

RESEARCH ARTICLE

The Antioxidant and Cytotoxic Effects of *Cosmos caudatus* Ethanolic Extract on Cervical Cancer

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Abstract

BACKGROUND: Oxidative stress is closely related to all aspects of cancer. *Cosmos caudatus* ethanolic extract (CCEE) has been proved to have antioxidant effect that inhibited cancer cell growth due to its bioactive compounds such as catechin, quercetin and chlorogenic acid. This study aimed to evaluate antioxidant and anticancer activity of CCEE and its compounds.

METHODS: Total phenol was measured according to the Folin-Ciocalteu method. Catechin, quercetin and chlorogenic acid contained in CCEE were identified by high-performance liquid chromatography (HPLC). Antioxidant activity was evaluated by 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)-reducing activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, and ferric reducing antioxidant power (FRAP) activity test. The cytotoxic activity of CCEE was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay on HeLa cells.

RESULTS: The result showed that total phenol of CCEE was 181.64 ± 0.93 μg Catechin/mg extract. ABTS-reducing activity test showed that catechin had the highest activity (2.90 ± 0.04 $\mu\text{g}/\text{mL}$), while CCEE had moderate activity compared to other compounds. FRAP activity test demonstrated that catechin had the highest activity (315.83 μM Fe(II)/ μg) compared to other compounds. DPPH scavenging activity of CCEE was 22.82 ± 0.05 $\mu\text{g}/\text{mL}$. Cytotoxicity test on HeLa cell showed that CCEE had lower activity (inhibitory concentration (IC_{50}) = 89.90 ± 1.30 $\mu\text{g}/\text{mL}$) compared to quercetin (IC_{50} = 13.30 ± 0.64 $\mu\text{g}/\text{mL}$).

CONCLUSION: CCEE has the lowest antioxidant activity compared to quercetin, catechin, and chlorogenic acid and has the lowest anticancer activity compared to quercetin. However, CCEE and its compounds has potential as antioxidant and anticancer properties.

KEYWORDS: antioxidant, anticancer, catechin, *Cosmos caudatus*, quercetin

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Introduction

Initiation, promotion and progression are the three multistage of cancer.(1,2) Oxidative stress is related to the cancer initiation and progression by increasing DNA mutations or inducing DNA damage, genome instability

and cell proliferation.(3) Wide spectrum of diseases, such as most type of cancer, are involving the role of reactive oxygen species (ROS).(4) Imbalance between production of free radicals and reactive metabolites called oxidants or ROS are the sign of oxidative stress, leading to damage of important biomolecules and cells which potentially affected on the whole organism.(5)

Cosmos caudatus locally known as 'Ulam raja' and widely used as traditional medicine in Southeast Asia, is a herb of the family Compositae. Some studies reported that *C. caudatus* contains some bioactive compounds such as ascorbic acid, quercetin, chlorogenic acid and catechin. These natural compounds have been reported to be excellent antioxidants.(6-8) *C. caudatus* is suggested to have high antioxidant capacity, antidiabetic activity, antihypertensive properties, anti-inflammatory responses, bone protective effect, antimicrobial activity and anticancer properties.(9,10) This research aimed to evaluate the antioxidant potency and cytotoxic effect of *C. caudatus* ethanol extracts. Therefore, we also used high performance liquid chromatography (HPLC) method to observe the compounds in the *C. caudatus* extracts based on standard compound.(11)

Methods

Plant Extract Preparation

Leaves of *C. caudatus* were collected from Cihideung, Lembang, West Java, Indonesia. The plants were identified by herbarium staff, Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The *C. caudatus* simplicia (300 g) was extracted with ethanol 70% using maceration technique. Ethanol filtrate was filtered, and waste was re-macerated in triplicate. Using RV 10 rotary evaporator (IKA Works, Wilmington, NC, USA) at 50°C, the filtrate was concentrated to obtain extract. The extract was stored at -20°C.(12-14)

HPLC Assay

C. caudatus ethanolic extract (CCEE) chemical profiling analysis was performed using HPLC. Quantification of CCEE was done using the standard chlorogenic acid (Chengdu Biopurify Phytochemical, Sichuan, China), catechin (Sigma Aldrich, Darmstadt, Germany) and quercetin (Sigma Aldrich). HPLC analysis used the Hitachi Pump HPLC L-6200, Hitachi L-4000 UV detector and Reverse Phase Column C-18 (Phenosphere ODS-2, Phenomenex, 4.6 mm x 250 mm). Acetonitrile 70% (Merck, Darmstadt, Germany) was used as mobile phase (isocratic) with flow rate of 1.0 mL/min. The samples were then dissolved in methanol 70% (1 mg/mL), filtered through a 0.22 µm syringe, and injected (20 µL) to the column. UV absorbance was measured at 254 nm.(11)

Total Phenolic Content Assay

Total phenolic content was measured according to the Folin-Ciocalteu method. Briefly 15 µL of samples was placed into microplate then added 75 µL of Folin-Ciocalteu's reagent 2.0 M (Merck), followed by 60 µL of sodium carbonate 7.5% (Merck). The mixture was incubated at 45°C for 15 minutes.(15,16) Subsequently, absorbance value was measured at 760 nm using microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Waltham, MA, USA). Total phenolic content expressed as catechin equivalent was calculated by the following formula:

$$C = \frac{c \times v}{m}$$

C: total content of phenolic compounds (µg/mg) of *C. caudatus* in catechin equivalent;

c: the concentration of catechin established from the calibration curve (µg/mL);

V: the volume of extract (mL);

m: the weight of extract (mg).

2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)-reducing Activity Assay

ABTS⁺ solution was produced by reacting 14 mM ABTS and 4.9 mM potassium persulfate (Merck) (1:1 volume ratio) for 16 hours in dark condition at room temperature. The mixture was then diluted with 5.5 mM PBS (pH 7.4) until the absorbance of the solution was 0.70±0.02 at wavelength 745 nm. In brief, 2 µL of various concentrations of sample (0.23, 0.47, 0.94, 1.88, 3.75, 7.50, 15.00 µg/mL; µM) was added to each well at 96-well microplate, then the fresh 198 µL ABTS⁺ solution (Sigma Aldrich) was added. Then, the plate was incubated for 6 minutes at 30°C and calculated its absorbance at 745 nm. The ratio of reducing ABTS⁺ absorbance in the presence of the sample relative to the absorbance in the absence of the sample (negative control) was determined as the inhibition percentage of ABTS radical (%). The calculation of the median Inhibitory Concentration (IC)₅₀ was also measured.(14,16,17)

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP reagent was prepared by mixing acetate buffer (10 mL) 300 mM, ferric chloride hexahydrate (Merck) (1 mL) 20 mM dissolved in distilled water, and 1 mL of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Sigma Aldrich) 10 mM dissolved in HCl 40 mM. In 96-well microplate, 7.5 µL of various concentrations of sample (1.17, 2.34, 4.69, 9.38,

18.75, 37.50, 75.00 $\mu\text{g/mL}$; μM) was mixed with 142.5 μL FRAP reagent, and incubated at 37°C for 30 minutes. The absorbance value was measured at 593 nm with Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific). The standard curve was created using FeSO_4 , between 0.019 and 95 $\mu\text{g/mL}$ FeSO_4 . The measurement results were expressed in μM $\text{Fe(II)}/\mu\text{g}$ extract.(14,16,18)

2,2-Diphenyl-1-picrylhydrazil (DPPH) Scavenging Assay

The DPPH scavenging assay was used to measure the radical scavenging activity of the samples.(14) Samples (50 μL) with various concentrations were added to each well in a 96-well microplate. It was followed by addition of 200 μL of DPPH (Sigma Aldrich) solution (0.077 mmol/L in methanol) into the well. The mixture was then incubated in the dark for 30 minutes at room temperature. The absorbance was read using a microplate reader at 517 nm wavelength. The radical scavenging activity was measured using the following formula:

$$\% \text{ Scavenging} = (\text{Ac} - \text{As}) / \text{Ac} \times 100$$

Ac = negative control absorbance (without sample).

As = sample absorbance.

Cytotoxicity Assay

The cervical cancer cells (HeLa (American Type Culture Collection (ATCC) CC-Chemokine Ligand 2 (CCL-2)) were obtained from Stem Cell and Cancer Institute, Jakarta, Indonesia. The cells were maintained in Dulbecco modified Eagle's medium containing 10% FBS (Invitrogen, California, USA), 100 U/mL penicillin (Invitrogen) and 100 mg/mL streptomycin (Invitrogen). Then, the cells were incubated at 37°C, 5% CO_2 .(12) Briefly, 5×10^3 of cells were seeded in 96 well-plates for 24 hours.(12,13) The medium was discarded, then 180 μL of fresh medium was added into each well. The cells were treated with 20 μL of *C. caudatus* ethanol extract in various concentrations (1000, 500, 250, 125, 62.5, 31.25, 16.125 $\mu\text{g/mL}$) and quercetin in various concentrations (200, 100, 50, 25, 12.5, 6.25, 3.125 μM). Dimethyl sulfoxide (DMSO) 10% was added in different well as blank. All samples and blank were set in triplicate and incubated for 24 hours. Untreated cells were employed as control. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI, USA) was used to determine cell viability.(12) Twenty μL MTS was added to each well. The plate was incubated in 5% CO_2 at 37°C for 4 hours.

The absorbance was measured at 490 nm with a microplate reader. The data were then presented as percentage of viable cells (%).(12,13)

Results

Total Phenolic Content

Total phenolic content of the sample was measured, this study show that CCEE has total phenolic content is 181.64 ± 0.93 μg Catechin/mg extract.

HPLC Assay

The compounds content of CCEE was evaluated using HPLC with quercetin, catechin and chlorogenic acid as standard. Figure 1 shows that quercetin, catechin, and chlorogenic acid had retention time at 1.64 minutes, 1.40 minutes and 1.30 minutes, respectively. CCEE has peak at 1.403 minutes, it is close with catechin peak (1.40 minutes) which was assumed as catechin. This HPLC assay indicated that CCEE contained catechin compound.

ABTS-reducing Activity

ABTS-reducing activity of CCEE, catechin, quercetin and chlorogenic acid can be seen in Figure 2A and Table 1. Figure 2A shows ABTS-reducing activity in concentration-dependent manner, where higher concentration of sample increased ABTS-reducing activity. At the highest concentration of sample (15 $\mu\text{g/mL}$), catechin has the highest percentage of ABTS-reducing activity (73.53%) compared to quercetin (62.10%), CCEE (24.94%) and chlorogenic acid (22.46%). This results indicated that CCEE had low ABTS-reducing activity among other compounds except chlorogenic acid.

Table 1. IC_{50} value of ABTS-reducing activity of CEE, catechin, quercetin and chlorogenic acid.

Sample	ABTS-reducing Activity	
	IC_{50} (μM)	IC_{50} ($\mu\text{g/mL}$)
CCEE	-	31.97 ± 1.42
Catechin	10.00 ± 0.15	2.90 ± 0.04
Quercetin	12.04 ± 0.16	3.64 ± 0.05
Chlorogenic Acid	35.94 ± 2.14	12.70 ± 0.76

*CCEE = *C. caudatus* ethanolic extract, ABTS = 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid). The data was presented as mean \pm standard deviation. The ABTS-reducing activity assay was measured in triplicate for each sample.

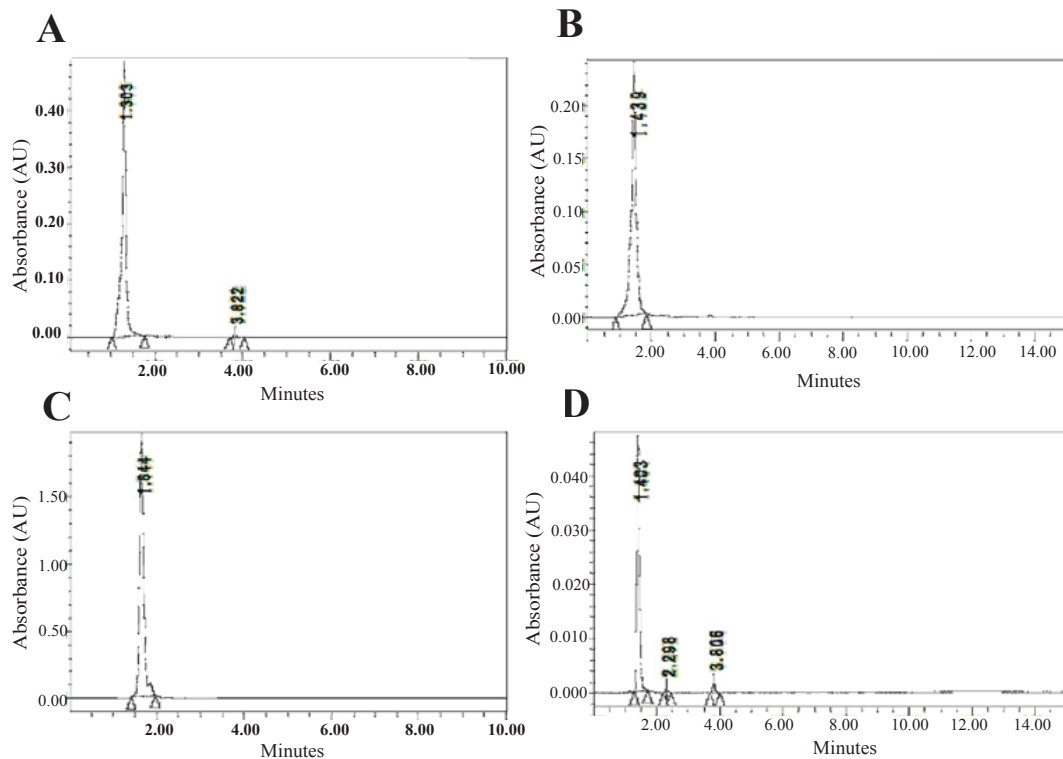


Figure 1. Chromatogram of extract and compounds with HPLC. A: Chlorogenic acid; B: Catechin; C: Quercetin; D: CCEE . *CCEE = *C. caudatus* ethanolic extract, HPLC = High-Performance Liquid Chromatography. This research was conducted in triplicate for each treatment.

Table 1 shows that catechin had the lowest IC_{50} value ($2.90 \pm 0.04 \mu\text{g/mL}$) compared to quercetin ($3.64 \pm 0.05 \mu\text{g/mL}$), chlorogenic acid ($12.70 \pm 0.76 \mu\text{g/mL}$), and CCEE ($31.97 \pm 1.42 \mu\text{g/mL}$). This finding supported the result of ABTS-reducing activity demonstrated the lowest activity of CCEE compared to other samples.

FRAP Activity

FRAP activity of CCEE, catechin, quercetin and chlorogenic acid can be seen in Figure 2B. The antioxidant activity of CCEE, catechin, quercetin and chlorogenic acid were evaluated using FRAP activity assay. Catechin had the highest activity ($315.83 \mu\text{M Fe(II)}/\mu\text{g}$) compared to

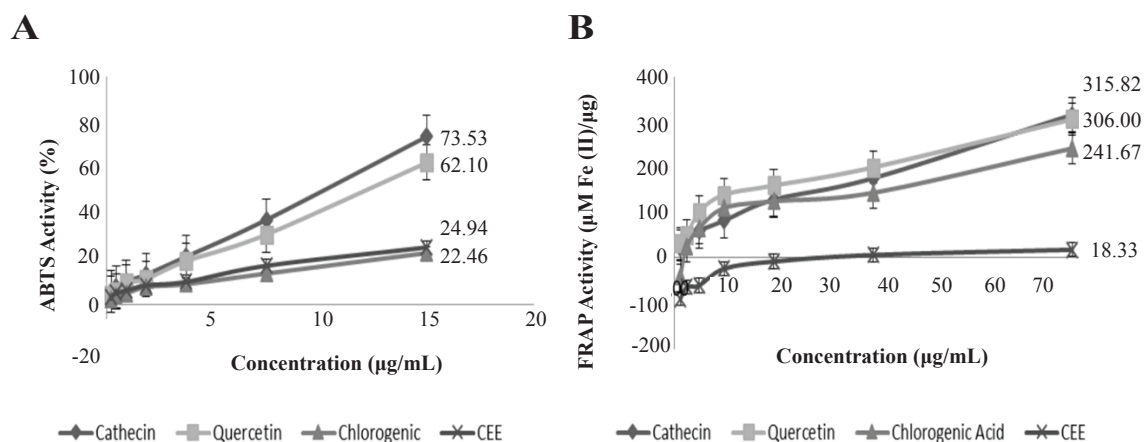


Figure 2. ABTS and FRAP activity of CCEE, catechin, quercetin and chlorogenic acid. *CCEE = *C. caudatus* ethanolic extract, ABTS = 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), FRAP = Ferric Reducing Antioxidant Power. This research was conducted in triplicate for each treatment. CCEE, catechin, quercetin and chlorogenic acid in ABTS assay were diluted in DMSO to reach the final concentration of 0.23; 0.47; 0.94; 1.88; 3.75; 7.50; 15.00 ($\mu\text{g/mL}$ for CEE and μM for compounds), while in FRAP assay were diluted in DMSO to reach the final concentration of 1.17; 2.34; 4.69; 9.38; 18.75; 37.50; 75.00 ($\mu\text{g/mL}$ for CCEE and μM for compounds).

quercetin (306.00 $\mu\text{M Fe(II)/}\mu\text{g}$), chlorogenic acid (241.67 $\mu\text{M Fe(II)/}\mu\text{g}$) and CCEE (18.33 $\mu\text{M Fe(II)/}\mu\text{g}$). This indicated that CCEE had the lowest antioxidant activity compared to other compounds (Figure 2B).

DPPH Scavenging Activity

The median IC_{50} of DPPH scavenging activity of CCEE, catechin, quercetin and chlorogenic acid can be seen in Table 1. It shows that the IC_{50} value of DPPH scavenging activity of CCEE (22.82 \pm 0.05 $\mu\text{g/mL}$) indicated antioxidant activity through scavenging DPPH free radical.

Cytotoxic Activity

Figure 3 shows the correlation between CCEE and quercetin concentration and its cytotoxicity on HeLa cell. Viability of cells decreased in concentration-dependent manner. The increased concentration was correlated with increased toxicity (<90% viable cells). The highest extract concentration (1000.00 $\mu\text{g/mL}$ and 200.00 μM) demonstrated the lowest of viability of cells by CCEE was 19.23% and quercetin 34.07%, respectively. CCEE and quercetin can inhibit the growth of HeLa cancer cell line with minimum inhibitory concentration (IC_{50}) values 89.90 \pm 1.30 $\mu\text{g/mL}$ and 43.99 \pm 2.15 μM (13.30 \pm 0.64 $\mu\text{g/mL}$), respectively (Table 2). This indicated that CCEE had lower cytotoxicity compared to quercetin.

Discussion

C. caudatus has been known as a potential herb that has antioxidant and anticancer activity.(19) *C. caudatus* has

Table 2. IC_{50} value cytotoxicity HeLa cells of CCEE and quercetin.

Sample	Cytotoxic Activity	
	IC_{50} (μM)	IC_{50} ($\mu\text{g/mL}$)
CCEE	-	89.90 \pm 1.30
Quercetin	43.99 \pm 2.15	13.30 \pm 0.64

*CCEE = *C. caudatus* ethanolic extract. IC_{50} of CCEE and quercetin was presented as $\mu\text{g/mL}$ and μM , respectively. The data was presented as mean \pm standard deviation. This research was conducted in triplicate for each treatment.

been reported to have high antioxidant capacity, mainly due to its polyphenol content.(7) The rich-phenolic foods are the sources of natural antioxidants.(19,20) The total phenolic content of CCEE in this study was 181.64 μg Catechin/mg extract. The result of other study showed that *C. caudatus* has high total phenolic content (1274 \pm 98 GAE mg/100 g fresh weight) in the acetone/water system.(7) The aqueous extract of *C. caudatus* has also been known to have the highest phenolic content.(21) Other study showed that the total phenolic content of *C. caudatus* ethanol extract (1144.6 mg/100g) was higher than *C. caudatus* water solvent (844.8 mg/100g).(22) High phytochemical contents, antioxidants, proteins, amino acids, vitamins and minerals are associated with risk reduction of free radical-related degenerative diseases.(23)

In this study, HPLC analysis was evaluated to determine compounds content of *C. caudatus*. Quercetin, catechin and chlorogenic acid were used as standards. Figure 1 shows that CCEE peaked at 1.403 minutes which was assumed as catechin. This indicated CCEE contain catechin compound.

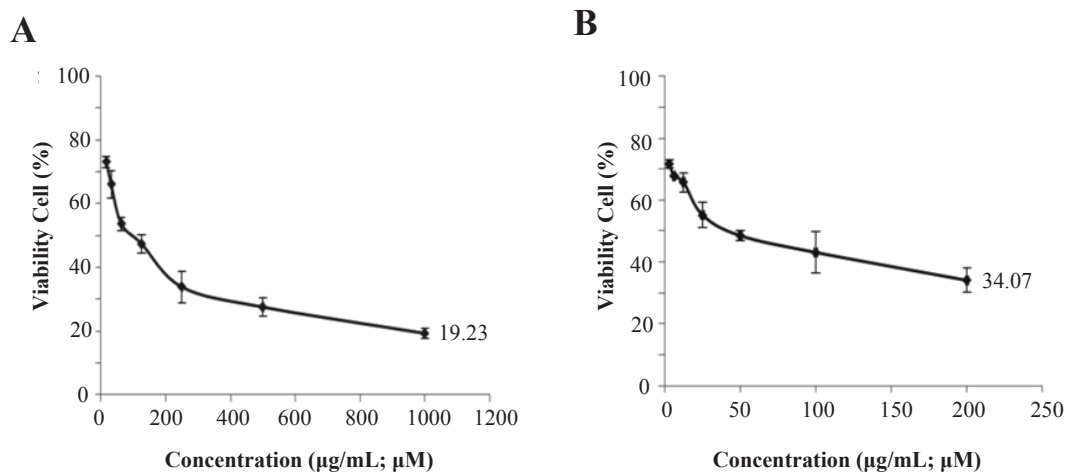


Figure 3. Viability of HeLa cell of CEE and quercetin. A: CEE; B: Quercetin. *CCEE = *C. caudatus* ethanolic extract. This research was conducted in triplicate for each treatment. CCEE was diluted in DMSO to reach the final concentration of 16.125; 31.25; 62.50; 125.00; 250.00; 500.00; 1000.00 ($\mu\text{g/mL}$); Quercetin was diluted in DMSO to reach the final concentration of 3.125; 6.25; 62.50; 12.50; 25.00; 50.00; 100.00 200.00 (μM).

Based on Noriham, *et al.*, study, *C. caudatus* ethanol extract measured by HPLC show presence of catechin.(24)

ABTS-reducing activity of CCEE had moderate activity compared to catechin, quercetin and chlorogenic acid. Based on the result above, CCEE had moderate activity compared to catechin, quercetin and chlorogenic acid, meanwhile in previous study, CCEE had the highest ABTS-reducing activity compared to other plants (4.71 $\mu\text{mol TE/g fw}$).(25) High antioxidants activity of *C. caudatus* was associated with the ability to reduce oxidative stress.(7) Another study also proved that *C. caudatus* had extremely high antioxidant compared to other plants through total antioxidant capacity (ascorbic acid equivalent antioxidant capacity (AEAC) value).(10)

In this research, DPPH scavenging activity of CCEE ($\text{IC}_{50}=22.82 \mu\text{g/mL}$), indicated that antioxidant activity through DPPH free radical scavenging activity. In our previous studies, the IC_{50} DPPH scavenging activity values of catechin were 7.02 $\mu\text{g/mL}$ (26) and 8.11 μM (27), while DPPH values of quercetin were 4.279 $\mu\text{g/mL}$ (28), 3.244 $\mu\text{g/mL}$ (29) and 19.200 $\mu\text{g/mL}$ (21). The FRAP activity value of CCEE was the lowest among other compounds. Some studies reported that *C. caudatus* had greater antioxidant activity than *Sauropus androgynus* (L) Merr and *Centella asiatica* in DPPH and FRAP assays.(30) Other study showed that *C. caudatus* had the greatest FRAP activity among other plants (25), also CCEE had the highest DPPH scavenging activity which was correlated with flavonoid content in the plants. *C. caudatus* had been reported to possess the highest flavonoid and phenolic content.(25) *C. caudatus* aqueous extract is a good source of antioxidant because it has the highest DPPH and FRAP values.(10) In another study, *C. caudatus* had the highest free radical scavenging potential extract (86.85%).(31) CCEE also showed beneficial activities in reducing number of parameters such as peroxyl value as an antioxidant. Phenolic content in CCEE plays a key role in scavenging free radicals which cause oxidative stress.(19) In addition, phenolic compounds have been shown to possess antioxidant ability which facilitates scavenging electrophiles and active oxygen species, slows down nitrosation and chelates metal ions to limit auto-oxidation, and increases the ability to adjust some enzyme actions.(32,33)

Tumorigenesis occurs due to the increasing free radicals that lead to DNA damage and mutation, apoptosis inhibition, cell cycle/proliferation stimulation, and DNA repair inhibition.(34) The role of ROS in cancer development can be determined in three different stages. Firstly, generating DNA damage including mutations and

structural alterations is the ROS first role, followed by the second stage which is the promotion stage where ROS blocks cell-cell communication leading to abnormal gene expression and modification of second messenger, resulting in increased cell proliferation or decreased cell apoptosis. Last stage, furthermore, is the progression of cancer caused by oxidative stress affecting further DNA alterations.(1) Free radicals can react with membrane fatty acids and form lipid peroxides, accumulation of which leads to production of carcinogenic agents.(35) In this study, CCEE had lower cytotoxicity on HeLa cell compared to quercetin. These results were confirmed by Lee and Vairappan that found a weak cytotoxic activity of the ethanolic extract of *C. caudatus* against P388 murine leukemia cells.(9) However, in other study, *C. caudatus* exhibited the highest DPPH free radical scavenging, ABTS-reducing activity, FRAP and inhibition of linoleic acid.(24)

Conclusion

C. caudatus and its compounds showed antioxidant activities as measured through ABTS-reducing activity, DPPH scavenging activity, and FRAP activity. CCEE has the lowest antioxidant activity compared to quercetin, catechin and chlorogenic acid. CCEE also has the lowest cytotoxic activity compared to quercetin. However, CCEE and its compounds has potential as antioxidant and anticancer properties.

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