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Full Length Research Paper

Antioxidative activity and catechin content of four kinds of *Uncaria gambir* extracts from West Sumatra, Indonesia

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Gambir cubadak (GC), gambir udang (GU), gambir riau mancik (GRm) and gambir riau gadang (GRg) are popular cultivars of *Uncaria gambir* in Siguntur, West Sumatra, Indonesia. The aqueous extract of gambir has been used as traditional medicine to treat diarrhea, sore throat and some studies have attributed it to the presence of antioxidant properties. The purpose of this study was to characterize the antioxidative activity and properties of four kinds of gambir extract. The antioxidative activity and properties were determined using 1,1-diphenyl-2picrylhydrazyl (DPPH) radical scavenging activity and HPLC. Reaction times of 2 min and 30 min were used to compare the measurement of DPPH. Cell viability was determined using a cytotoxicity assay. Results indicated that the four kinds of *U. gambir* provided antioxidants that were effective and safe. Total polyphenol contents of GC, GU, GRm, and GRg were 13.86, 13.60, 13.58 and 13.90 g 100 g^{-1}, respectively. Catechin is a major component of gambir extracts. GC and GRm contain caffeic acid (0.99 and 0.98 µg ml^{-1}), while in GU and GRg, it is not detected. The catechin contents of GC, GU, GRm and GRg was 104.5, 101.2, 99.4 and 108.5, 104.5 µg ml^{-1}. In conclusion, the results indicated that GC, GU, GRm and GRg, showed similar tendencies as antioxidants. The presence of catechin influences the antioxidant properties and the reaction time of 30 min was recommended for the measurement of DPPH scavenging activity because of slow acting antioxidant material in gambir.

Key words: Antioxidant, catechin, DPPH, *Uncaria gambir*.

INTRODUCTION

Gambir (*Uncaria gambir*) is a member of the Rubiaceae family and contains an officially recognized pharmacological compound (Heitzman et al., 2005). Gambir is the aqueous extract of the leaves and young twigs of *U. gambir*. The species are widely distributed in tropical regions, which vary based on region. Gambir extract has been used for the treatment of diarrhea and as an astringent medicine in Asian countries (Taniguchi et al., 2007b). Interest in the use of complementary medicine against potent oxidants, to alleviate inflammatory conditions and improve health conditions is increasing in developed countries (Marshall, 2000). There are four cultivars of gambir in Siguntur, West Sumatra, Indonesia, with the local names: Gambir cubadak (GC), gambir udang (GU), gambir riau mancik (GRm) and gambir riau gadang (GRg). The leaf type of GC is bigger than that of GU, GRm and GRg. GU leaves are partially red in color. GRm leaves are smaller in size than those of GRg (Figure 1).

The *Uncaria* genus has been instrumental in the discovery of natural medicinal products (Heitzman et al., 2005; Sandoval et al., 2002; Taniguchi et al., 2007a). Studies have revealed that certain *Uncaria* species contain tannins and condensed tannins with antioxidant properties, which are responsible for some of *uncaria*'s

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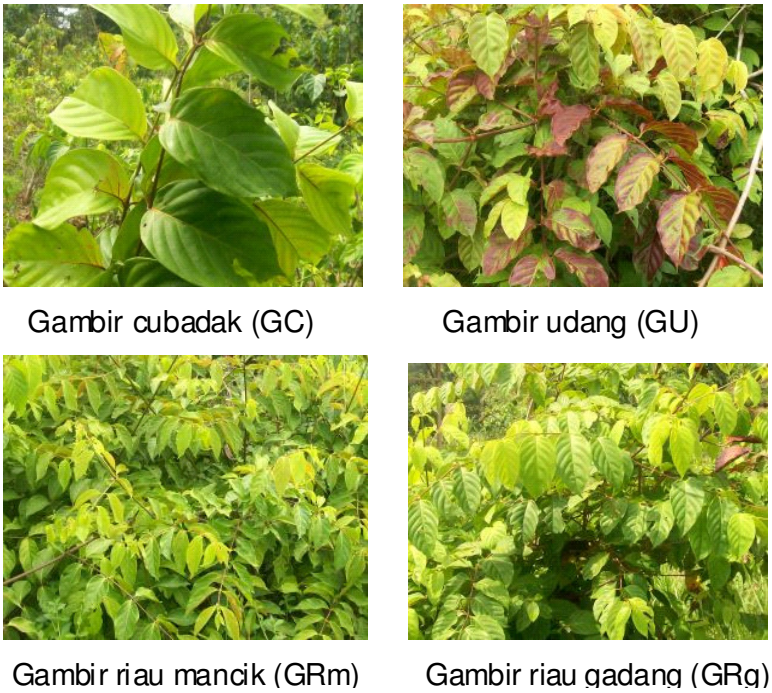

Figure 1. Photographs of GC, GU, GRm and GRg.

pharmacological effects (Desmarchelier et al., 1997). Catechin is a group that occupies an intermediary position in the tannin hierarchy as a family of catechin tannins (Bhat et al., 1998). The presence of catechin in green tea and fermented tea is associated with health protective and cancer preventive properties in animal models, due to its antioxidant activity (Sang et al., 2002).

The focus of this research is to compare the antioxidative activity of four kinds of *U. gambir* from Siguntur, West Sumatra. There are four predominant species of *U. gambir*, which are used interchangeably in traditional medicine for their antioxidant properties (Laus, 2004). However, there is a lack of scientific data comparing the efficacy of the four cultivars of gambir from West Sumatra and the potential differences among them. The results of this paper are very important for the gambir farmers in West Sumatra. The determination of the highest antioxidants activity among four kinds of gambir, increase the prize and indirectly increase the farmers income. To fill this need, we have established a research program focusing on the four species of gambir, with the purpose of determining potential differences in their antioxidant activities and properties. Caffeic acid measurement could be more valuable to antioxidant property of gambir. Caffeic acid is a member of antioxidants in *Uncaria* species (Heitzman et al., 2005). For 1,1-diphenyl-2picrylhydrazyl (DPPH) measurements, the original Blois method recommended a reaction time of 30 min; shorter times have also been used. Using a modified Blois method, we compared DPPH radical scavenging activity reaction times of 2 and 30 min.

MATERIALS AND METHODS

Materials

Raw materials

Gambir extracts (GC, GU, GRm and GRg) were obtained from a gambir farmer in Siguntur, West Sumatra, Indonesia.

Chemicals and reagents

DPPH was obtained from Wako Pure Chemical Industries, Ltd, Osaka, Japan. Dulbecco's phosphate buffered saline (DPBS) was obtained from Invitrogen, California, United States. Gallic acid (for total polyphenol standard curve) was obtained from Katayama Chemical, Osaka, Japan. Sodium carbonate, ethanol and methanol were purchased from Wako Pure Chemical Industries, Ltd. Epicatechin, catechin, and caffeic acid were purchased from Wako Chemical Industries, Ltd. HPLC-grade solvents were degassed in an ultrasonic bath before use.

Preparation of gambir extract

Gambir extract was prepared using a traditional method. Gambir leaves and stems were boiled for 1.5 h and then pressed to obtain the extract. Next, the viscous extract was placed in a 'paraku' (a container specifically for the viscous gambir extract made from wood, 3 m × 30 cm × 10 cm (L×W×H)) for 24 h . The extract was then molded and sun-dried for approximately 3 days.

DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the

method of Blois (1958) with a slight modification. Briefly, a 200 µM DPPH solution of DPPH radical solution in 99.5% ethanol was prepared (3.94 mg in 50 ml 99.5% ethanol), and then 300 µl of this solution was mixed with 150 µl of 200 mM MES(2-Morpholinoethanesulfonic acid, monohydrate) buffer, 150 µl distilled water, 150 µl of 50 mM MES buffer and 1 µl of gambir extracts (15 µl (concentration 0.25 mg ml^{-1}), 30 µl (0.50), 45 µl (0.75), 60 µl (1.00), 90 µl (1.50), 120 µl (2.00), 180 µl (3.00), 240 µl (4.00) and 300 µl (5.00)) with total volume, 900 µl. For control absorbance, DPPH was substituted with the 99.5% ethanol solution.

For gambir extract preparation, 10 mg of gambir extract was diluted with 99.5% ethanol up to a volume of 25 ml. Four milliliters of this extract was added to 1 ml of 200 mM MES buffer, 0.2 ml of distilled water, and 0.8 ml of 50% ethanol. The reactions were allowed to proceed for 2 and 30 min at room temperature. The absorbance of the samples was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radicals by the sample and was calculated using the following formula:

% DPPH radical scavenging activity = [(control absorbance – extract absorbance) × 100
/ control absorbance]

Total polyphenol content

100 mg of gambir sample was resuspended in 10 ml ethanol. From the solution, 0.125 ml of extract was added to 1.5 ml of distilled water and 0.123 ml of phenol and incubated for 6 min at room temperature. After incubation, 1.25 ml of sodium carbonate (7%) and 1 ml of distilled water was added. The solution was incubated for 90 min at room temperature, and absorbance was measured at 760 nm (Singleton et al., 1999; Sakanaka et al., 2005). The standard curve consisted of 6 mg of gallic acid in 10 ml distilled water.

Separation and analysis of gambir extract

The gambir extract was resuspended in ethanol. A Hitachi (Tokyo, Japan) liquid chromatographic system, consisting of a D-2500 Chromato-Integrator, an L-7100 pump, an L-7420 UV-Vis spectrophotometric detector, an L-7300 column oven, and a degasser (Gastorr-720, FLOM, Tokyo, Japan), was used. The gambir extract (5 µl) was subjected to HPLC analysis using a Develosil ODS-HG-5 column (4.6 i.d. ×150 mm, Nomura Chemical, Aichi, Japan) with a guard column (4.0 i.d. × 100 mm, Develosil ODS-HG-5) and at a flow rate of 0.7 ml/min. The elution was performed using a linear gradient system with just solvent: MeOH/H_2O/acetic acid (10:88:2 v/v). The gradient was achieved within 30 min. Absorbance at 280 nm was monitored. Catechin and epicatechin contents were determined from the peak area of the samples with reference to calibration of authentic samples. Absorbance at 325 nm was used for caffeic acid.

Cytotoxicity assay

IEC-6 cell (Intestinal Epithelial Cell line no. 6) was utilized for toxicity test. The cells were cultured on a 96 wells plate in which mixed standard fetal bovine serum (Thermo Fisher Scientific Inc., USA) and DMEM (Invitrogen, USA) and used for the toxicity test after 48 h cultivation. For toxicity test, the cells were washed by 100 µl of PBS (Invitrogen, USA) at room temperature and 100 µl of FBS/DMEM was cultured with sample for 24 h. Sample concentrations were 1, 10, 25, 50, 75, 100, 150 and 200 µg/ml. Cells were added with 10 µl of WST-1 (Roche, USA) and incubated for 4 h. Absorbance of cells were measured by absorption spectrometer at 440 nm.

RESULTS AND DISCUSSION

DPPH radical scavenging activity and total polyphenol content

We measured the DPPH radical scavenging activity of gambir extract, since this method has been widely used to test the ability of compounds to act as free radical scavengers and evaluate antioxidative activity. The DPPH radical scavenging activity method gives a strong absorption at 517 nm by visible spectroscopy (purple color). Experiments were conducted to assess the capacity of GC, GU, GRm and GRg extracts to scavenge stable free radicals (DPPH). The DPPH radical scavenging activity was correlated with concentration. Although the original method recommends a reaction time of 30 min, shorter times have also been used. Therefore, we compared reaction times of 2 and 30 min.

Based on the DPPH data from the 2-min reaction time, the four kinds of gambir extract (GC, GU, GRm and GRg,) exhibited very strong antioxidant activity (Figure 2). The DPPH radical scavenging activity for 0.25 to 5.0 mg/ml GRm, GRg, GC and GU was 22.10 to 92.78, 20.38 to 93.07, 22.84 to 92.63 and 20.79 to 90.50%, indicating that values increased with increasing concentrations. Total polyphenol content of gambir ranged from 13.58 (GRm) to 13.90 (GRg) g 100 g^{-1}, while GU and GC were 13.60 and 13.86 g 100 g^{-1}, respectively (Table 1). The evaluation of gambir antioxidant activity is increasingly important, since it has been found that phenolic compounds are one of the most effective antioxidants. The high total phenolic content in gambir might be due to the presence of catechins. The antioxidative properties of catechins are manifested particularly by their ability to inhibit and scavenge free radicals (Apea-Bah et al., 2009). The concentrations of 3.00 to 5.00 mg ml^{-1} have almost the same antioxidant activity, but for 0.25 to 2.00 mg ml^{-1}, the antioxidant activity increased based on the concentration of the sample. The results showed that GC, GU, GRm and GRg antioxidant activity was concentration-dependent. With respect to DPPH radical scavenging activity, the results indicated that GC, GU, GRm and GRg, showed similar tendencies as antioxidants. The results of the 30-min reaction time differed from the 2-min reaction time (Figure 3). Among the four kinds of gambir, the DPPH radical scavenging activity rate for the 1.00, 1.50, 2.00, 3.00, 4.00 and 5.00 mg ml^{-1} samples was comparable, ranging from 92.0 to 93.1%. The DPPH radical scavenging activity values of GC GU, GRm, GRg, indicate, again, the efficiency of catechins as free radical scavengers. According to William et al. (1986) and Evelyn et al. (1960), tannins are classified into hydrolysable and condensed tannins based on their structure. Hydrolyzable tannins are characterized

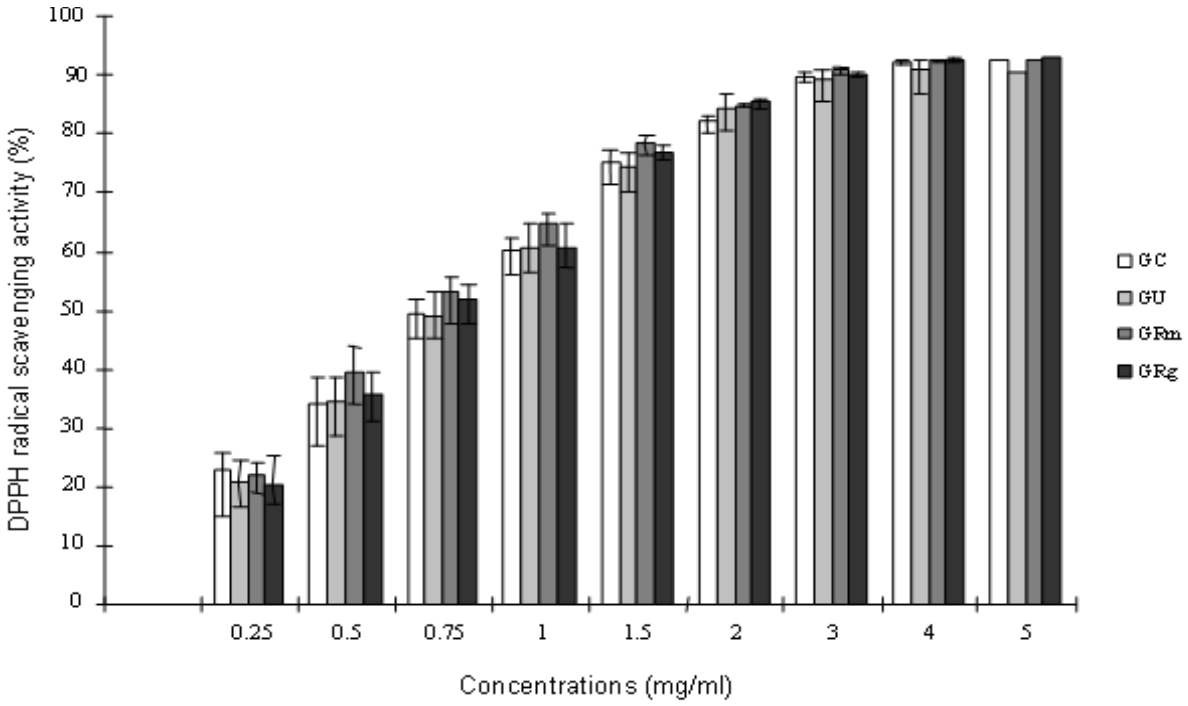

Figure 2. Gambir DPPH radical scavenging activity at a reaction time of 2 min. Gambir concentration were 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4 and 5 mg ml^{-1} for GC, GU, GRm and GRg. The data are the means ± s.d of three replications. GC: Gambir Cubadak; GU: Gambir Udang; GRm: Gambir Riau mancik, and GRg: Gambir Riau gadang.

Table 1. Total polyphenol content of GC, GU, GRm and GRg.

Gambir	Polyphenol (g 100 g^{-1})
Gambir Cubadak (GC)	13.86 ± 0.11
Gambir Udang (GU)	13.60 ± 0.19
Gambir Riau mancik (GRm)	13.58 ± 0.10
Gambir Riau udang (GRg)	13.90 ± 0.17

The data are the means ± s.d of three replications.

by having several gallic acid groups, or acids clearly derivable from gallic acid, united by ester linkage to a central glucose residue. Condensed tannins are polymers of flavan-3-ols (catechins) or flavan-3,4-diols or a combination of both. Catechins are a major component of condensed tannins, considered to be recalcitrant (Arunachalam et al., 2003). A reaction time of 30 min resulted in greater DPPH scavenging activity than the 2-min reaction time; this difference reflects the optimal reaction of catechins and slow acting antioxidant material in gambir. Radicals obtained from anti-oxidants with molecular structures are stable species which then stop the oxidation chain. The reaction mechanism between the antioxidants and DPPH depends on the structural conformation of the antioxidant Some compounds react very quickly with DPPH, but the majority of the compounds tested, the reactions are slower and the mechanisms seem to be more complex (Bondet et al., 1997).

Separation and analysis of gambir extract

Separation of catechin, epicatechin and caffeic acid was carried out using HPLC (Figure 4). The catechin content of gambir ranges from 99.4 to 108.5 µg ml^{-1} (Table 2). The antioxidant activity was correlated directly with catechin concentration. There is another report that shows catechin content of gambir from Indonesia ranged from 0.22 to 0.76 /g and epicatechin 1.40 to 23.90 mg/g (Taniguchi et al., 2007b). Gambir was formerly cultivated extensively in Indonesia as a commercial source of tanning materials and catechins are the most abundant polyphenolic constituent in the plant (Das and Griffiths, 1967; Taniguchi et al., 2007b).

Among the four kinds of gambir, the epicatechin a caffeic acid contents were detected at very low concentrations. Therefore, catechin, as compared to epicatechin and caffeic acid, shows potential as the source

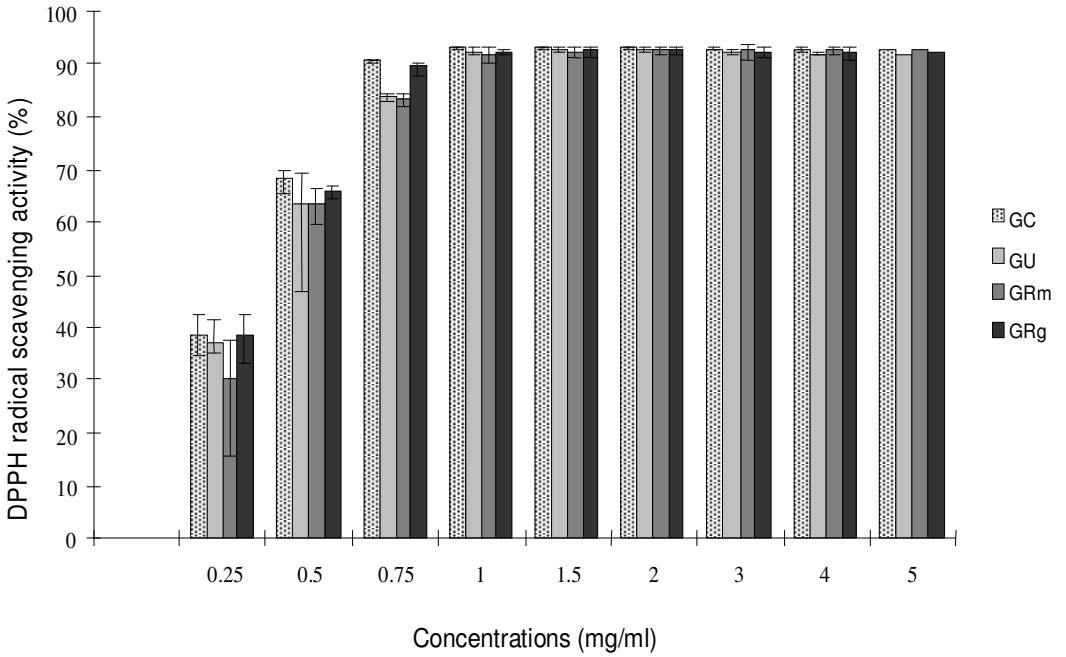

Figure 3. Gambir DPPH radical scavenging activity at a reaction time of 30 min. Gambir concentration were 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4 and 5 mg ml^{-1} for GRm, GRg, GC and GU. The data are the means ± S.D of three replications. GC: Gambir Cubadak; GU: Gambir Udang; GRm: Gambir Riau mancik; GRg: Gambir Riau gadang.

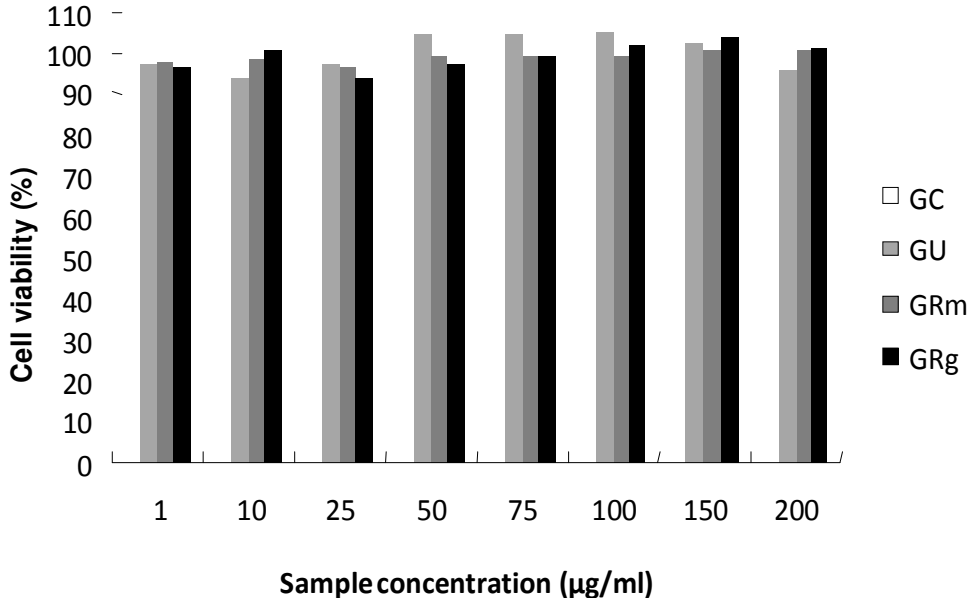

Figure 4. Cytotoxicity assay of gambir extracts. The effect of various concentrations of GC, GU, GRm and GRg on IEC6 cell viability was determined. I : Standard deviation of 3 times experiment.

of the antioxidant activity of gambir extracts in Siguntur, West Sumatra. Catechin is the only major flavonoid in *U. gambir*, and epicatechin and/or caffeic acid are/is minor flavonoids with difference among 4 kinds of gambir.

Cytotoxicity assay

Cytotoxicity is a measure of cellular viability and is used to assess whether compounds have a toxic effect.

Table 2. Concentration of catechin, epicatechin and caffeic acid in gambir extracts.

Gambir extract	Catechin (µg/ml)	Epicatechin (µg/ml)	Caffeic acid (µg/ml)
GC	104.5	0.80	0.99
GU	101.2	0.62	-
GRm	99.4	0.49	0.98
GRg	108	0.74	-

Gambir extract is used interchangeably in traditional medicine. It was revealed that in a cytotoxicity assay using IEC-6, the antioxidant in gambir was safe (Figure 4). The range of normalized gambir values ((gambir/ control) × 100) were as follows: GC: 96.3 to 103.8%; GU: 94.4 to 104.6%; GRm: 96.6 to 100.3%, and GRg: 93 to 103.7%. The gambir extract showed no negative effects against IEC-6, as indicated by more than 93% live cells (for concentration 1 to 200 µg/ml). The result showed different tendency with grape seed extract (GSE), where GSE concentration from 3.9 to 15.6 µg/ml did not affect IEC-6 cell viability but higher concentrations (62.5 to 1.000 µg/ml) reduced cell viability (Cheah et al., 2009).

Conclusion

Although different in appearance, the four cultivars of *U. gambir* of Siguntur, West Sumatra, Indonesia (GC, GU, GRm and GRg) displayed similar antioxidant activities and excellent antioxidant properties, with a remarkably potent ability to scavenge free radicals. The study has shown that 30-min reaction time for DPPH measurement is recommended because gambir contain slow acting antioxidant material. The DPPH radical scavenging activity ranged from 92.0 to 93.1% and the catechin content ranged from 99.4 to 108.5 µg/ml. Total polyphenol content was as follows: 13.86 (GC), 13.60 (GU), 13.58 (GRm) and 13.90 g/100 g (GRg). Catechin was identified as the major bioactive compound in gambir. Of notable importance is that the antioxidant in gambir is safe.

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