Determination of total phenol, condensed tannin and flavonoid contents and antioxidant activity of *Uncaria gambir* extracts

Penentuan kandungan fenol total, tannin terkondensasi dan flavonoid dan aktiviti antioksidan ekstrak *Uncaria gambir*

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Abstract

Uncaria gambir, a well known Southeast Asia plant have been previously used as an alternative medicine for treatment such as diarrheal, sore throat and spongy gums. Due to its useful properties, in this study we have analysed the total phenol, condensed tannin, flavonoid content and antioxidant activity of *Uncaria gambir* in three different solvent extracts. Characterization and quantification analysis using Fourier Transform Infrared (FTIR) spectroscopy and reverse phase-high performance liquid chromatography (RP-HPLC) has confirmed that the major chemical constituents of *Uncaria gambir* are mainly catechins. It was revealed that the ethyl acetate gambir extract gives the highest catechin content and antioxidant activity compared with other solvent extracts.
Key words: *Uncaria gambir*, antioxidant activity, condensed tannin, flavonoid

Abstrak

Uncaria gambir, tumbuhan herba asli dari Asia Tenggara telah digunakan sebelum ini secara meluas sebagai ubat alternatif untuk penyakit cirit-birit, sakit tekak dan juga gusi berdarah. Oleh kerana kepelbagaian sifatnya, dalam kajian ini kami telah menganalisa kandungan fenol total, tannin terkondensasi dan flavonoid dan aktiviti antioksidan *Uncaria gambir* dalam tiga ekstrak pelarut. Pencirian dan analisis kuantitatif menggunakan spektroskopi inframerah pengubah Fourier (FTIR) dan fasa balik-kromatografi cecair prestasi tinggi (RP-HPLC) telah membuktikan bahawa konstitusi kimia utama *Uncaria gambir* adalah katekin. Ekstrak gambir menggunakan etil asetat telah didapati memberikan kandungan katekin dan aktiviti antioksidan yang tertinggi berbanding ekstrak pelarut lain.
Kata kunci: *Uncaria gambir*, aktiviti antioksidan, tannin terkondensasi, flavonoid

Introduction

Uncaria gambir, can be found mostly in countries such as Malaysia and Indonesia. Some might call it as Gambir, Gou Teng, Asen'yaku, Cat's Claw, Una de Gato, and Pale Catechu (Taniguchi *et al*., 2007a; Remington and Wood, 1918; Chong, 2009). Gambir plant can grow about eight feet high and has oval shape of leave around 8 to 14 cm in length with 4 to 5 pairs or nerves (Kim Suan, 2009). The flowers also originate at the base of the leaves with each pair of leaves may have a pair of globular inflorescences. According to Hadad *et al*. (2009), gambir plant can be grown only at certain condition, which is the plant must be grown at 200 to 800 meter above sea level with rainfall around ±3.3 mm per year and humidity around 70 to 85 %. Any types of soil can be used for gambir plantation with the pH range from 4.8 to 5.5.

In 1923, Freudenberg and Purrmann has isolated two active polyphenol from dried aqueous extract of gambir which is (±)-catechin and (+)-epicatechin (Nonaka and Nishioka, 1980). Further works done by Nonaka and Nishioka (1980), led to the isolation of seven new biflavonoids which is gambiriin A1, A2, A3, B1, B2, B3 and C from gambir. Characterization of these seven new biflavonoids has been done using mass spectrum (Nonaka and Nishioka, 1980), ^{1}H and ^{13}C NMR (Taniguchi *et al*., 2007b), reverse phase-high performance liquid chromatography, RP-HPLC and gel permeation chromatography, GPC (Taniguchi *et al*., 2007a). Besides catechin, gambir contains a few amount of quercetin, which is a colouring agent that makes the gambir extract appears in yellow (Hayani, 2003; Idris, 2007). Quantitative analysis of gambir done by Taniguchi *et al*. (2007a), have shown that the total flavan content by using the vanillin-acid estimation method ranged from 24 to 79 % while analysis using RP-HPLC techniques revealed that catechin content is around 76 %, epicatechin content is 1.5 %, and 1 % each for the content of gambiriin B1, B3 and A1 . The analysis indicated that catechin was the most abundant constituent in gambir. Besides RP-HPLC, Hayani (2003) have studied the catechin content in three different extraction method using spectrophotometer. From the study, she concluded that the extraction of gambir using a hot plate had showed higher percentage of catechin content with the range around 81 to 88 %.

The anti-oxidative properties of catechin in gambir have attracted researchers to study deeper on its applications. Traditionally, gambir has been used for skin tanning, colouring in textile and chewing (Hayani, 2003; Remington and Wood, 1918; Zamarel and Risfaheri, 1991). Recently, gambir often used as remedies for diarrhea and sore throat (Taniguchi et al., 2007b). Pambayun *et al*. (2007) have studied the antibacterial properties of various extracts of gambir and it was reported that gambir extract gave highest inhibition effect on the Gram-positive bacteria such as *Streptococcus mutans*, *Staphylococcus aureus* and *Bacillus subtilis*. Gambir also exhibits the plant pesticide properties by inhibiting the growth of *Phytophthora cinnamomi* fungi in cinnamon plant and preventing leave spot disease that caused by *Fusarium sp* in *Citronella crop* leaves (Nasrun *et al*., 1997; Idris, 2007). Moreover, Hazwan and Jain (2010) have reported the potential of gambir extract as corrosion inhibitor for mild steel in acidic solution.

Methodology

Gambir extraction

Raw gambir samples were obtained from Medan, Indonesia. The *Uncaria gambir* cubes were ground into fine powder and screened through a 250 μm sieve. Parts from this gambir (5.0 g) were dissolved in 80 °C of distilled water (100 mL). The mixture was shaken at 200 rpm (IKA® KS 260, Sweeden) for 1 hour. Then it was transferred to a centrifuge tube and centrifuged for 5 minutes to obtain a clear solution. The undissolved gambir was removed by filtering them through vacuum filter and the mother liquors were treated with n-hexane (50 mL, QRëC) three times in order to remove fat and oil from the extracts. The purified aqueous extracts of gambir were freeze-dried (Labconco, USA). The resulting aqueous extract powder (1.0 g) was then dissolved in three different solvent which is ethyl acetate, methanol and hot water (50 mL, QRëC). Then, the extracts were concentrated at 50 °C under reduced pressure in a rotary evaporator (Heidolph, Germany). The concentrated extracts than were dried overnight in the oven (50 °C) and finally, the dried extracts were ground into a fine powder.

Total phenols determination

Total phenolic content of *Uncaria gambir* extract was determined by Folin Ciocalteu reagent with gallic acid as a standard (McDonald *et al*., 2001). For this method, 0.5 mL (1:10, m/v) of each extract or gallic acid standard (Fluka) was mixed with 5 mL (1:10, v/v diluted with distilled water) of Folin Ciocalteu reagent (Merck) and 4 mL (1 M) aqueous Na_2CO_3 (HmbG). The standard solutions of gallic acid were prepared with concentration 20, 40, 60, 80, and 100 ppm. The absorbance of the mixtures was measured at 765 nm (U 200 Hitachi, Japan) after kept in dark for 2 hours. The total phenolic contents were expressed as gallic acid equivalent, GAE (mg g^{-1}).

Total condensed tannin determination

Slight modification was applied to the method proposed by Garro Galvez *et al*. (1996) and Yazaki *et al*. (1985) for the determination of total

condensed tannin in gambir extract samples. A 100.0 mg of sample was dissolved in 10.0 mL distilled water. Then 2.0 mL of 5 M HCl and 2.0 mL of 37 % formaldehyde (Merck) were added and the mixture was heated under reflux for 1 hour. The reacted mixture was then filtered while hot through vacuum suction. The reddish precipitate was washed with 10.0 mL hot water 5 times. The precipitate then dried in desiccators of silica gel and weighted. The yield was expressed as percentage of the weight of the starting material.

Total flavonoids determination

An aliquot (1.0 mL) of extracts (0.5 g dried gambir extracts in 50.0 mL methanol) was added into 10.0 mL volumetric flask containing 4.0 mL distilled water. To the flask, 0.3 mL 5 % $NaNO_3$ (Merck) was added and after 5 minute, 0.3 mL 10 % $AlCl_3$ (R&M Chemicals) was added. At the 6^{th} minute, 2.0 mL of 1 M NaOH (R&M Chemicals) solution was added and the total volume was made up to 10.0 mL with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. (+)-catechin hydrate (Sigma Aldrich) was used as standard solution to plot the calibration curve (10 ppm to 100 ppm). The total flavonoid content was expressed as mg g^{-1} catechin equivalent (CE). Samples were analysis in triplicate.

Fourier Transform Infrared Spectroscopy (FTIR)

KBr disc in FTIR analysis were prepared with the concentration of the sample in KBr (R&M Chemicals) were in the ratio of 1:20. About 200 mg of KBr were placed into a mortar and were ground until there is no evidence of crystalinity. 10 mg of (+)-catechin hydrate sample was placed into the mortar and again ground it until a fine powder was formed. Excessive grinding of the potassium bromide was prevented, since it will lead to the powdered potassium bromide to be very fine. The finely powdered potassium bromide will absorb more humidity (hygroscopic) from the air and therefore lead to an increased background in certain ranges. The KBr sample mixture was then transferred to KBr press assembly, where the KBr disc was produced for FTIR analysis with Perkin–Elmer System 2000 FTIR. These methods were applied to all the (+)-catechin hydrate samples from different organic solvents extraction (methanol, hot water and ethyl acetate).

Quantification of catechin content in gambir extracts using HPLC

The HPLC grade solvent of methanol and acetonitrile (QRec) and analytical grade of acetic acid (QRec) were used in this analysis. The sample of gambir extract was analysed on a Shimadzu AD-VP, using Chromolith SemiPrep RP-18 column (100-10 mm, Merck) at a flow rate of 0.5 mL min^{-1} and detection at 280 nm using a UV detector. The chromatogram of a standard mixture of (+)-catechin hydrate, (-)-epicatechin, (-)-epicatechin gallate, (-)-gallocatechin and (-)-epigallocatechin (all purchased from Sigma Aldrich) was obtained by the gradient elution as described by Cheong *et al*., (2005). Two different mobile phases were prepared accordingly: Solvent A, acetonitrile/water (50:50 v/v) with 0.1 % acetic acid; solvent B, acetonitrile/water (5:95 v/v) with 0.1 % acetic acid. All mobile phase were filtered with 0.45 μm PTFE Milipore (Whitman) filter paper and degassed with sonicator for 10 minutes.

Quantification of flavonoids

Five stock solutions were made by dissolving 1 mg of an individual standard material in 5 mL methanol and stored in a refrigerator at 4 ºC. A small amount (1 mL) of each stock solution was taken and the 5 aliquots were mixed and diluted (5 mL of methanol) to give a wide range of standard mixture. The optimized gradient elution program was followed the standard separation of flavonoid (Cheong *et al*., 2005). An exact amount of 1 mg raw gambir was diluted in 5 mL methanol. Each standard and raw gambir samples were filtered through 0.45 μm filter paper before injection. The chromatogram of raw gambir was obtained under the same chromatographic condition, and the retention times were compared to those of standard mixture to identify the corresponding flavonoid. The flavonoid was confirmed by spiking the standard component in the raw gambir. Of each sample, 20 μL was injected and elution time was 20 minutes.

Quantitative determination of catechin

The calibration curves (peak area versus concentration) of (+)-catechin hydrate was obtained for a wide concentration range (10 to 60 ppm), and a good linear relationship was observed. Each gambir extracts (50 ppm) were injected in the same condition and the concentration range was roughly determined from the linear correlations.

DPPH radical scavenging assay

DPPH radical scavenging assay was performed by modifying the method of Blois (1958). Briefly, 1 mL of 0.1 mM solution of DPPH in methanol was mixed with 3 mL of 50 ppm gambir extracts solution in water. After 30 min incubation the absorbance was measured at 517 nm using colorimeter. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The result expressed in percentage of DPPH radical scavenging calculated according to the following equation (Pinelo *et al.*, 2004):

% DPPH radical scavenging =

$[(A_0 - A_1)/A_0] \times 100$……(1)

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample of gambir extracts. Ascorbic acid was used as common standard for antioxidant compound.

Result and discussions

Total phenols determination

Uncaria gambir extracts showed different total phenolic content for each solvent with different polarity index. Folin Ciocalteu assay method has been used to compare the presence of phenolic compounds extracted by different type of solvents. The reduction of Folin Ciocalteu reagent by phenolic ion will change its solution colour into blue (Prior *et al.*, 2005). The reduction of complex will increase when the extract contain more phenolic compounds. Thus, the colour will be darker and the absorbance will be higher (Arbianti *et al.*, 2007). Ethyl acetate has lower polarity index followed by methanol and water as the highest polarity index. The results shows that ethyl acetate extract contain the highest total phenolic content (113.43 mg g^{-1}) followed by methanol extract (99.25 mg g^{-1}) and water extract (76.75 mg g^{-1}) as the lowest. Although, ethyl acetate has the lowest polarity index, it has higher molecular weight compared to methanol and hot water. It is strongly believed that the higher the molecular weight of the solvent, the lower the polarity which allows other substances of about the same molecular weight to be easily extracted. This also can be correlated to "like dissolve like" or "polarity versus polarity" principles as both catechin and ethyl acetate have high molecular weight (Uma *et al.,* 2010). Since, catechin contains higher molecular weight, thus ethyl acetate is tend to be the most effective solvent for extraction.

Total condensed tannin determination

Formaldehyde has also been used as a reagent in the determination of tannin. The aim of Stiansy test is to determine the percentage condensed tannin in gambir extract that can reacted with formaldehyde, it will attack the benzene ring of catehcin or condensed tannins to form phenol-formaldehyde resins (Garro Galvez *et al.*, 1996). Table 1 showed the percentage of Stiasny precipitate, which ethyl acetate gave the highest percentage, 93.12 (% wt). Gambir extracted with methanol and hot water showed lower Stiasny precipitate compare with ethyl acetate which were 75.35 and 66.96 (% wt), respectively.

Total flavonoids determination

Colorimetric determination of flavonoid content in plant material based on color reaction with $AlCl_3$ reagent is well-known method (Zhi Shen *et al.*, 1999). The aluminum chloride forms acid stable complexes with the hydroxyl group of the flavonoids and flavonols (Ayesha *et al.*, 2009; Chang *et al.*, 2002). Table 2 showed the catehcin equivalent, CE of different solvents extraction. Obviously, ethyl acetate extraction gave the highest CE value of 93.31. This indicated that the ethyl acetate extracted total flavonoid effectively from gambir. When the polarity of solvent increased from methanol to water (Polarity index: 5.2 to 10.0), the CE was decreased from 70.94 to 60.85. This phenomenon suggested that flavonoid extraction from gambir performed well with solvents polarity below 5.2 which ethyl acetate has the polarity index about 4.5.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra for (+)-catechin samples (in KBr disks) are shown in Fig. 1. The catechin samples were extracted by using three different types of organic solvents, extraction by methanol (a), extraction by water (b), and extraction by ethyl acetate (c). All the spectra of the catechins in different types of organic solvents investigated shared certain spectral similarities. A broad band due to the OH stretch is observed near 3420 cm^{-1}. A band due

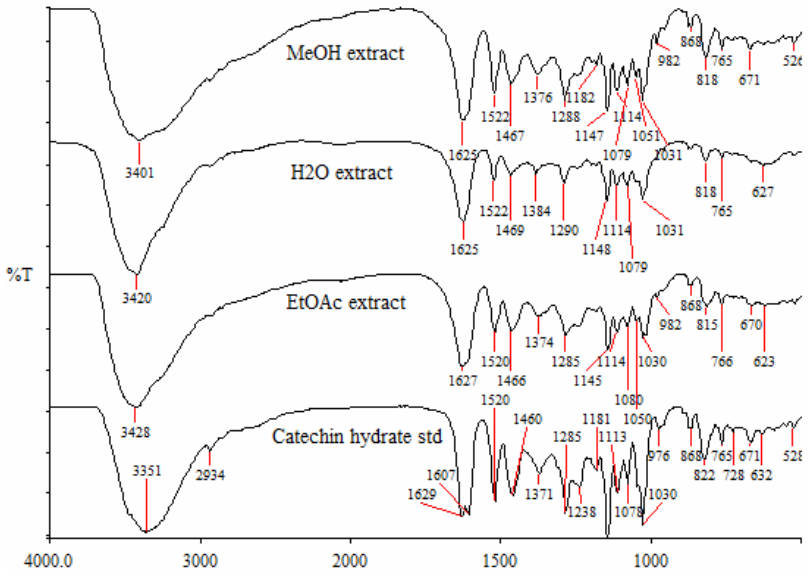

Fig. 1. FTIR spectrum for all *U. gambir* extract with (+)-catechin hydrate as a standard.

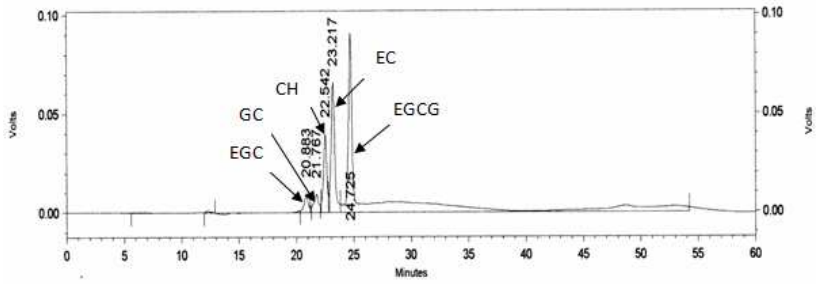

Fig. 2. The HPLC chromatogram of five flavonoid standards mixture.

to the aromatic ring quadrant stretch is observed at 1625 cm^{-1}, and one due to the aromatic semicircle stretch at 1520 cm^{-1}. At 1290 cm^{-1}, a band due to the OH deformation of the aromatic alcohol is observed, and at 1182 cm^{-1}, a band corresponding to the CO stretch of an aromatic alcohol is observed. Near 1080 cm^{-1}, another band due to an aromatic ring stretch is observed. An aliphatic secondary alcohol, CO stretch, is observed near 1030 cm^{-1}. The bands due to the aromatic OH wags are observed between 900–750 cm^{-1}; the frequency of the bands due to the OH wags depend on the substitution of the aromatic rings (Robb *et al.*, 2002). Table 3 are shown characteristic wave number regions for different groups which are, or might be, present in the tested catechin samples. (Ramos-Tejada, *et al.*, 2002).

Quantification of catechin content in gambir extracts using HPLC

Qualitative determination of flavonoids

Fig. 2 shows the chromatogram containing a mixture of five flavonoid standards ((+)-catechin hydrate, (-)-epicatechin, (-)-epicatechin gallate, (-)-gallocatechin and (-)-epigallocatechin) eluted at a flow rate of 0.5 mL min^{-1}. A good separation of all five flavonoid standards can be observed from the

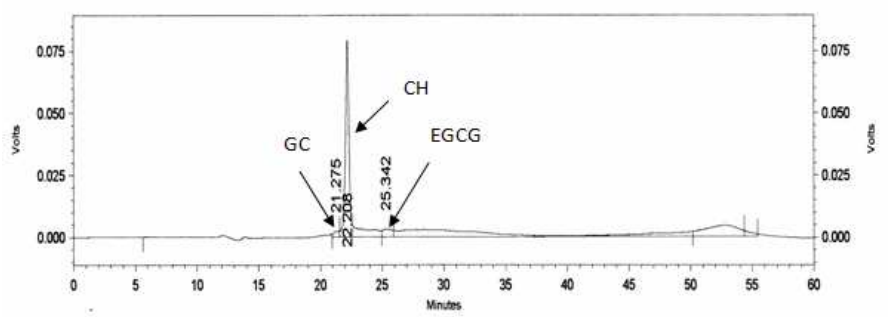

Fig. 3. The HPLC chromatogram of raw *U. gambir*.

Table 1: The percentage of condensed tannins in gambir extracted by different solvents.

Solvents	Staisny precipitate, (%)
Ethyl acetate	93.12
Methanol	75.35
Hot water	66.96

Table 2: The total flavonoids content of three gambir extracts by different polarity solvents.

Solvents	mg catehcin equivalent, (CE)
Ethyl acetate	93.31
Methanol	70.94
Hot water	60.85

chromatogram (Fig. 2) where further identification shows that all five flavonoid standards appears at different retention time but it seems to be closed with each other ((-)-epigallocatechin: 20.883 min; (-)-gallocatechin: 21.767 min; (+)-catechin hydrate: 22.542 min; (-)-epicatechin: 23.217 min; (-)-epicatechin gallate: 24.725 min). In turn, Fig. 3 shows the chromatogram of raw gambir under the same condition. Here, a sharp and symmetrical peak can be observed at 22.208 min retention time and two weak peaks appear at 21.275 min and 25.342 min retention time. As for confirmation, these retention times were compared with the retention time obtained from the analysis of five flavonoid standards and it can be reveal that a strong peak at 22.208 min was equivalent with the retention time for standard (+)-catechin hydrate while the two weak peaks (retention time at 21.275 min and 25.342 min) was equivalent with the standard of (-)-gallocatechin and (-)-epigallocatechin gallate (Fig. 4).

Quantitative determination of catechin

The calibration curve of standard (+)-catechin hydrate is given in Fig. 5. A very good linear relationship was observed for this standard when different concentration of (+)-catechin hydrate (from 10 ppm to 60 ppm) was used to plot a standard calibration curve. From the above analysis, it was known that standard (+)-catechin hydrate appears around 22.5 min retention time. The catechin content in gambir extracts were calculated using the linear equation obtained from the curve (y = 26809x + 54608). The percentage of catechin content in gambir extracted by different solvents was shown in Table 4. This experiment shows that ethyl acetate gambir extract gives the highest catechin content (87.33 % wt), followed by methanol (59.47 % wt), hot water (42.75 % wt) gambir extract. The quantitative analysis was in a good agreement with other test which proved that ethyl acetate gambir extract will gives a very high catechin content compare with other solvent extract. Hence, we can conclude that

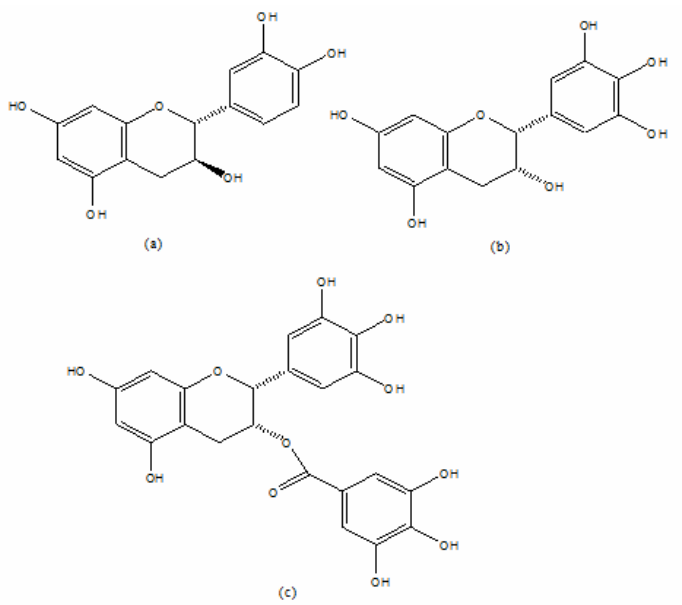

Fig. 4. The chemical structure of, (a) catechin, (b) gallocatechin, (c) epigallocatechin gallate.

Table 3: Characteristic peak bands on FTIR-spectra for different groups.

Group	**Wave numberregion (cm^{-1})**	**Characteristic**
O-H	3600–3200	H- bonded broad andstrong
C-H	3100–3000	aromatic medium
C-H	3000–2850	alkane medium, sharp (stretch)
C-C	1600–1400	Aromatic medium-weak, series of sharp bands
C-O	1820–1670	ester and carbonyl generally strong, conjugated lower
-C-H	1480–1350	alkane variable (bending)
C-O	1300–1000	alcohol and ether strong, ester two bands or more
1,2-disubstituted	1200–900	benzene ring, three peaks, two medium, one strong
1,3-disubstituted	1100–700	Benzene ring, four peaks, two medium, two strong

HPLC can serve as one of the best method used for the qualitative and quantitative determination of gambir extract.

DPPH radical scavenging assay

The antioxidant activity of phenolic compounds is mainly due to redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, heavy metal chelators and hydroxyl radical quenchers (Rice-Evans *et al.*, 1997). The study shows that the ethyl acetate extract has highest radical scavenging activity which is 88.63 % at 50 ppm followed by methanol extract of 85.98 % at 50 ppm concentration. The minimum antioxidant activity was shown by hot water

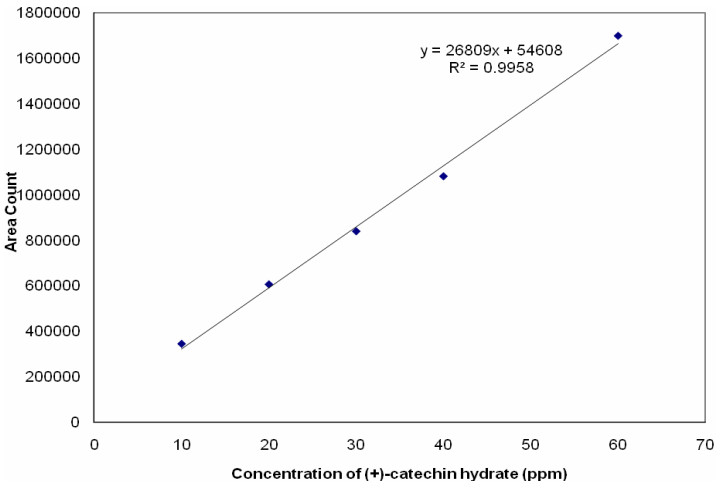

Fig. 5: The calibration curves of standard (+)-catechin hydrate by HPLC-UV system.

Table 4: The content of catechin determined by HPLC method.

Sample extract	Area Count	Content of catechin (% wt. catechin equivalent, CE)
Ethyl acetate	1225166	87.33
Methanol	851721	59.47
Hot water	795407	55.26

extract (82.23 %) at the same concentration. The color change from purple to yellow indicated the absorbance decreased when the DPPH radical was scavenged by an antioxidant through donation of hydrogen to form a stable DPPH-H molecule. A stable diamagnetic molecule formed by the acceptance of an electron or hydrogen radical from the antioxidant compound (Matthaus, 2002). It was also seen that the ascorbic acid compared to extracts exhibited good H-donor activity which has 90.29 % radical scavenging activity at 50 ppm concentration. It can be inferred from this that gambir extracts may scavenge the radicals by other mechanisms also, in addition to the donation of hydrogen.

Conclusions

U. gambir have been successfully extracted with three different solvent and it was revealed that ethyl acetate gambir extract gives the highest total phenol, condensed tannin and flavonoid content compared with other solvent extract.

Characterization using FTIR confirmed that most of the active functional group exhibits in gambir extracts were almost identical with standard catechin.

Quantitative determination of catechin using RP-HPLC has shown that ethyl acetate gambir extract gives the highest catechin recovery.

Antioxidant activity test also revealed that the ethyl acetate gambir extract gives the highest phenolic antioxidant activity.

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