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(+)-Catechin and (-)-Epicatechin contents and antioxidant activity

C. Jadwiga Grabarkiewicz-Szczfsna, W. Karl Hult

Department of Plant Pathology, Agricultural University, 02-766 Warszawa, Poland

ABSTRACT

Catechu is considered as one of the most potent herbal medicines use for anti-diarrhea and anti-ulcer. A simple and reliable method to determine (+)-catechin and (-)-epicatechin contents in commercial black and pale catechu was performed by high performance liquid chromatography (HPLC). The antioxidant activities, total phenolic, and total tannin contents of commercial black catechu and pale catechu in Thailand markets were studied. The extracts of some black catechu and all pale catechu showed high antioxidant activities, total phenolic, and non-tannin phenolic contents whereas total tannin contents were quite low. Some black catechu samples were found to be rich source for (+)-catechin and (-)-epicatechin. High (+)-catechin contents were found in all pale catechu whereas (-)-epicatechin were detected at very low concentrations. The main validation parameters of HPLC method were determined. The method was linear over a range of 5-200 μ g/ml with high coefficients (r^2> 0.99) for both (+)-catechin and (-)-epicatechin. The method also showed good recovery, good repeatability and intermediate precision (%RSD < 3). The results demonstrated that greater amount of phenolic contents lead to more potent antioxidant effect. Moreover, HPLC method can be applied to determine (+)-catechin and (-)-epicatechin contents in plant materials.

Keywords: cutch, gambir, catechin isomers, antioxidantion,non-tannin phenolic content

INTRODUCTION

Black catechu and pale catechu are well-known crude drugs which have been previously used as an alternative medicine for treatment diarrhea and sore throat. They are componentsof Ya-Leong-Pid-Smut, Thai traditional medicine from the list of Herbal Medicine Product A.D. 2006. In commerce, catechu is applicable to black catechu and pale catechu. Black catechu or cutch is the solid extract obtained from the heartwood of *Acacia catechu* (Linn.f.) Willd.(Mimosaceae),a common tree of India, Pakistan, Myanmar, and Thailand. It is a round mass with blackish-brown, shining, heavy, and hard. It is odorless but has a strong astringent taste. Pale catechu or gambir is the solid extract prepared from the leaves and stems of *Uncariagambir* (Hunter) Roxb.(Rubiaceae), a shrubby plant that mostly found in Southeast Asia. It is generally a small cylinder of pale reddish-brown color, light, and friable. Its taste is bitter and astringent [1-5].

Catechin is a polyphenol antioxidant plant metabolite which extracted from *A. catechu* and *U. gambir*. Several recent studies are reports on the application of HPLC method for quantitation and isolation of catechin which presented in *A. catechu* and *U. gambir* [6-9]. (+)-Catechin ($C_{15}H_{14}O_6$) and (-)-epicatechin ($C_{15}H_{14}O_6$) are the most common optical isomers that found in nature [10]. The structures of catechins were illustrated in Figure 1. For this reason, these 2 compounds were selected as markers in present study. Nowadays, the antioxidant activities of *A. catechu* and *U. gambir* are reported [8, 11-13]. However, there are few reports about the antioxidant activity, total phenolic content, total tannin content and quantitation of chemical constituents in commercial black and pale catechu. Consequently, this present study was attempted to investigate the antioxidant activities, total phenolic

content, total tannin contents as well as to determine (+)-catechin and (-)-epicatechin contents of commercial black and pale catechu in Thailand markets.

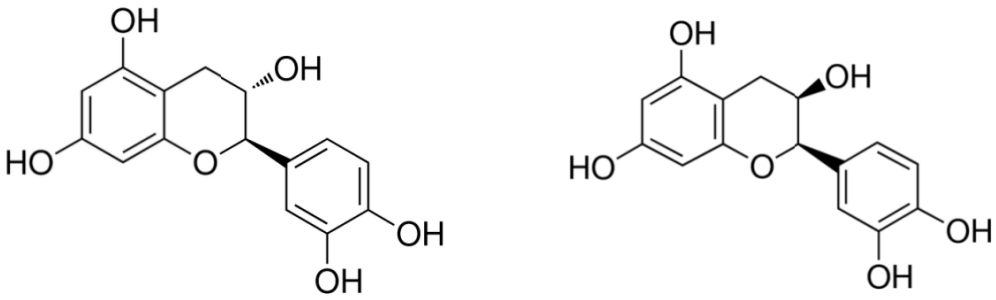


Figure 1 The structure of (+)-catechin (Left) and the structure of (-)-epicatechin (Right)

EXPERIMENTAL SECTION

Sample Collection

Twenty two samples of black catechu and 20 samples of pale catechu were purchased from Thai traditional drug stores located at four regions of Thailand. All sets of crude drugs were authenticated by Associate Professor Dr. NijsiriRuangrungsi.

One milligram of each sample was mixed with 1 ml of water. The mixture was diluted to evaluate the antioxidant activities, total phenolic, and total tannin contents at concentration of 100 µg/ml.

Chemicals and materials

2, 2-diphenyl-1-picylhydrazyl (DPPH), iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), sodium carbonate (Na_2CO_3), sodium acetate ($C_2H_3NaO_2$), hide powder, (+)-catechin hydrate (CAS no. 225937-10-0, purity ≥ 98 %), (+)-catechin (CAS no. 154-23-4, purity ≥ 99 %), and (-)-epicatechin (CAS no. 490-46-0, purity ≥ 98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iron (III) chloride ($FeCl_3{\cdot}6H_2O$) and ferrozine were purchased from Ajax Finechem (New Zealand) and Fulka (USA) respectively.Ethylenediaminetetraacetic acid (EDTA) and Folin-Ciocalteu reagent were obtained from Merk (Darmstadt, Germarny). HPLC grade methanol and acetonitrile were obtained from RCI Labscan, Thailand. Formic acid was purchased from Fisher Scientific (Leicestershire, UK). Ultra-pure water was prepared by SNW ultra-pure water system (NW20VF, Heal Force). The filters were 46 mm x 0.45 µm nylon membrane filters (National Scientific, TN) and 13 mm x 0.45 µm PTFE membrane syringe filters (ANPEL Scientific Instrument, China).

Antioxidant activity

2, 2-diphenyl-1-picylhydrazyl (DPPH) assay

Five hundred microliters of sample at concentration of 100 µl/ml in water was mixed with 500 µl of 120 µM DPPH solution in methanol. The mixture was left to stand for 30 min in the dark at room temperature. The reduction of the DPPH radical was determined by measuring theabsorbance at 517 nm using a UV-spectrophotometer (UV-1800 model, Shimadzu, Kyoto, Japan). A blank sample contained the same amount of distilled water and DPPH solution. Catechin hydrate was used as a positive control. All samples were performed in triplicate. Percent scavenging activity was calculated from the following equation:

$$\text{Scavenging activity (\%)} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})/ \text{Absorbance}_{\text{control}}] \times 100$$

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed in 96 well plates to assess antioxidant power. FRAP reagent was prepared according to the method of Benzie and Strain [14]. Briefly, the FRAP reagent was prepared by mixing 100 ml of 300 mM acetate buffer pH 3.6 with 10 ml of 10 mM TPTZ dissolved in 40 mMHCl and 10 ml of 20 mM $FeCl_3{\cdot}6H_2O$ dissolved in distilled water. Freshly prepared reagent was warmed at 37 ˚C before used. Twenty five microliters of each sample (100 µ g/ml) was mixed with 175µ l of the FRAP reagent for 30 min under the dark conditions. The absorbance was measured at 593 nm using a microplate reader (BiochromAsys UVM 340). Aqueous solutions of $FeSO_4{\cdot}7H_2O$ in the range of 0.1-1.0 mM were used for calibration curve. Results were expressed in mM Fe (II)/mg of dry sample. In order to make comparison, catechin hydrate was also tested under the same conditions as standard antioxidant compound. Triplicate measurement were carried out.

Metal ion chelation activity

The chelating activity of sample on Fe^{2+} was measuredaccording to the method of Dinis*et al*. [15]. Briefly, 150 µl of the sample at concentration of 100 µl/ml in water was incubated with 7.5 µl of 2 mM $FeCl_2$ for 5 min. Then 30 µl of 5 mMferrozine was added to the mixture. After 10 min, the absorbance of ferrous ion-ferrozine complex at 562 nm was read using a microplate reader. EDTA was served as positive control. All determinations were performed in triplicate. The ability of the sample to chelate ferrous ion was calculated using the following equation:

Chelating activity (%) = [(Absorbance $_{control}$ – Absorbance $_{sample}$)/ Absorbance $_{control}$] x 100

Total phenolic content

The total phenolic content of sample was determined using the Folin-Ciocalteu reagent. Eight hundreds microliters of sample extracts (100 µg/ml) and 200 µl of 15% Folin- Ciocalteu reagent were added in the test tube then adjusted the volume to 2.0 ml with distilled water. The mixture was left for 5 min. After that, 1.0 ml of Na_2CO_3 (0.106 g/ml) was added. The incubation was performed in the dark at room temperature for 60 min. The absorbance was measured at 756 nm using a UV-spectrophotometer. The total phenolic content in all sample extracts were expressed as micrograms of catechin equivalents (CE) per 100 µg dry weights of crude drug (DW). All samples were performed in triplicate.

Total tannin content

The total tannin content was estimated by Folin-Ciocalteuassay. Briefly, 3.5 mg of hide powder was weighed, and then 5 ml of sample (100 µg/ml) was added in the test tube. The mixture was shaken for 60 min afterwards centrifuged for 10 min and finally the supernatant was collected. The supernatant has only simple phenolic compounds other than tannins. The tannins would have been precipitated along with the hide powder. The phenolic content of the supernatant was then measured following the same procedure describe above. The content of non-tannin phenols was expressed as micrograms of catechin equivalents (CE) per 100 µg dry weights of crude drug. Total tannin content was determined by subtraction of non-tannin phenolic content from total phenolic content. All samples were performed in triplicates.

Quantification of (+)-catechin and (-)-epicatechin

Instrumentation and chromatographic conditions

HPLC analysis was performed using a Shimadzu DGU-20A3 HPLC (Shimadzu, Japan) equipped with a binary solvent delivery system, an auto-sampler, a column temperature controller, and a photo diode array detector (Shimadzu SPD-M20A, Shimadzu, Japan). System control and data analysis were processed with Shimadzu LC Solution software.

The chromatographic separation was accomplished with an Inersil ODS-3 column (5 µm x 4.6 x 250 mm) and an Inertsil ODS-3 HPLC guard column (5 µm x 4.0 x 10 mm). The binary mobile phase consisted of0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The mobile phases were filtrated through 0.45 µm nylon membrane filters and degassed using an ultrasonic bath before analysis. The isocratic program was set at 20% B for 15 min with a flow rate of 1 ml/min. The column temperature was maintained at 40 ˚C and the injection volume was 1 µl. The detection wavelength was set at 280 nm.

Preparation of standard solution

The stock solution of (+)-catechin and (-)-epicatechin were prepared by dissolving 1 mg of each compound in 1 ml of methanol. Then, the solution was filtered through a 0.45 µm PTFE membrane syringe filter.

Preparation of sample

One miligram of black catechu or pale catechu was dissolved in 1 ml of methanol and votexed for 1 min. The mixture was filtered through a 0.45 µm PTFE membrane syringe filter before chromatographic analysis.

Method validation

The tests of linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, specificity, and robustnesswere evaluated according to the ICH guideline for validation of analytical method [16].

Linearity

Linearity was determined by the calibration curves that obtained from the HPLC analysis of (+)-catechin and (-)-epicatechin. The calibration curves of these two compounds were fitted by linear regression. The stock solutions of (+)-catechin and (-)-epicatechin were dissolved in methanol to give concentrations of 5, 10, 50, 100, and 200 µg/ml for evaluate the calibration curves.

Limit of detection (LOD) and Limit of quantitation (LOQ)
LOD and LOQ were calculated based on the residual standard deviation of a regression lines (σ) and the slope of the calibration curve (S) as follows:
LOD = $3.3(\sigma)/S$
LOQ = $10(\sigma)/S$

Precision
The precision of black catechu and pale catechu were evaluated at 2 levels including repeatability and intermediate precision. Nine determinations covering the specific range (50, 100, and 150 µ g/ml, 3 replicates each) were evaluated and analyzed on one day and three consecutive days.The data were expressed as percent relative standard deviation (% RSD).

Accuracy
The accuracy of black catechu and pale catechu were determined by recovery method. The crude extract was spiked with (+)-catechin (50, 100, and 150 µg/ml) and (-)-epicatechin (50, 100, and 150 µg/ml) then percent recoveries were calculated by comparing the measured amount of catechins with the theoretical one.

Specificity
The specificity was evaluated by peak purity test.

Robustness
The robustness was determined for variations in flow rates (0.995 and 1.005 ml/min) and variations in column temperature (39 and 41 ˚C). The percentage of RSD was calculated to evaluate whether the flow rate and temperature variations altered the results of HPLC.

RESULTS AND DISCUSSION

Antioxidant activity
The different mechanisms of antioxidant activities including free radical scavenging of DPPH in the DPPH assay, the reduction of ferric ion in the FRAP assay and the chelation of ferrous ions in the metal ion chelating activity were evaluated. The results from DPPH assay and FRAP assaywere related in almost samples; whereas the results from metal ion chelating activity were reversal with the results from these 2 assays. The percentage of free radical scavenging varied from 2.62-75.47 in black catechu and 74.18-77.06 in pale catechu. FRAP values of black catechu and pale catechu were of 0.0-0.57 and 0.169-0.389 mM FeSO4/100 µ g DW respectively. The percentage of chelating activity of black catechu and pale catechu were varied from 1.97 - 16.12 and 0.65 - 7.59 respectively. The percentage of free radical scavenging, chelating activity and FRAP value of catechin hydrate were found to be 82.66 ± 0.24, 2.59 ± 1.87 and 0.542 ± 0.003 mM FeSO4/100 µg DW.

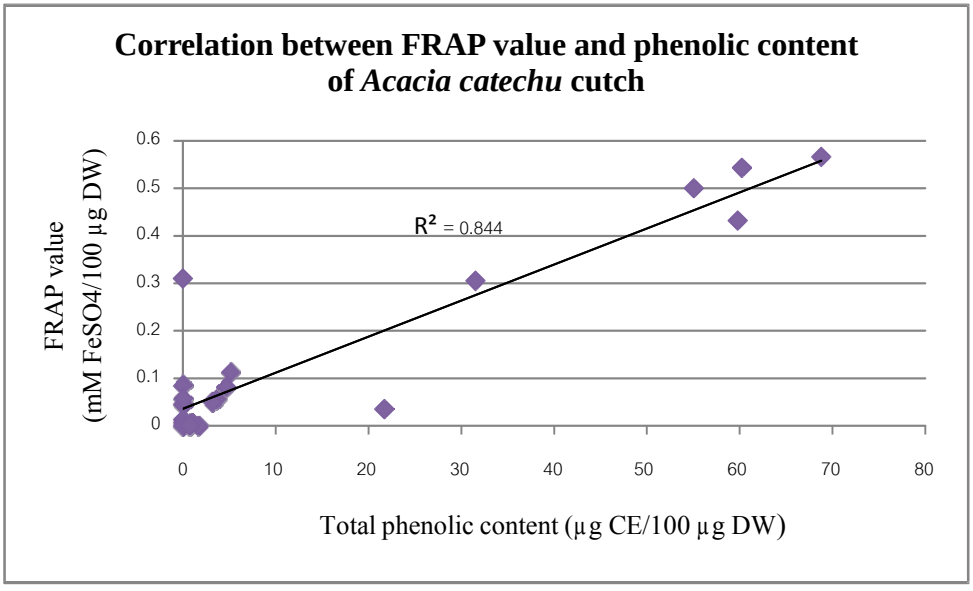


Figure 2 Correlation between FRAP value and phenolic content of black catechu

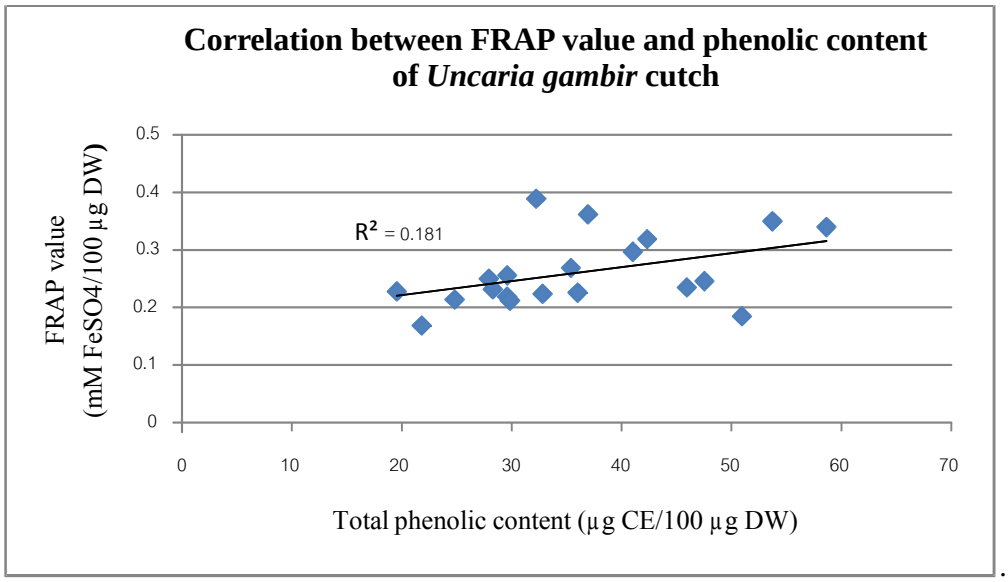


Figure 3 Correlation between FRAP value and phenolic content of pale catechu

Total phenolic, non-tannin phenolic and tannin contents of commercial black catechu ranged from 0.0-68.77, 0.0-67.51, and 0.0-4.43 µg CE/100 µg DW respectively. For pale catechu, total phenolic, non-tannin phenolic, and tannin contents ranged from 19.55-58.64, 18.21-58.18, and 0.09-1.54 µg CE/100 µg DW.

The extracts of some black catechu and all pale catechu showed high antioxidant activities,total phenolic, and non-phenolic contents whereas total tannin contents were quite low. The values of antioxidant activities, total phenol, non-phenol, and total tannin were found to be different for different sources of the sample. These might be due to the impurity of both commercial black catechu and pale catechu [17]. Nevertheless, their phenolic contents were correlated with the antioxidant power as shown in Figure 2 and Figure 3. The results were consistent with the finding of various research that showed positive correlations between total phenolic content and antioxidant activity [18-21].

Quantification of (+)-catechin and (-)-epicatechin

Figure 4HPLC chromatograms of black catechu

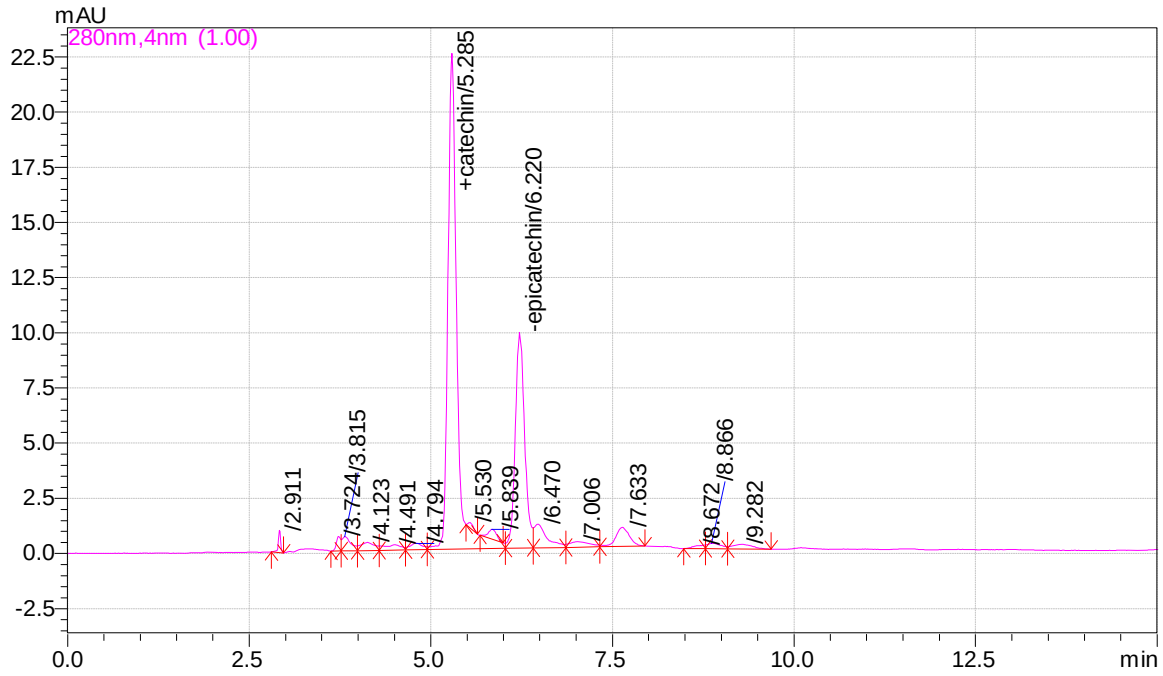
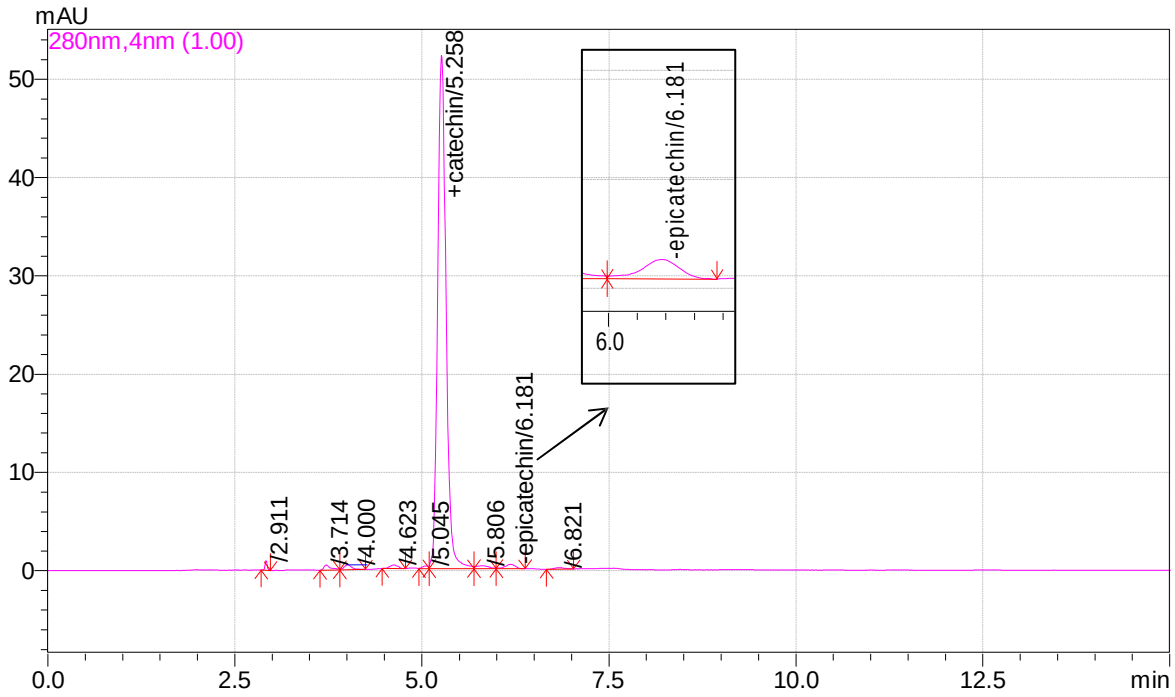


Figure 5HPLC chromatograms of pale catechu



(+)-Catechin and (-)-epicatechin quantification in commercial black and pale catechu were performed by HPLC analysis. The HPLC chromatograms of black catechu and pale catechu extracts (Figure 4 and Figure 5) showed both (+)-catechin and (-)-epicatechin peaks. Some black catechu samples were found to be rich source for (+)-catechin and (-)-epicatechin. The concentrations of (+)-catechin and (-)-epicatechin in black catechu were range from 0 – 236.28 µg/mg and 0 – 160.12 µg/mg of crude drug respectively. High (+)-catechin contents (183.90 – 633.78 µg/mg of crude drug) were found in allpale catechu samples whereas (-)-epicatechin were detected at very low concentrations (0 – 9.30 µg/mg of crude drug).However, the results were in accordant with recent studies [6-9]. (+)-Catechin and (-)-epicatechin contentsof some black and pale catechu samplescannot be determined quantitatively due to low concentration (< LOQ).Varied concentration ofboth (+)-catechin and (-)-epicatechin in different sources of the sample might be due tothe impurity of both commercial black catechu and pale catechu. Previous study reported that black catechu was adulterated with clay and pale catechu was adulterated with quart [17].

According to ICH guideline, the tests of linearity, LOD, LOQ, precision, accuracy, specificity, and robustness should be performed for the validation of an analytical method. (+)-Catechin and (-)-epicatechin at 5 concentration levels were investigated for linearity of the HPLC method. The calibration curves of both standard compounds were linear in the range of 5-200 µg/ml. The regression equation of (+)-catechin and (-)-epicatechin were $y = 746.29x - 2203.3$ and $y = 517.61x - 652.07$ respectively. Good correlation coefficient (r^2) was obtained ($r^2 \geq 0.99$) in this study. The LOD values, taken as the lowest concentration of analyte in a sample which can be detected were found to be 4.80 µg/ml for (+)-catechin and 5.14 µg/ml for (-)-epicatechin. The LOQ values, taken as the lowest concentration of analyte in a sample which can be quantitively determined were 14.54 µg/ml for (+)-catechin and 15.57 µg/ml for (-)-epicatechin.The precision of black catechu and pale catechu extracts were conducted as % RSD of 9 determinations covering the specific range. The accuracy was determined by recovery test. The results of precision and accuracy of (+)-catechin and (-)-epicatechin of back catechu and pale catechu extracts were illustrated in Table 1 and Table 2 respectively.

Table 1 Precision and accuracy of (+)-catechin and (-)-epicatechin in black catechu extract

Compound	Spike concentration (µg/ml)	%RSD		% recovery
		Repeatability precision (n = 9)	Intermediate precision (n = 3)	(n = 3)
(+)-Catechin	50	0.20	1.11	98.2
	100	0.36	1.10	97.6
	150	0.26	0.68	82.0
(-)-Epicatechin	50	0.26	0.67	96.0
	100	0.43	1.58	102.6
	150	0.14	0.91	110.0

Table 2 Precision and accuracy of (+)-catechin and (-)-epicatechin in pale catechu extract

Compound	Spike concentration (µg/ml)	%RSD		% recovery
		Repeatability precision (n = 9)	Intermediate precision (n = 3)	(n = 3)
(+)-Catechin	50	0.16	1.86	111.8
	100	0.68	1.44	96.9
	150	0.27	1.73	80.0
(-)-Epicatechin	50	0.79	2.46	114.3
	100	0.26	1.23	91.3
	150	0.29	2.71	102.4

The percent RSD of repeatability and intermediate precision were found to be less than 3 which revealed that the HPLC method was precise [22]. The recoveries of (+)-catechin and (-)-epicatechin in black catechu were rangedfrom82.0 - 98.2% and 96.0-110.0% respectively. For pale catechu, the recoveries of (+)-catechin were 80.0 – 111.8% and the recoveries of(-)-epicatechin were 91.3 – 114.3%. According to ICH guideline, good agreement of recovery was ranged from 80 - 120% with the required for complex matrices [16]. Hence, the results indicated that this method was accurate for (+)-catechin and (-)-epicatechindetermination in black catechu and pale catechu.The specificity was performed by peak purity checking. The peak purity test is useful to show that the analyte chromatographic peak is not attributable to more than one component. The results showed peak purity index of (+)-catechin and (-)-epicatechinwere more than 0.99 which can be suggested that no impurity detected in those peaks.The robustness should be investigated during the analysis of HPLC method, and it should demonstrate the reliability of analysis with the respect to deliberate variation in the parameters of the method [16, 23]. This present study revealed that there were no differences (%RSD < 5) in the area of the curve and retention time of (+)-catechin and (-)-epicatechin when the flow rate of mobile phase was varied from 0.995 – 1.005 ml/min and the column temperature was varied from 39 – 41 ˚C. The results suggested that the HPLC method proved to be robust for (+)-catechin and (-)-epicatechin analyzed, under the condition evaluated.

CONCLUSION

The present study proposed the first reports of antioxidant activities as well as the contents of (+)-catechin and (-)-epicatechin from commercial black catechu and pale catechu in Thailand. This study demonstrated high antioxidant activities related to non-tannin phenolic content in all pale catechu but a few black catechu samples. It revealed the inferiority of black catechu crude drug in Thai markets leading to insufficient phenolic components and inefficient antioxidant potential. In addition, HPLC method can be applied to determine (+)-catechin and (-)-epicatechin content in plant materials.

REFERENCES

[1] JM Neligan. Medicines, Their Uses and Mode of Administration,6thEdition, Longman, London, **1864**,61-62.
[2] AmericanPhysician. The Eclectic and General Dispensatory: Comprehending a System of Pharmacy, MateriaMedica and Receipts for the Most Common Empirical Medicine,Towar and Hogan, Philadelphia, **1827**,81.
[3] FN Howes. Vegetable Tanning Materials, 1st Edition, Great Britain at the Chapel River Press, London, **1953**, 19-237.
[4] GB Wood; F Bache. The Dispensatory of the United States of America, 12th Edition, J.B. Lippincott, Philadelphia, **1869**, 191-194.
[5] RW Hemingway; PE Laks. Plant Polyphenols: Synthesis, Properties, Significance, 12th Edition, Springer, New York, **1992**, 758-760.
[6] T Lakshmi; M Anitha; R Rajendran,*J. Pharm. Sci. & Res.*, **2012**, 4(3), 1764-1767.
[7] D Shen; W Qingli; W Mingfu; Y Yonghong; EJ Lavoie; JE Simon,*J. Agric. Food Chem.*, **2006**, 54, 3219-3224.
[8] T Anggraini; A Tai; T Yoshino; T Itani,*Afr. J. Biochem. Res.*, **2011**, 5(1), 33-38.
[9] MJ Kassim; MH Hussin; A Achmad; NH Dahon; TK Suan; HS Hamdan,*MajalahFarmasi Indonesia*, **2011**, 22(1), 50-59.
[10] M Kofink; M Papagiannopoulos; R Galensa,*Molecules*, **2007**, 12, 1274-1288.
[11] A Sameena; K Manisha; T Manisha,*BioMedRx*, **2013**, 1(1), 109-114.
[12] VG Devi; A John; RS Devi; VA Prabhakaran,*Int. J. Pharm. Pharm Sci.*, **2011**, 3(2), 108-111.
[13] FB Apea-Bah; M Hanafi; RT Dewi; S Fajriah; A Darwaman; N Artanti; P Lotulung; P Ngadymang; B Minarti,*J.Med. Plant. Res.*, **2009**, 3(10), 736-757.
[14] IFF Benzie; JJ Strain,*Anal.Biochem.*,**1996**, 239, 70-79.
[15] TCP Dinis; VMC Madeira; LM Almeida,*Arch. Biochem. Biophys.*,**1994**, 315, 161-169.
[16] http://www.ich.org/products/guideline/quality/article/quality-guideline.html
[17] K Mujarin; P Rattanakul; J Siripee; T Dechathiwong,*FDA J.*, **2009**, 39-46.
[18] FQ Alali; K Tawaha; T El-Elimat; M Syouf; M El-Fayda; K Abulaila; SJ Nielsen; WD Wheatons; JO Falkinham; NH Oberliesy,*Nat. Prod. Res.*, **2007**, 2(12), 1121-1131.

[19] D Gupta; B Bleakley; RK Gupta,*J. Med. Plant. Res.*, **2011**, 5(13), 2736-2742.
[20] R Kumar; S Hemalatha,*J. Chem. Pharm. Res.*, **2011**, 3(1), 259-267.
[21] D Laloo; ANSahu,*J. Chem. Pharm. Res.*, **2011**, 3(1), 277-283.
[22] http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf
[23] NR Pai; SS Patil,*J. Chem. Pharm. Res.*, **2013**, 5(7), 121-128.