between total flavonoids content and their antioxidant activity. This will help to find new sources of safe and inexpensive natural antioxidants to use them in food and pharmaceutical preparations to replace synthetic antioxidants.

MATERIALS AND METHODS

Chromatographic materials and Chemicals

TLC foils (precoated) Silica gel 60 GF254, 0.2 mm and TLC foils (precoated) Cellulose F, 0.2mm, were purchased from Merck, Darmstadt, Germany. Sephadex LH-20 was purchased from Pharmacia Biotech, Uppasala, Sweden. DPPH and (±) catechin was purchased from Sigma Chemical Company, USA. Aluminum chloride was purchased from sd fine-chemicals. All other chemicals were of analytical grade.

Plant materials

Red heartwood of A. catechu Willd was collected from Jhapa district in January 2012. Fresh young and old leaves twigs and stems of *C. sinensis* and C. sinensis var. *assamica* were collected from the tea gardens of Panchthar, Ilam and Jhapa districts in February 2012. They were authenticated by Prof. R. P. Chaudhary, Central Department of Botany, Tribhuvan University, Kathmandu, Nepal. Voucher specimens were deposited at the Research Centre for Applied Science and Technology, RECAST, Tribhuvan University.

Extraction of A. catechu heartwood and isolation of catechin

The fine chips of the heartwood of A. catechu was boiled with water. The water was evaporated and the dried extract was subjected to liquid-liquid extraction using ethylacetate and water. The ethylacetate phase was dried in a rotator evaporator to get a viscous mass. The ethylacetate extract was subjected to gel column chromatography on Sephadex LH-20 using methanol. Altogether four major fractions were collected. The fractions were examined for the presence of catechin by TLC analysis in different solvent systems and the chromatogram was visualized by spraying with vanillin-HCl reagent which produce dark pink colour against white background, is a specific reagent for the detection of condensed tannins. The catechin accumulated fraction was further subjected to Sephadex LH-20 column chromatography using methanol and three major fractions were collected. The fraction containing catechin with minor impurities was purified by Sephadex LH-20 column chromatography using 50% aqueous methanol. The white powder thus obtained was further purified by recrystallization with hot water.

Extraction of *C. sinensis* and *C. sinensis* var *assamica*

An amount of 20 g each of the different parts (old leaves, young leaves and stem) of dried and powdered plant samples were extracted with methanol in a soxhlet extraction apparatus. The residue was extracted with 50% aqueous methanol under reflux. Similarly, each 20 g of different parts of dried and powdered samples were percolated with 70% acetone and subjected to ultrasound-assisted extraction. The extracts were filtered and the solvent was evaporated in a rotary evaporator under reduced pressure.

Determination of Total Flavonoid Content in different extracts

Preparation of standard

The total flavonoid content was determined by aluminum chloride colorimetric assay (16). Various concentrations of standard catechin (0.75mg/mL, 0.5mg/mL, 0.25mg/mL and 0.125mg/mL) were prepared. An aliquot of 1 mL catechin of each concentration in methanol was added to 10 mL volumetric flask containing 4 mL of 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 catechin. The average absorbance values obtained at different concentrations of catechin were used to plot the calibration curve.

Preparation of sample

Various concentrations of the extracts (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 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 catechin equivalents (CE) per gram of sample in dry weight (mg/g). Total flavonoid content is calculated by using the formula: $C = cV/m$ where, C = total flavonoid content mg CE/g dry extract, c = concentration of catechin 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 flavonoid content was calculated.

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

Antioxidant activity of the selected extracts was assessed (17) using DPPH free radical. 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 extracts 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

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

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 flavonoid content, TFC was determined by plotting IC_{50} (µg/mL) against TFC (mg/g).

RESULT AND DISCUSSION

Characterization of catechin from *A. catechu*

Repeated column chromatograph of the ethylacetate phase of the water extract of A. catechu over Sephadex LH 20 yielded catechin which was purified by recrystallization with hot water. Thin layer chromatography behavior and the melting point of the isolated catechin is in good agreement with the authentic catechin. The UV spectrum of the isolated and authentic catechin showed absorption bands at 220 and 277 nm. The IR spectra of the catechin has a broad band around 3400-2600 cm^{-1} region corresponding to the aliphatic and aromatic C-H, phenolic and alcoholic O-H stretching. Other stretching's were comparable with IR spectra of authentic catechin.

Total flavonoid contents in *C. sinensis* and *C. sinensis* var. *assamica*

Total flavonoid contents in the extracts were 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. Total Flavonoid content of the extracts was calculated from the regression equation of calibration curve (y=0.002x; R^2=1) and expressed as mg catechin equivalents (CE) per gram of sample in dry weight (mg/g).

Total flavonoid content differs according to the nature of the extract and the plant parts used. However, topography of the collection site is not significant. The total flavonoid content in methanol and 70% acetone extracts were relatively high when compared with 50% aqueous methanol extract in all samples collected from Ilam, Jhapa and Panchthar. Similarly, the total flavonoid present in the young leaves and the stem collected from all three geographical regions were relatively high in comparison to the old leaves. The highest amount of flavonoid was detected in the methanol extract of the stem collected from Ilam (192.18 mg catechin/g sample) and the lowest amount was detected in 50% aqueous methanol extract of old leaves collected from Jhapa (19.21 mg catechin/g sample). The methanol and 70% acetone extracts of young leaves collected from Ilam, Jhapa and Panchthar contain nearly the same amounts of flavonoids ranged from 116.08-140.86 mg catechin/g sample. On the other hand, the total flavonoid contents in the methanol and 70% acetone extracts of stem collected from three different regions showed little variation and ranged from 103.57-192.18 mg catechin/g sample. All the three extracts of the old leaves collected from all three regions showed the presence of relatively low amount of total flavonoids than the stem and young leaves. However, 70% acetone extract of old leaves showed the presence of somehow higher amount of flavonoid (84.82 mg catechin/g sample) than the methanol extract (70.30 mg catechin/g sample). The total flavonoid content in different parts of Camellia plant is given in Table 1.

Tea, particularly green tea is a source of polyphenols and thus acts as potential antioxidants. Many reports are available about the total flavonoid content and the

Table 1: Total Flavonoid Content in different parts of various *Camellia* plants

Plants	Parts	Collection site	Methanol (mg/g)	50% Methanol (mg/g)	70% Acetone (mg/g)
C. sinensis	Young leaves	Ilam	129.4625 ± 3.4841	48.25 ± 2.5851	140.8625 ± 3.9053
C. sinensis		Jhapa	134.125 ± 1.2316	30.25 ± 4.0234	123.5125 ± 2.7673
C. sinensis var *assamica*		Panchthar	116.0875 ± 2.5245	45.5125 ± 2.0532	132.45 ± 3.1635
C. sinensis	Old leaves	Ilam	70.3 ± 4.428	30.6375 ± 2.9999	84.6625 ± 2.9093
C. sinensis		Jhapa	62.55 ± 4.3557	19.2125 ± 3.8721	78.2125 ± 6.4719
C. sinensis var *assamica*		Panchthar	40.575 ± 2.4273	31.275 ± 2.2354	84.825 ± 4.9073
C. sinensis	Stem	Ilam	192.187 ± 1.4117	37.55 ± 2.5705	142.612 ± 7.9321
C. sinensis		Jhapa	124.20 ± 1.5953	55.962 ± 2.8960	141.15 ± 4.5401
C. sinensis var *assamica*		Panchthar	103.575 ± 0.7361	48.5625± 0.9092	121.925 ± 1.9917

antioxidant activity but the result varies depending on the assay method. The aluminum chloride colorimetric assay of 50% aqueous methanol extract Camellia, green tea revealed the presence of 19.17 mg catechin/g sample [18] and water extract revealed 47 mg epicatechin/g sample[19]. These values are very low when compared with our results. The total polyphenol contents and antioxidant activities depends on the geographical regions of growth, season of collection, storage condition and extraction methods.

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 become paired in the presence of free radical scavenger of antioxidant agent, DPPH radicals get reduced to corresponding hydrazine, DPPH-H form[20] and the solution gets decolorized from its initial deep violet to light yellow color. The degree of fall in the absorbance is measured spectrophotometrically and is proportional to the concentration of the antioxidant.

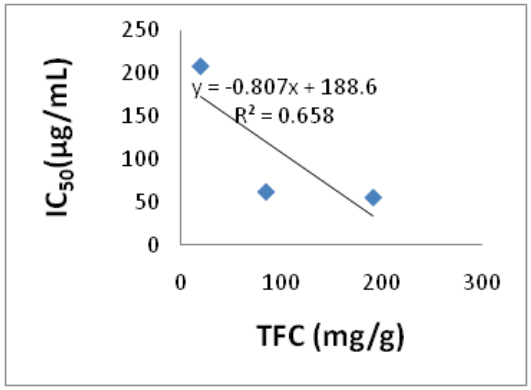

Fig 1: Correlation between radical scavenging activity and total flavonoid content.

Selected extracts, methanol extract of C.sinensis (stem/ Ilam, TFC 192.18), 50% methanol extract of C. sinensis (old leaves/Jhapa, TFC 19.21) and 70% acetone extract of C. sinensis var. assamica (old leaves/Panchthar, TFC 84.82) were assessed 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 DPPH$^{\cdot}$ radicals against the samples. The IC_{50} values of various extracts were calculated from the percentage inhibitions at various concentrations and found to be 55.47, 209.13 and 62.13 µg/mL respectively. The extract containing high amount of flavonoids showed high radical scavenging activity.

The correlation between antioxidant activity and TFC had been determined by plotting IC_{50} (µg/mL) against TFC (mg/g). The relationship between total flavonoid contents and free radical scavenging activity, FRSA of the samples is shown in Fig 1. A direct correlation between radical scavenging activity (IC_{50}) and TFC of the samples was demonstrated by linear regression analysis. The relationship between radical scavenging activity (Y) and TFC (X) revealed coefficient of determination R^2 of 0.658. These result suggested that flavonoid compounds contributed 65.8 % to free DPPH radical scavenging of the extracts.

It is well known that green tea is a potential source of natural antioxidant and its antioxidant power is correlated to the total phenolic content. The antioxidant activity of phenolics is due to their redox properties which allow them to act as reducing agents, hydrogen donors, singlet oxygen quencher and metal chelators[21-22]. Synthetic antioxidants used in food industry such as butylatedhydroxy anisole (BHA) exhibit genotoxic and carcinogenic effect, while butylatedhydroxy toluene (BHT) is proven to cause hemorrhage[23-25]. Thus, the natural antioxidants from plants are of greater interest in preventive medicines and food industry[26] and their activity is considered to be multifunctional to prevent oxidation in complex food system [27].

CONCLUSION

The results of the investigation indicated that the different parts of Camellia plants are the rich source of high value polyphenol compounds as natural antioxidants. These compounds were easily extractable with methanol and 70% acetone rather than 50 % methanol. Therefore, not only the terminal three leaves of shoots of Camellia plants but the twigs and stems can also be used for preparing tea and other home herbal remedies which may have possible beneficial implications in human health such as in the treatment and prevention of cancer, cardiovascular disease and other pathologies.

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