Phytochemical composition and antioxidant capacity of *Cordia dichotoma* seeds

Shuge Tian[1,2], Feng Liu[3], Xuejia Zhang[1,2] and Halmuart Upur*[1]
[1]Xinjiang Key Laboratory of Famous Prescription and Science of Formulas, Urumqi, Xinjiang, China
[2]College of TCM, Xinjiang Medical University, Urumqi, Xinjiang, China
[3]College of Chemistry and Chemical Engineering, Xinjiang Normal University, Urumqi, Xinjiang, China

Abstract: This study aims to determine the phytochemical composition and antioxidant activity of air-dried *Cordia dichotoma* seeds. Total polyphenolic content was analyzed via the Folin-Ciocalteu method. Total triterpenoid content and amino acids was analyzed colorimetrically. The rosmarinic acid content was examined using high-performance liquid chromatography tandem mass spectrometry. The ethanolic extracts contained polyphenolic compounds (1.0%), triterpenoids (0.075%), amino acids (1.39%), and rosmarinic acid (0.0028%). The results from this study indicate that *C. dichotoma* seeds are a rich source of polyphenolic compounds and amino acids, which can be used for quality assessment. The ethanolic extract of *C. dichotoma* seeds has good antioxidant capacity.

Keywords: *Cordia dichotoma* seeds; phytochemical composition; antioxidant activities; SOD.

INTRODUCTION

It has been proved that almost all of the organisms have the ability of antioxidant, but this ability can not completely prevent oxidative damage (Nuhu Alam *et al.*, 2010). The oxidizing diseases caused by free radicals, resulting in lipid peroxides and reactive oxygen species (ROS). The three main forms of ROS are the superoxide anion radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2) (Reena Singh *et al.*, 2010; Reyhaneh Sariri *et al.*, 2011). Excessive production is implicated in aging and in many diseases, including inflammation, cancer, cardiovascular disease, osteoporosis, and degenerative diseases (Reyhaneh Sariri1 *et al.*, 2011; Liang Linghong and Wu Xiangyang, 2012; Hedia Hannachi *et al.*, 2011). Because antioxidants are very important for us in human health; the antioxidant supplements and foods that contain antioxidants are useful in helping the human body reduce oxidative damage (Reyhaneh Sariri *et al.*, 2011; Liang Linghong and Xiangyang, 2012). Many natural antioxidants from food and medicine are used industrially to improve product shelf life, such as polyphenols, flavonoids et al. (Shirin Adel Pilerood and Jamuna Prakash, 2011). The four methods commonly used in antioxidant activity assays are 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity (RSC), superoxide anion-scavenging capacity (superoxide-ASC), hydroxyl-RSC, and superoxide dismutase (SOD)-like activity (Aysel Aricioglu *et al.*, 2001). DPPH has been used by investigators to assay the antioxidant activity of *Cordia dichotoma* (Zhang, Z.G. *et al.*, 2007).

Abnormal Savda Munziq (ASMq) (Halmurat Upur *et al.*, 2011), an herbal formulation in traditional Uighur medicine, is widely used in the Xinjiang Region of China, especially for treating and preventing cancer (Abdiryim Yusup, Halmurat Upur *et al.*, 2005), diabetes, cardiovascular disorders, and chronic asthma (Murat Kizaibek *et al.*, 2012). ASMq is an herbal formula composed of 10 medicinal herbs: *C. dichotoma*, *Ziziphus jujuba*, *Glycyrrhiza uralensis*, *Anchusa italica*, *Foeniculum vulgare*, *Euphorbia humifusa*, *Lavandula angustifolia*, *Melissa officinalis*, and *Alhagi pseudoalhagi* (Murat Kizaibek *et al.*, 2009). Research shows that *C. dichotoma* is an important component of ASMq (Murat Kizaibek *et al.*, 2009); thus, studying *C. dichotoma* has great significance.

Cordia dichotoma L. (Boraginaceae), commonly known as Lasaura/Lasura, is a tree found in tropical and subtropical regions. *C. dichotoma* is a medium-sized tree grows in sub-Himalayan tracts and outer ranges, its fruits are globose, yellowish-brown, pink or black, and pulpy (Anjana Patel *et al.*, 2011). The medicinal attributes of *C. dichotoma* have been known for years. The seeds of the plant are anti-inflammatory (Sharma *et al.*, 2010). The fruits of the plant are used as an astringent, expectorant, anthelmintic (Anurag Mishra and Ganesh Prasad Garg, 2011), purgative, and diuretic (Kuppast and Vasudeva Nayak, 2005). The methanolic extract of *C. dichotoma* possesses antioxidant, cognitive enhancing, and antiepileptic properties. *C. dichotoma* seeds contain α-amyrins, betulin, octacosanol, fatty acids and flavonoids. α-Amyrin and toxifolin 3, 5, dirhamnoside show significant anti-inflammatory activity (Reena Singh *et al.*, 2010). The present study aims to investigate the antioxidant profile of ethanolic extract of *C. dichotoma* seeds using different antioxidant models.

**Corresponding author:* e-mail: tsgyz@sina.com

MATERIALS AND METHODS

Materials

Cordia dichotoma seeds was obtained from the air-dried fruit, purchased from Xinjiang Maidisen Uyghur Medicine Co., Ltd. Standard rosmarinic acid was obtained from Sigma-Aldrich (USA). Antiscorbic acid, galic acid were obtained from the Tianjin Reagent Co. (Tianjin, China). Reverse phase HPLC grade methanol was supplied by Fisher Scientific (USA) and water was obtained from a Millipore Q3 ultra-pure water system (Millipore, USA). Ortho-oxybenzoic acid was purchased from Sigma Co. (USA), SOD kit (China, Nanjing Jiancheng Bioengineering Institute, 20091201), cAMP (USA, Sigma Co, 064K1441), Folin–Ciocalteau reagent, 2,2-diphenyl-1-picryl hydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO,29802KI). All other organic solvents used in the study were analytical grade.

Extraction procedure

Ethanol extraction

C. dichotoma seeds were pulverized into fine powder using a stainless steel blender. The dried and powdered plant materials (3 g) were extracted once with 45 mL of 80% ethanol for 20 min by ultrasound. The ethanolic extract was then filtered and ethanol was added to a final volume of 100mL.

Purification process

The ethanolic extract was concentrated via rotary evaporation, dissolved in 30 mL of chloroform, transferred into a separator funnel, extracted four times with 15mL of saturated sodium bicarbonate and chloroform, and mixed with sodium bicarbonate solution. The pH of the mixture was adjusted to 2-3 using 6 mol/mL hydrochloric acid. The mixture was extracted four times with 15mL of chloroform. The chloroform extract was pooled, washed with water. The chloroform solution was dried with anhydrous sodium sulfate, filtered, and washed thrice with 15 mL of water. The washing liquid and filtrate was pooled and evaporated to dryness under a vacuum. The residue was mixed with anhydrous ethanol solution to a final volume of 50 mL. After shaking, the product solution was obtained.

Aqueous extract

C. dichotoma seeds were pulverized into fine powder using a stainless steel blender. Exactly 2 g of the powder (2 g) was placed in a 250 mL round-bottomed flask. Boiling water (100 mL) to conduct reflux extraction for 1h. The resulting solution was filtered while hot. The reflux extraction was repeated twice with 50 mL of boiling water for 0.5 h and filtered while hot. The filtrates from the three-reflux extractions were pooled and concentrated into 100 mL as the test solution.

Total polyphenolic content (TPC)

The total phenolic content (TPC) of the 80% ethanolic extract was determined using the Folin-Ciocalteau method. We mixed 0.5 mL of the respective extract with 1mL of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 1 min. Then, 2 mL of sodium carbonate (20% Na_2CO_3) solution was added. Distilled water was added to a final volume of 10mL and the mixture was allowed to stand at room temperature for 10 min. The absorbance of the supernatant was recorded at 760 nm using a spectrophotometer. The results were compared with similarly prepared standards with known gallic acid concentrations. All samples were analyzed in triplicate.

Total triterpenoid content (TTC)

The total triterpenoid content (TTC) was determined via colorimetry using the following procedure. Briefly, 1.0 mL of the purified extract was placed in a test tube in a boiling water bath, and cooled. Then, we mixed the solution with 0.5 mL of 5% vanillin-glacial acetic acid solution and 0.8 mL of concentrated sulfuric acid solution. The test tube was heated in a water bath at 60°C for 15 min, and then cooled to room temperature. After adding a constant volume of glacial acetic acid to scale, the samples were shaken. The absorbance of each sample solution was measured at 553 nm, using a UV-vis spectrophotometer. Uralic acid (1.2 mg/mL to 7.2 mg/mL) was used as a standard. All samples were analyzed in triplicate.

Total free amino acid content (TFAAC)

Total free amino acid content (TFAAC) was determined by the ninhydrin reaction method of Rosen (1957) (Asagbra, Okafor, Onawola, Etoamaihe, Olatope, 2012). We added 1mL of water extractions to 1 mL of sodium carbonate buffer salts and 2 mL of 2% ninhydrin solution in a tube. The tubes were heated in a boiling water bath for 15 min and allowed to cool at room temperature for 15 min. After adding a constant volume of water to scale, the tubes were shaken. Each sample solution was measured at 566 nm, using a UV-vis spectrophotometer. Arginine (4 μg/mL to 16 μg/mL) was used as a standard. All samples were analyzed in triplicate.

HPLC-MS/MS analysis of rosmarinic acid

High-performance liquid chromatography (HPLC) system (Agilent Technologies 1100, USA) was equipped with a binary pump, a thermos tatted column compartment, a degasser, and a control module. The extracts were analyzed using HPLC on a C18 reverse phase column (4.6 mm×250 mm; Agilent). The solvent gradient used in this study was formed through solvent (A methanol and B 0.03% phosphoric acid in water). The linear gradient was used for chromatographic separation: 0 min, 70% B; 6 min, 55% B; 20 min, 45% B. The run time was 30min. The injection volume was 5 μL. The solvent flow rate was maintained at 1.0 mL/min. All analyses were carried out at 30°C. The spectra were recorded at 330 nm. The

method of external standard was used for quantification. We identified rosmarinic acid in the ethanolic extract by matching its retention times and the spectral characteristics to those of a single HPLC run of a known rosmarinic acid standard (figs. 1-2).

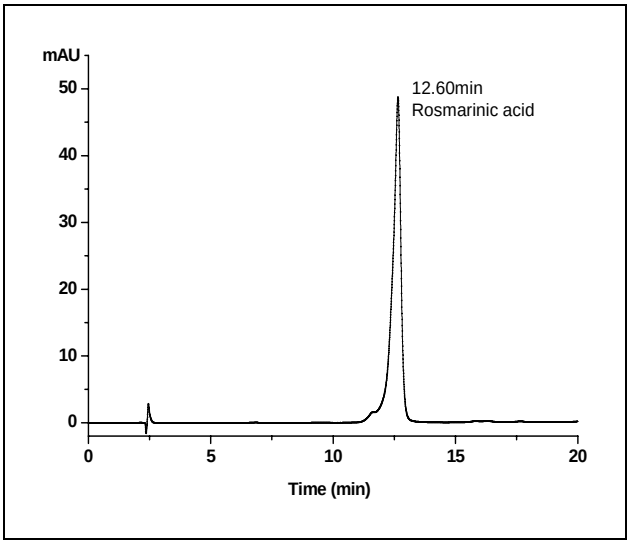

Fig. 1: Chromatography of Rosmarinic acid.

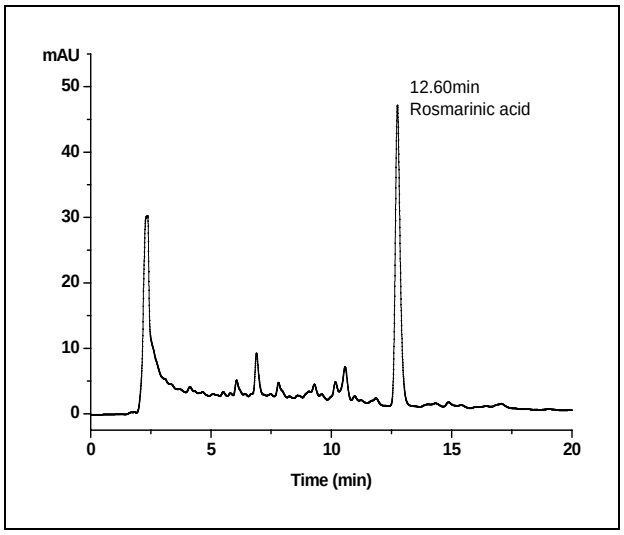

Fig. 2: Chromatography of *C. dichotoma* seeds.

The HPLC system was coupled with an Esquire 6310 Ion Trap LC/mass spectrometry (MS) system (Agilent Technologies, USA) and an 1100 UV detector (Agilent Technologies, USA). The MS parameters included electrospray ionization, scanning system for negative ion mode; dryer temperature of 350°C, dryer pressure of 55psi, N_2 dryer flow of 12 L/min, capillary voltage of 3500V; rosemarinic acid level of the mass spectrum scan ranging fromm/z 350 to m/z 370; secondary ion mass spectrometry m/z 359, cataclastic voltage of 0.35 V, and scanning range of m/z 100 to m/z 400 (figs. 3 and 4).

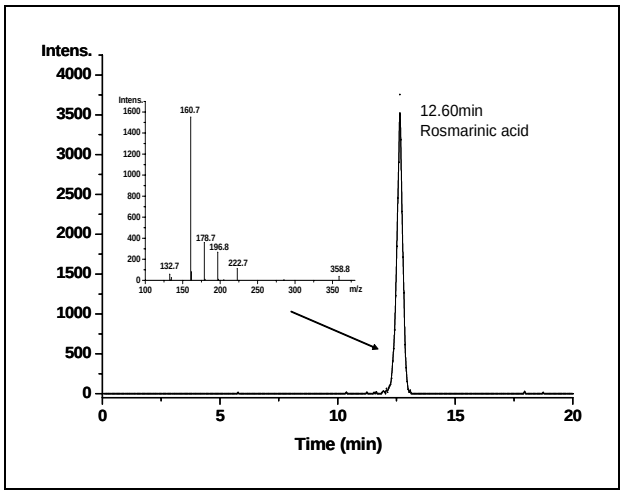

Fig. 3: ESI-MS/MS of Rosmarinic acid.

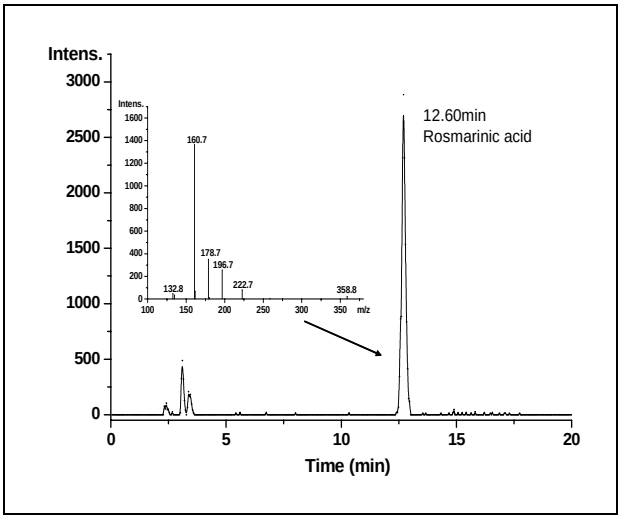

Fig. 4: ESI-MS/MS of Rosmarinic acid were abtained by *C. dichotoma* seeds.

DPPH-RSC

First, a 4 mL test tube with 2 mL of the ethanol DPPH-solution was determined marked as A_0, measured it at 540 nm with a spectrophotometer. Ethanol (4 mL) was added and measured as a blank. Then, the *C. dichotoma* seed ethanolic extract was diluted to 20, 40, 60, 80, 100, 120, and 140 μg/mL. Second, a 4 mL test tube with 2mL of the ethanol DPPH-solution and add 2 mL of the extracts at different concentrations. After shaken and allowed to reacting for 30 min stand at room temperature in the dark, the absorbance was determined marked as A_x (Deepak HB , Chandrasekaran CV *et al.*, 2012; Aikemu Ainiwaer; Yusup Abdiryim *et al.*, 2012). The decrease in absorbance of the resulting solution was monitored at 540 nm using a spectrophotometer. The VC standard solutions (20, 40, 60, 80, 100, 120 and 140 μg/mL) in ethanol was prepared under the same conditions.

$$\%RSA= \{[A_0 - (A_x - A_{x0})]/A_0\} \times 100\%$$

Superoxide-ASC

Superoxide anion, a one-electron reductant of molecular dioxygen, is harmful to cellular components and functions as a precursor of other ROS, such as singlet oxygen and hydroxyl radical (Keita Saito *et al.*, 2008). In this assay, 3 mL of Tris-HCl buffer solution (pH = 8.21, 0.05 mol/L) was heated in a water bath at 30 °C for 20 min. Then, we added 0.5 mL of the respective concentrations (20, 40, 60, 80, 100, 120, 140 μg/mL/) of *C. dichotoma* seed extracts and 3 mL of phloroglucinol (25 mmol/L) to the solution, which was heated in a water bath at 30 °C for 4 min. Finally, we added 1 mL of HCl solution (8 mmol/L) as a sample. The absorbance was determined at 420 nm using a spectrophotometer. Distilled water was used as the blank instead of the extracts of different polarities (Liao Haibing, *et al.*, 2012; Zheleva-Dimitrova, DZ, 2013). The VC standard solution (20, 40, 60, 80, 100, 120 and 140 μg/mL) in distilled water was prepared under the same conditions.

$$\%ASC = [A_0 - (A_x - A_{x0})/A_0] \times 100\%$$

Hydroxyl –RSC

Hydroxyl radicals were generated by adding iron (II) salts to a reaction mixture. In this assay, sample, 1.0 mL of ferrous sulfate solution (4.5 mmol/L) was added to 1.0 mL of salicylic acid ethanol solution (4.5 mmol/L). We added 1mL of *C. dichotoma* seed extracts at different concentrations (20, 40, 60, 80, 100, 120 and 140 μg/mL), respectively, and 1.0 mL of hydrogen peroxide solution (4.4 mmol/L) to the solution, which was held in a water bath at 37°C for 0. h. We used 1.0 mL of distilled water as the damage liquid instead of the extracts. Distilled water (1.0 mL) and hydrogen peroxide solution were used as the blank instead of the extracts. The absorbance was determined at 510 nm using a spectrophotometer (UV-Cintra-40, GBC, Australia). The VC standard solution (20, 40, 60, 80, 100, 120 and 140 μg/mL) in distilled water was prepared under the same conditions.

$$\%RSC = [A_0 - (A_x - A_{x0})/A_0] \times 100\%$$

Determination of SOD-like activity

The SOD-like activity of the 80% ethanolic extracts from the *C. dichotoma* seeds was measured by an SOD kit. We added 1 mL of the different concentration (20, 40, 60, 80, 100, 120 and 140 μg/mL) samples of the *C. dichotoma* seed extracts to the reaction system. The mixture was shaken to uniformity. The reaction system was allowed to stand at 37°C for 40 min, and then 2mL of the developed was added to the mixture. After reacting for 10 min at room temperature, the absorbance, marked as A_x, was measured at 550 nm using a spectrophotometer. We simultaneously added 1 mL of distilled water to the reaction system as a blank, marked as A_0. We added the equivalent volume of distilled water to replace the other reagents, except samples to eliminate the influence sample coloration, marked as A_{x0}. The standard solution of VC (20, 40, 60, 80, 100, 120 and 140 μg/mL), which was resolved by distilled water, was prepared under the same conditions. All samples were analyzed in triplicate. The results were expressed as SOD-like activity (%RSC).

$$\%SOD = [A_0 - (A_x - A_{x0})/A_0] \times 100\%$$

RESULTS

Total polyphenolic content (TPC)

The Folin-Ciocalteu method (TANG jin-hua, DING wen-huan, *et al.*,2012) measures phenolic content in terms of the reduction in the reagent through the formation of a dark blue complex that can be determined at 760 nm against a gallic acid standard. Table 1 shows the TPC of the *C. dichotoma* seeds concentrated in 80% ethanolic extract (1.00%). The standard curve prepared using gallic acid was A=139.5C + 0.03371, r=0.9994 (A: absorbance, C: concentration), which shows good linearity at concentrations ranging from 0.001 mg/mL to 0.007 mg/mL.

Total triterpenoid content (TTC)

The vanillin-glacial acetic acid-perchloric acid colorimetry method was used to determine the triterpenoid acid content, which was determined at 553 nm against an ursolic acid standard. Table 1 shows that the total triterpenoid content of *C. dichotoma* seeds is concentrated in the 80% ethanolic extract (0.075%). The TPC of *C. dichotoma* has been reported, but the triterpenoid acid content of *C. dichotoma* seeds is still unknown. The standard curve prepared using ursolic acid was A=0.02524C-0.005, r=0.9994 (A: absorbance, C: concentration), ranging from 1.2mg/mL to 7.2mg/mL.

Total free amino acid content (TFAAC)

The total free amino acid content of the extract was determined through colorimetry using ninhydrin. Thus, the free amino acid content measured at 566 nm against arginine was used as a standard. Table 1 shows that the total free amino acid content of *C. dichotoma* seeds is concentrated in the aqueous extract (1.39%). The standard curve prepared with arginine was A=26.1071C- 0.07224, r=0.9999 (A: absorbance, C: concentration), which ranges from 4μg/mL to 16μg/mL.

HPLC-MS/MS analysis of rosmarinic acid

Rosmarinic acid was detected in 80% ethanolic extracts of *C. dichotoma* seeds at 340 nm using an HPLC diode array detector. Separation was achieved using a gradient mobile phase of 0.03% phosphoric acid in water and methanol. The retention time of the standard rosmarinic acid was 12.6 min. The presence of rosmarinic acid was verified by comparing the retention time, absorbance spectrum, and fragmentation pattern with those of authentic standards with that of ethanolic *C. dichotoma* seed extract. Under HPLC-MS, rosmarinic acid was precisely identified in

ethanolic *C. dichotoma* seed extract. Table 1 shows that the rosmarinic acid content of *C. dichotoma* seeds is concentrated in the ethanolic extract (0.0028%). The standard curve linearity using HPLC method was determined at five concentration levels ranging from 1.9 μg/mL to 9.5 μg/mL for rosmarinic acid, A=343.6080 C-158.4941, r=0.9996 (A: absorbance, C: concentration).

DPPH-RSC

In this study, DPPH was used to determine the free radical-scavenging activity of the ethanolic *C. dichotoma* seed extracts (table 2). DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reductive capability of the DPPH radical is determined by the decrease in its absorbance at 540 nm, induced by antioxidants. The maximum absorbance of stable DPPH radicals in ethanol was at 540 nm. The decrease in the absorbance of the DPPH radicals is caused by scavenging the radicals by hydrogen donation. This process is visually noticeable as a change in color from purple to yellow. Thus, DPPH is usually used as a substrate to evaluate the anti-oxidative activity of antioxidants. These results indicate that both *C. dichotoma* seed extracts noticeably affect the scavenging of free radicals. Free radical-scavenging activity also increased with increasing concentration. At 20 μg/mL to 140 μg/mL, the scavenging abilities of VC, the 80% ethanolic extract, and the purified 80% ethanolic extracts on the DPPH radical ranged from 64.13±8.82, 65.63±1.48 and 469.90±5.33, respectively (table 2).

Superoxide-ASC

The decrease in absorbance at 420 nm with the presence of antioxidants indicates the consumption of superoxide anions in the reaction mixture. The superoxides in two *C. dichotoma* seed extracts were compared with that of VC. The higher concentration required to scavenge the radicals meant lower antioxidant activity. The 80% ethanolic extracts exhibited the highest superoxide anion scavenging activity (table 2).

Hydroxyl-RSC

Hydroxyl radical, the most toxic radical, nonspecifically oxidizes all classes of biological macromolecules including lipids, proteins, and nucleic acids at virtually diffusion-limited rates. Hydroxyl radical result in many diseases including arthritis, atherosclerosis, cirrhosis, diabetes, cancer, Alzheimer's disease, emphysema, and aging. The order of activity of the extracts was as follows: VC >80% ethanolic extract > purified 80% ethanolic extract.

Determination of SOD-like activity

SOD is very important because it provides a cellular defense mechanism against toxic oxygen by catalytically scavenging O_2^- (Nejati-Yazdinejad M *et al.*, 2013). The results of this study are summarized in table 5. The order of antioxidant activity was as follows: VC >80% ethanolic extract > purified 80% ethanolic extract.

DISCUSSION

Quantification of photochemical constituents

The results from this study indicate that *C. dichotoma* seeds are a rich source of polyphenols, triterpenoids, amino acids, and rosmarinic acid. The TPC, TTC and TFAAC of the extract were analyzed through colorimetry. The rosmarinic acid content was examined using high-performance liquid chromatography tandem mass spectrometry. The results indicate that *C. dichotoma* seeds are a rich source of polyphenolic compounds, amino acids. The HPLC-MS is very suitable for the rapid characterization of the phytochemical composition in plant extracts.

In-vitro antioxidant activity

At the concentrations tested of scavenging DPPH radical, the results indicate that the 80% ethanolic extracts possessed good activity, whereas the purified 80% ethanolic extracts showed poor activity. Except for the

Table 1: Phytochemical composition for *C. dichotoma* seeds

Total phenolic content (%)	Total triterpenoid content (%)	Total free amino acids content (%)	Rosmarinic acid content (%)
1.00±0.8	0.075±1.33	1.39±1.10	0.0028±1.93

The data are presented as mean ± SD for three replicates

Table 2: Antioxidant activities of the two extracts for *C. dichotoma* seeds and positive control.

Extract and positive control	DPPH-RSC assay[a]	Superoxide-ASC assay[a]	Hydroxyl-RSC assay[a]	SOD assay[a]
VC	64.13±8.82	416.04±3.03	44.78±0.96	39.15±0.50
80% ethanol extract	65.63±1.48	118.46±4.97	153.95±4.17	269.01±2.29
purified 80% ethanol extract	469.90±5.33	505.03±9.40	8356.19±14.42	58895.25±6.6

The data are presented as mean ± SD for three replicates. [a]IC50 values in μg/mL.

80% ethanolic extracts, the *C. dichotoma* seed extracts exhibited no significant differences in superoxide anion scavenging capacity. Furthermore, almost no significant differences in superoxide anion scavenging capacity were observed among the purified 80% ethanolic extracts. VC exhibited the highest capacity to scavenge hydroxyl radicals compared with the two *C. dichotoma* seed extracts (table 2). The results of SOD-like activity indicate that the superoxide scavenging activities were reflected by the polyphenol content.

CONCLUSION

The results from this study indicate that *C. dichotoma* seeds are a rich source of polyphenols, triterpenoids, amino acids, and rosmarinic acid, which can be used for quality assessment. This study demonstrates that the HPLC-MS is very suitable for the rapid characterization of the phytochemical composition in plant extracts. *C. dichotoma* seeds antioxidant status assessment will facilitate the identification and quantification of the active ingredient, which will help protect people against free radical damage and oxidative stress-related diseases. The proposed method provides a fast and reliable quality control for the crude drug, which is used as a medicinal plant in many countries. Thus, *C. dichotoma* seeds are a good source of antioxidant agents available in everyday life.

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