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ฤทธิ์ต้านออกซิเดชันและฤทธิ์ความเป็นพิษต่อเซลล์ของสารสกัดพืชท้องถิ่นของไทย

ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF THAI LOCAL PLANT EXTRACTS

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บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อสกัดสารออกฤทธิ์จากพืชพื้นบ้านในประเทศไทยทั้งหมด 8 ชนิด โดยใช้ตัวทำละลายเอทานอลและเฮกเซนศึกษาฤทธิ์ต้านอนุมูลอิสระ ปริมาณสารประกอบฟีนอลิกทั้งหมด ปริมาณสารประกอบฟลาโวนอยด์ พบว่าสารสกัดพืชในตัวทำละลายเอทานอลให้ร้อยละผลผลิตสูงกว่าเฮกเซนอย่างมีนัยสำคัญ พบว่าสารสกัดหยาบหญ้าแห้วหมู (*Cyperus rotundus*) ในตัวทำละลายเอทานอลมีปริมาณสารประกอบฟีนอลิกทั้งหมดสูงสุดเท่ากับ 11.617±0.289 มิลลิกรัมสมมูลของกรดแกลลิก/กรัมน้ำหนักแห้ง และสารประกอบฟลาโวนอยด์มีค่าสูงที่สุดมีค่าเท่ากับ 7.973±0.231 มิลลิกรัมสมมูลของกรดแกลลิก/กรัมน้ำหนักแห้ง นอกจากนั้นสารสกัดหญ้าแห้วหมู (*C. rodundus*) มีฤทธิ์ต้านอนุมูลอิสระ DPPH และ ABTS ได้สูงสุด มีค่า IC_{50} เท่ากับ 0.408±0.011 และ 0.402±0.0071 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ นอกจากนั้นสารสกัดหยาบหญ้าแห้วหมูในตัวทำละลายเอทานอลมีปริมาณสารคาเทชิน m/z 289.1 และแอนโทไซยานิน m/z 463.2 เมื่อวิเคราะห์ด้วยลิควิดโครมาโทกราฟี-แมสสเปกโทรเมตรี

(LC-MS) โดยปริมาณคาเทชินนี้มีความสำคัญในการยืนยันฤทธิ์ต้านอนุมูลอิสระในงานวิจัยนี้ได้ศึกษาความเป็นพิษของหญ้าแห้วหมู (*C. rodundus*) ต่อเซลล์ NCI-H187 โดยมีค่า IC_{50}42.47 ± 2.66 ไมโครกรัม/มิลลิลิตร และยังพบความเป็นพิษต่อเซลล์ของกระชายดำ (*Kaempferia parviflora*) ต่อเซลล์มะเร็งช่องปาก (KB cell line) และเซลล์มะเร็งปอด (NCI-H 187 cell line) โดยมีค่า IC_{50} ที่ 60.24±1.73 และ 27.22±1.04 ไมโครกรัม/มิลลิลิตร ตามลำดับ จากผลการทดลองชี้ให้เห็นว่าหญ้าแห้วหมู (*C. rodundus*) และกระชายดำ (*K. parviflora*) เป็นแหล่งสารต้านอนุมูลอิสระที่ดีและเป็นพืชที่มีศักยภาพในการต้านเซลล์เซลล์มะเร็งช่องปากและเซลล์มะเร็งปอด

คำสำคัญ: ต้านอนุมูลอิสระ; ความเป็นพิษต่อเซลล์; หญ้าแห้วหมู; กระชายดำ

Abstract

The purpose of this study was to analyze the total phenolic, total flavonoids, antioxidant, cytotoxic activity and antioxidant components of crude extracts from eight species of Thai local plants using different solvents such as ethanol and hexane. Ethanol showed significantly higher percentage extraction yields than hexane. The contents of total phenolic and total flavonoids in the crude ethanol extract of *Cyperus rotundus* (CRE) were the highest at 11.617±0.289 mg GAE/g dry extract and 7.973±0.231 mg QE/g dry extract, respectively. The free radical scavenging of crude ethanol extracts of *C. rotundus* was the highest compared to the other plants and showed IC_{50} values in the DPPH assay and ABTS assay of 0.408±0.011 mg/ ml and 0.402±0.0071 mg/ml, respectively. Moreover, crude ethanol extracts of *C. rotundus* indicated the presence of catechin (*m/z* 289.1) and anthocyanin (*m/z* 463.2), as analyzed by the LC-MS method. The catechin content was important in corroborating antioxidant activity. Studies of the in *vitro* cytotoxic activity of the crude ethanol extracts of *C. rotundus* against the NCI-H187 cell line showed IC_{50} values of 42.47±2.66 µg/ml. In addition, the crude ethanol extract of *Kaempferia parviflora* against KB and NCI-H 187 cell lines showed IC_{50} values of 60.24±1.73 and 27.22±1.04 µg/ml, respectively. These results indicated that the traditional medicinal plants *C. rotundus* and *K. parviflora* are excellent sources of antioxidants and potential cytotoxic agents against KB and NCI-H 187 cell lines.

Keywords: Antioxidant; Cytotoxic; *Cyperus Rotundus; Kaempferia Parviflora*

Introduction

In Thailand, medicinal herbs or plants have been used for the curing of substantial diseases for many decades. These plants are highly efficacious, inexpensive, safe and readily available [1]. They comprise many phytochemical constituents, which are known to be biologically active compounds and liable to present various pharmacological activities. The bioactive secondary metabolites of plants are significant sources of natural antioxidants [2]. Antioxidants are substances that can inhibit the oxidation of a substrate, or slow damage to cells caused by free radicals even at low concentrations. Antioxidants are separated into two clusters based on their mechanism of action, preventing antioxidants or chain breaking by chain propagation. Antioxidant

systems can protect against the uncontrolled formation of free radicals, motivate oxygen species or inhibit their reactions with biological formations [1]. Free radicals is originated as by-products of biological reactions or from exogenous factors. Phenolic compounds are plant secondary metabolites that can resist the oxidation of low-density lipoprotein (LDL) due to their antioxidant properties, which are imparted by the constituent hydroxyl groups that scavenge free radicals. Phenolic compounds possess key pharmacological activities such as antioxidant, anti-carcinogenic and anti-diabetic. Flavonoids are a group of more than 4,000 polyphenolic compounds among plant secondary metabolites which have properties comprised the free radical scavenging, anti-inflammatory action and inhibition of hydrolysis [1-2].

Cancer is a major global public health concern. Chemotherapy has many side effects, ranging from nausea to bone marrow damage and the development of multidrug resistance (MDR). So, the components of plants may supply alternative cancer treatments. Isolated natural products may function as pharmacologically active compounds against cancer. Therefore, the identification of modern natural products that overwhelm preferable efficacy against cancer, but hardly harmful effects has become desirable. Some of the compounds that have already been isolated from plants include vincristine, vinblastine and paclitaxel. The research on plant products encouraged the development of cancer drugs [3]. Although it is possible to chemically synthesize the flavonoid compounds, the use of toxic chemical solvents limited the synthesis of components. On the other hand, LC-MS was a major role in biochemistry and could compete with conventional liquid chromatography and other techniques such as immunoassay [4].

Objectives

The aim of this study was to analyze the antioxidant content, total phenolic, total flavonoid and cytotoxicity that identified the antioxidant components of crude extracts from eight species of Thai local plants using different solvents.

Methods

1. Preparation of crude extracts

Eight plant species, ground into powder, were purchased from pharmacy shops in Thailand (Table 1). Five hundred grams of plant powder were macerated with 95% ethanol and hexane solvents for 3 days at room temperature (37^{o}C). The extract solutions were sieved over Whatman filter paper No.1. The filtrates were then concentrated by evaporation (R-200 rotary evaporator; Büchi, Flawil, Switzerland) and frozen in a freezer (Cool Safe; Labogene, Lynge, Denmark). The percentage yields of crude extract powders were calculated by the following equation:

$$\text{Percentage yield} = [\text{Extract weight} / \text{Dried plant weight}] \times 100$$

Table 1 Thai local plants used in this study.

Botanical names	Common names	Plant part
Kaempferia parviflora	kra-chai-dam.	rhizome
Camellia sinensis	Green tea	leaves
Cyperus rotundus	Nut grass	rhizome
Hesperethusacrenulata	Tanaka	stem
Houttuyniacordata.	PluKaow	leaves
Morindacitrifolia	Baiyo	leaves
Thunbergialaurifolia	Rangjued	vine
Tiliacoratriandra	Thaoyanang	leaves

2. Determination of Antioxidant activity

2.1 Phenolic Content (TPC)

Total phenolic content was examined by Folin-Ciocalteu colorimetric method with trivial modification [5]. The 1000 ppm concentration was used for the analysis of all the samples. The reaction mixtures were prepared by mixing 0.05 ml extract sample and added to a 15 ml tube containing 0.95 ml of deionized water; 5 ml of 10% Folin-Ciocalteu was added to the mixture followed by 4 ml of 7.5% Na_2CO_3. Afterward, the mixtures were matured at room temperature for 30 min and their absorbance was measured at 725 nm using a spectrophotometer. The TPC is shown in terms of gallic acid equivalents (mg of GAE/g dry extract) using the following equation derided on the calibration curve:

$$Y = 0.0002x+0.0211;\ R^2 = 0.9995$$

where x was the absorbance and Y was the mg GAE/g dry extract.

2.2 Total Flavonoid Content (TFC)

Total flavonoid contents were defined by the colorimetric method [6]. Aliquots (0.5 ml) of dilute extracts were pipetted into a 1.5 ml tube containing 2 ml of deionized water and mixed with 0.15 ml of 5% $NaNO_2$. After 5 min, 0.15 ml of a 10% AlCl3.6H2O solution was added and the mixture was permitted to place for another 5 min. Then, 1 ml of 1 M NaOH was added. The interaction solutions were merged and the absorbance was determined at 415 nm. The experiment was tripled. TFC was shown in the form of estimated quercetin (mg QE / g of the extract) using the following equation, according to the calibration graph:

$$Y = 0.005x + 0.0012;\ R^2 = 0.9987$$

where x is light absorption and Y is mg QE / g in dry extraction.

2.3 DPPH-radical-scavenging activity

The antioxidant activity of all extracted samples was invented using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals based on the brands-Williams and Al [7] with slight modification. In this assay, a reaction

mixture containing the 1000 ppm stock sample solution was prepared and diluted to obtain final concentrations of 300, 500, 700, 900 and 1000 ppm in methanol. The 1.5 ml of sample solution was added to 1.5 ml of a 0.2 mM DPPH solution, and the resulting solution was then mixed by vertex. Samples were stored at room temperature for 30 min, then the absorbance was recorded at 517 nm using a UV-VIS recording spectrophotometer (UV-2401PC, Shimadzu). Deionized water was used as a reference. Trolox (Sigma®, St. Louis, MO, USA) was used as standards. The scavenging activity of DPPH radicals was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(Abs_c - Abs_s)/Abs_c] \times 100$$

Where Abs_s was the absorbance of extract sample and Abs_c was the absorbance of control

2.4 ABTS radical scavenging activity

The total antioxidant capacity of plant extracts was carried out using the thiazoline-6-sulfphonic acid diammonium salt (ABTS) radical cation method [8]. ABTS solution (3.0 ml, absorbance of 0.700) was used with 1 ml of each extract and mixed completely. The reaction composition stayed in the dark at room temperature for 6 min, and then the absorbance was noted at 734 nm using the UV visible spectrophotometer. Trolox is a positive control, ethanol is the negative control, and the extract without ABTS served as the blank. The ABTS radicals scavenging activity was calculated using the following equation:

$$\text{ABTS scavenging effect (\%)} = [(Abs_c - Abs_s)/Abs_c] \times 100$$

Where Abs_s is the sample absorbance and Abs_c is the negative control absorbance

3. Intention of LC-MS analysis

Liquid chromatography-mass spectrometry (LC-MS) is now a routine technique with the development of electrospray ionization (ESI) providing a simple and robust interface. It can be applied to a wide range of biological molecules and the use of tandem MS and secure isotope internal standards allows extremely sensitive and accurate analysis [4]. The eight ethanol crude extracts were identified antioxidant composition using Qtrap LC-MS (Dionex ultimate 3000, Bruker Amazon SL) and column: ACE 3 C18- AR (150 x 3.0 I.D.; 5 µm). The mass spectrometer is set up for negative ESI. The MS parameter were capillary voltages 4500 V, drying gas temperature 220^{o}C, drying gas flow rates 7.0 L/min, and nebulizer gas pressure 2.00 bar. The mass scan began at 70 to 2,000 m/z. In addition, the mobile phase consisted of 1% acetic acid (A) and acetonitrile (B) and the gradient elution for each sample was as follows: (0 min) 5% acetonitrile, (40min) 20% acetonitrile, (45 min) 35% acetonitrile, (50 min) 50% acetonitrile, (60-70min) 75% acetonitrile, (75-95min) 80%- acetonitrile, and (100-120 min) 5% acetonitrile. Elution was carried out at a flow rate of 0.3 ml/min. The injection volume was 10 µl.

4. Determination of cytotoxic activity

Cytotoxicity against the NCI-H187 cell line was measured by using the Resazurin microplate assay (REMA) [7]. The NCI-H187 cell line (ATCC CRL-5804) comes from small-cell lung carcinoma. To each well 5

µl of test compound and 45 µl of cell suspension were added. The plate was then embraced at 37 °C in a damped incubator with 5% CO_2 for 5 days. After that, 12.5 µl of 0.0625 mg/ml resazurin solution was added to each well and then incubated at 37 °C for 4 hours before measuring the absorbance at 530 nm and 590 nm. Ellipticine and doxorubicin were positive control and 0.5% DMSO was a negative control. The percentage of cytotoxicity was intended by the following equation:

$$\% \text{ Cytotoxicity} = [1-(FU_T/FU_C)] \times 100$$

Where FU_T and FU_C are the mean fluorescent unit from cells treated with the test compound and 0.5%DMSO, respectively

4.1 Cytotoxicity against the KB cell line by using the Resazurin microplate assay (REMA) [8]

KB cell line (ATCC CCL-17) was obtained from epidermoid carcinoma of the oral cavity. In brief, to each well 5 µl of test component and 45 µl of cell suspension were added. The plate was then matured at 37 °C in a damped incubator with 5% CO_2 for 3 days. Subsequently, the resazurin solution (12.5 µl of 0.0625 mg/ml) was added to each bore and the plate was incubated at 37 °C for 4 hours. Fluorescence was measured at 530 nm excitation and 590 nm emission wavelengths by using the bottom-reading mode of the fluorimeters. The sign was deducted with the blank before calculation. Ellipticine and doxorubicin were used as positive control and 0.5% DMSO was a negative control. The percentage of cytotoxicity was calculated by the following equation:

$$\% \text{ Cytotoxicity} = [1-(FU_T/ FU_C)] \times 100$$

Where FU_T and FU_C are the mean fluorescent unit from cells treated with the test compound and 0.5% DMSO, respectively.

4.2 Cytotoxicity against the Vero cell line by Green fluorescent protein (GFP) detection [9]

The African green monkey kidney cell line (Vero, ATCC CCL-81) with plasmid carried gfp gene (pEGFP-N1, Clontech) was generated as a GFP-expressing Vero cell line. The cell line was collected in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2.2 g / L sodium bicarbonate and 0.8 mg/ml geneticin, and fermented at 37 °C. It was then incubated under 5% CO_2 conditions. A cell suspension concentration of 3.3x104 cells/ml in 5 µl was diluted with 45 µl of 5% DMSO in a microplate and matured for 4 days at 37°C with 5% CO_2. Fluorescence was measured in a base-reading manner with excitation and emission wavelengths of 485 and 535 nm. Ellipticine was a positive control. The fluorescent signal of day 4 was deducted with that of day 0 ahead of calculation.

$$\% \text{ Cytotoxicity} = [1-(FU_T/ FU_C)] \times 100$$

Where FU_T and FU_C are the mean fluorescent unit from cells treated with the test compound and 0.5% DMSO, respectively.

5. Statistical analysis

The sample was diagnosed one by one in triplicate and the information was shown as mean ± S.D. Analysis of variance (ANOVA) with the Duncan's test presented remarkable differences at the 95% confidential level ($p < 0.05$) using the statistical software SPSS version 22.

Results

1. Extraction yield

Table 2 presents the yield of sixteen crude extracts with ethanol and hexane. The CSE gave the highest percentage yield of 2.093±0.138. While, the HCRH showed the lowest percentage yield. For all plants, ethanol indicated major higher percentage yields compared with hexane because it is non-polar minor components (quinines tannins, and terpenes) from the plants. The efficient distillation of plants depends on the solvent, time and size fragment of the plant. However, the extraction yields and resulting physicochemical properties of plant extracts are stably based on the nature of the solvent, the extraction method, extraction pressure, temperature and pH, which can affect the solubility of the solute to be extracted [10].

Table 2 The yields of eight crude extracts with ethanol and hexane solvents.

sample	Extraction solvents	Sample code	% yield
Kaempferia parviflora	Ethanol	KPE	2.038±0.089^{h}
Kaempferia parviflora	Hexane	KPH	0.888±0.028^{c}
Camellia sinensis	Ethanol	CSE	2.093±0.138^{h}
Camellia sinensis	Hexane	CSH	1.461±0.043^{f}
Cyperus rotundus	Ethanol	CRE	0.852±0.068^{c}
Cyperus rotundus	Hexane	CRH	0.607±0.042^{b}
Hesperethusacrenulata	Ethanol	HCRE	0.545±0.011^{b}
Hesperethusacrenulata	Hexane	HCRH	0.328±0.019^{a}
Houttuyniacordata.	Ethanol	HCE	1.475±0.032^{f}
Houttuyniacordata.	Hexane	HCH	1.364±0.013^{f}
Morindacitrifolia	Ethanol	MCE	1.720±0.017^{g}
Morindacitrifolia	Hexane	MCH	0.980± 0.028^{d}
Thunbergialaurifolia	Ethanol	TLE	1.013±0.011^{d}
Thunbergialaurifolia	Hexane	TLH	0.501±0.018^{f}
Tiliacoratriandra	Ethanol	TTE	1.579±0.065^{f}
Tiliacoratriandra	Hexane	TTH	0.849 ±0.11^{c}

Remark: 1. Data expressed as means ± standard deviations of three independent extractions (n = 3)

2. a-f Different characters in the same column demonstrate significant differences ($p < 0.05$)

2. Antioxidant activity

2.1 Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Phenolic compounds have radical antioxidant activity associated with their ability to dispose of free radicals, and chelate metals. The antioxidant activity will decrease oxidized intermediates in the chain reaction [11]. Total Phenolic matter was determined by comparing with standard gallic acid. The effects of total phenolic components for sixteen crude extracts ranged from 1.550±0.173 to 11.617±0.289 mg GAE/g dry extract (Table 3). The highest phenolic contents were detected in CRE, which showed values of 11.617±0.289 mg GAE/g dry extract. In addition, the phenolic composition of HCE exhibited a value of 11.283±0.76 4mg GAE/g dry extract, which is higher than the report of Tian *et al*. [12] showed the value to be only 0.024 ±0.44 mg GAE/g dry extract. In this present study, most of the plants extracted with ethanol had phenolic contents higher than those of the hexane extract due to the property in solubility of phenol in organic solvents with high polarity. The phenolic extraction in plants always yields a mixture of various classes of phenols [13]. Furthermore, our results showed CRE had the highest phenolic content because it contains higher amounts of reducing agents than observed in other sample extracts. Therefore, electron donors of CRE could react with free radicals to change them into stable products and stop the free radical chain reactions. Many plants may have phenolic compounds that exhibit antioxidant activity because their hydroxyl groups can scavenge ability. Besides, the phenolic compounds in plants may have various molecular structures depending on the species. So, the efficient procedure for the extraction of antioxidants from various plant species may be different. The electron-donated substituents in the ring structure represented the antioxidant potential of phenolic compounds due to the amount and arrangement of the hydroxyl groups. In agreement with other research indicated that higher antioxidant activity is correlated with higher phenolic compounds [5].

Results of total flavonoid content (TFC) (Table 3) showed that the TFC of sixteen crude extracts varied considerably from 2.462±0.154 - 7.973±0.231 mg in names as quercetin equivalents/mg of dry extract. Most plants extracted with ethanol had a higher total flavonoid content than plants extracted with hexane. Because the structure of flavonoids, contains –OH and –COOH functional groups, they are extracted by the polar solvent in the sample [14]. In 2009, Bruijn *et al*. [15] reported that a positive relationship was found between the flavonoid contents and solvent correlative polarity, so flavonoids are rather polar compounds. The highest total flavonoid content was found in CRE and exhibited the values of 7.973±0.231 mg QE/g dry extract; this was higher than the total flavonoid components in ethanol extracts of *C. rotundus* (6.44-13.77 mg CE/g of dry matter). Krishna and Renu [16] showed that the TFC of ethanol extracts of *C. rotundus* was 1.96 mg/g.d.w. HCE showed the values of 6.062±0.154 mg QE/g dry extract, which was the same as presented by Chen *et al*. In addition, Chen *et al*. [14] reported the result of an *in vitro* antioxidant test which indicated that the flavonoids in *Houttuynia cordata* had a sturdy deducting ability and the ability to reduce was comparable to vitamin C with identical mass concentrations. Flavonoids in *Houttuynia cordata* exhibit weak superoxide anion radical

scavenging ability; on the contrary, the hydroxyl radical driving ability of flavonoids is stronger [31]. In addition, KPE showed values of 4.240 mg QE/g dry extract, which was more than the result of Vichitphan *et al*. [17] that reported the flavonoid content of *K. parviflora* wine (0.24 mg/ml). TTE showed a total flavonoid content value of 3.751±0.204mg QE/g dry extract, which was more than the water extracts of *Tiliacora triandra* (2.01±0.073 mg QE/g dry extract). Moreover, the TFC values of plants may vary according to the solvent used, as different phenolic compounds may be dissolved to variable degrees in different solvents. However, the activity of antioxidants may be occurring from phenolic acids, phenolic diterpenes, volatile oils and flavonoids [11].

Table 3 Total phenolic and total flavonoid contents of sixteen crude extracts.

Sample	Sample code	Total phenolic (mg GAE/g dry extract)	Total flavonoid (mg QE/g dry extract)
Kaempferia parviflora	KPE	4.482±0.055^{f}	4.240±0.000^{h}
Kaempferia parviflora	KPH	4.617±0.577^{f}	3.129±0.308^{d}
Camellia sinensis	CSE	4.417±0.058^{f}	3.266±0.080^{e}
Camellia sinensis	CSH	3.467±0.029^{d,e}	4.062±0.385^{g}
Cyperus rotundus	CRE	11.617±0.289^{j}	7.973±0.231^{l}
Cyperus rotundus	CRH	5.300±0.115^{g}	4.507±0.133$^{h}_{i}$
Hesperethusa crenulata	HCRE	3.448±0.003^{d,e}	3.266±0.080^{e}
Hesperethusa crenulata	HCRH	3.415±0.061^{d,e}	3.784±0.069^{f}
Houttuynia cordata	HCE	11.283±0.764^{j}	6.062±0.154^{k}
Houttuynia cordata	HCH	1.550±0.173^{a}	2.462±0.154^{a}
Morinda citrifolia	MCE	10.650±0.265^{i}	4.906±0.220^{j}
Morinda citrifolia	MCH	2.883±0.115^{b,c}	2.951±0.077^{c}
Thunbergial aurifolia	TLE	10.117±0.764^{h}	4.596±0.278^{i}
Thunbergial aurifolia	TLH	3.217±0.252^{c,d}	2.907±0.231^{c}
Tiliacora triandra	TTE	3.848±0.176^{e}	3.751±0.204^{f}
Tiliacora triandra	TTH	2.467±0.029^{b}	2.642±0.003^{b}

Remark: 1. Data expressed as means ± standard deviations of three independent extractions (n = 3).

2. a-f Different characters in the same column demonstrate significant differences (p < 0.05).

2.2 Antioxidant activity

Radical scavengers were assessed by their reactivity regarding constant free radical DPPH and ABTS. The DPPH and ABTS are normally used to determine the total antioxidative condition of many biological samples due to their constant reproducibility and relief of quality control [15]. DPPH assay is complex due to

analyzing substrates, mixtures of dozens of compounds with various functional groups, polarities, and chemical behaviors. Additionally, DPPH is a strong free radical and its spare electron delocalizes over the whole molecule. The free radical scavenging activity of ABTS represented that its mechanism of execution was as a hydrogen donor and it terminated the oxidation procedure by converting free radicals to more stable products. ABTS is appropriate for lipophilic systems in that the antioxidant is added to the preformed radical cation produced by the one-electron oxidation of ABTS. The effect of the antioxidant on ABTS is reflected to be due to the methoxy groups [18]. The IC_{50} of a compound is inversely related to its antioxidant capacity, as it expresses the quantity of antioxidants required to decrease the DPPH and ABTS concentrations by 50%. Lower values of IC_{50} show that the extract has higher antioxidant activity. The reducing capacity of substances evaluates the ability to donate hydrogen atoms and interfere with the free radical chain reaction [19]. In this study, CRE exhibited the highest free radical scavenging with IC_{50} values of 0.404±0.0071 and 0.408±0.0046 mg/ ml for the ABTS and DPPH assays, respectively. This is similar to the report of Kamala *et al*. [20] that presented the DPPH radical scavenging activity of methanol extracts of *C. rotundus* compared with ascorbic acid as the standard; the extract showed a scavenging activity of 65%, while at the same concentration, the ascorbic acid was 70%. This study presented that the proton–donating ability of extracts can serve as free radical scavengers. Moreover, the free radical scavenging of TTE was evidenced by the IC_{50} value of 0.438 ± 0.011 mg/ml in DPPH assay (Figure1). It was the same as the extracts of *Tiliacora triandra* by petroleum ether, dichloromethane, ethyl acetate, methanol and water which had IC_{50} values of 113.81+0.8542, 75.57+1.6791, 15.02+0.4654, 9.63+0.5628 and 16.19+0.4523 ppm for the DPPH assay, respectively. Besides, CRH and KPE are interesting items because of their high radical scavenging activity [21].

3. LC-MS analysis

The eight types of crude ethanol extracts were analyzed by LC-MS to determine the antioxidant composition. The results reported indicate that the six ethanol plant extracts were positively identified. The data of retention times (TR), relative peak area and Mass Spectra data of the antioxidant compounds found in the crude extracts are shown in Table 4, 5. For MS analysis the negative ion mode of ESI was selected, because it provided extensive structure information for most flavonoids and phenolic acids [6]. The results showed catechin and anthocyanin were detected in CRE at m/z~289.1 and m/z~463.2, which had a relative peak area of 0.195 and 5.461, respectively. Only rutin was detected in KPE and MCE from mass spectra at m/z 609.2 had a relative peak area of 0.005 and 1.65, respectively. CSE and HCE detected rutin (*m/z* 609.2) and anthocyanin (*m/z* 463.2) from mass spectra with a relative peak area of 3.128 and 1.38, respectively. Both rutin (*m/z* 609.3) and gallic acid (*m/z* 168.9) were detected in TTE from mass spectra and had a relative peak area of 0.155 and 0.02, respectively. However, the TLE and HCRE did not detect the antioxidant compounds.

The results indicated that CRE had the highest antioxidant activity due to catechin which is an effective scavenger. The catechins are promptly and widely metabolized and demonstrate their antioxidant activity in

vivo [8]. The potency of catechins as antioxidants can be inferred by their ability to inhibit the oxidation of low-density lipoprotein, and lower plasma cholesterol levels and platelet aggregation. Besides, anthocyanin had an effect to the amount of antioxidant compounds as our results showed CSE (1.38%), CRE (%5.461) and HCE (%12.786). These results showed high antioxidant activity. Catechins are classified as monomer flavan-3-ol [18]. Besides, anthocyanin, rutin and gallic acid were detected and are major phenolic compounds [2] that vary in quality and quantity between different plant species.

Table 4 Antioxidant composition of eight crude extracts with ethanol.

Sample	Compound name	$[M-H]^-$ *m/z*	TR (min)
KPE	Rutin	609.2	31.9
CSE	Rutin	609.2	31.9
	Anthocyanin	463.2	33.7
CRE	Catechin	289.1	23.0
	Anthocyanin	463.2	37.2
HCRE	ND	ND	ND
HCE	Rutin	609.2	31.9
	Anthocyanin	463.2	33.2
MCE	Rutin	609.2	31.7
TLE	ND	ND	ND
TTE	Rutin	609.3	31.8
	Gallic	168.9	55

Table 5 Relative peak area of eight crude extracts with ethanol.

Compound name	Relative peak area							
	KPE	CSE	CRE	HCRE	HCE	MCE	TLE	TLE
Catechin	-	-	0.195	-	-	-	-	-
Anthocyanin	-	1.38	5.461	-	12.786	-	-	-
Rutin	0.005	3.128	-	-	1.038	1.65	-	0.155
Gallic	-	-	-	-	-	-	-	0.02

4. Cytotoxic activity

In vitro, cytotoxicity activities of sixteen crude extracts were evaluated against human epidermoid carcinoma of the oral cavity (KB) and human small cell lung cancer (NCI-H187) cell lines by performing the Resazurin Microplate Assay (REMA) [8]. Cytotoxicity against the African green monkey kidney cell line (Vero cells) was detected using the Green Fluorescent Protein (GFP) based assay [9]. Ellipticine and Doxorubicilin

were included as reference substances. The result of cytotoxicity is present in Table 6. KPE and KPH indicated cytotoxicity against KB with IC_{50} values of 60.24±1.73 and 63.43±1.10 µg/ml, respectively. The other samples showed no cytotoxicity against the KB cell line. In 2009, Wanich and Yenjai [21] reported the structural modification of 5,7-dimethoxyflavone isolated from *K. parviflora* provided two nitro and seven amino derivatives. All compounds were measured for cytotoxicity against KB cell lines using the colorimetric method. Compound 2 (5,7-Dimethoxy-8-nitroflavone and Compound 3 (5,7-Dimethoxy-6-nitroflavone) showed no cytotoxicity against the KB cell line, which is weaker than our results (IC_{50} value of 60.24±1.73 µg/ml). Besides, KPE and KPH showed cytotoxicity against NCI-H 187 cell lines with IC_{50} values of 27.22 ±1.04 and 28.54 ± 0.96 µg/ml respectively. In addition, Wanich and Yenjai [21] reported their results of the biological evaluation of *K. parviflora* which suggested that the toxicity was possibly due to the presence of amino and hydroxyl groups, which are located at the ortho position. Hossain *et al*. [22] presented that *K. parviflora* showed strong cytotoxicity and caused cell death via an apoptotic pathway that involved loss of mitochondrial transmembrane potential. In addition, the ethanol extract of *K. parviflora* (5-100 mg/ml) suppressed HL-60 cell (Promyelocytic leukemia) growth and decreased viability in a dose- and time-dependent manner; the fifty-percent inhibitory concentrations (IC_{50}) at 24, 48 and 72 h were 25.5, 18.5 and 14.5 mg/ml, respectively. In contrast, Yenjai *et al*. [23] reported that nine flavonoids isolated from *K. parviflora* presented no cytotoxicity against KB (oral human epidermoid carcinoma), and NCI - H187 (human small cell lung cancer) cell lines, and showed weak cytotoxicity against HepG2 (liver hepatocellular carcinoma) and T47D (human ductal breast epithelial tumor) cell lines. Besides, *Kaempferia* includes chalcone derivatives, cyclohexane oxide derivatives, cinnamates, flavonoids, diterpenes, and monoterpenes. The potential use of the plants is for cancer treatment because their bioactive compounds presented high cytotoxic effects against some cancer cell lines [9]. In addition, HCRE and HCRH were significantly cytotoxic against NCI-H 187 with IC_{50} values of 25.12± 0.43 and 27.91± 0.75 and µg/ml, respectively, but exhibited potent toxicity. It has been previously reported that the ethanol extract of the bark powder of Thanaka (*Hesperethusa crenulata*) stem bark is cytotoxic against the human skin melanoma A-375 cell line, showing an IC_{50} value equal to 12.81±0.16 µg/ml [24]. According to the classification of the cytotoxicity for natural ingredients, *K. parviflora, Camellia sinensis, C. rotundus* and *Hesperethusa crenulata* extracts could be categorized as potentially cytotoxic (10 µg/ ml < IC_{50}< 100 µg/ml), and the *Houttuynia cordata* extract could be categorized as potentially harmful (100 µg/mL < IC_{50}< 1,000 µg/ml) to NCI-H 187 cells line. In addition, the extract of *K. parviflora* could be categorized as potentially cytotoxic to KB cell lines. In contrast, the extraction of *Morinda citrifolia, Thunbergial aurifolia and Tiliacora triandra* did not show cytotoxicity against any cell lines. On the other hand, it has cytotoxicity to NCI-H187-Small cell lung cancer. KB cell induced the interaction of oxidative stress in DNA. This process may lead to cellular death finally [25]. However, the reduced cytotoxicity of flavonoids may be attributed to their methylation. The cytotoxicity of phenolic derivatives was significantly affected by the position of the methyl group which plays an important role in cytotoxic activity [9]. In addition,

cytotoxicity against normal cells should be of concern. Therefore, cytotoxicity of these sixteen crude extracts was evaluated against Vero cells shown in Table 4., CRH, HCE, HCH, MCE, MCH, TLE, TLH, TTE and TTH exhibited no cytotoxicity against these cell lines. CSE and CSH showed IC_{50} values of 74.72±0.46 and 192.35±1.67 µg/ml against Vero cells which is about 2- and 5-fold lower toxicity than NCI-H187 cell lines, respectively. In the case of HCRE, it demonstrated an IC_{50} value of 138.12 ±0.98 µg/ml against Vero cells, which is about 6-fold lower toxicity than the NCI-H187 cell line. HCRH demonstrated an IC50 value of 140.71 ±1.71 µg/ml against Vero cells, which is about 5-fold lower toxicity than the NCI-H187 cell line. However, our results presented that *K. parviflora* showed cytotoxicity against KB and NCI-H 187 cell line and *Camellia sinensis, C. rotundus* and *Houttuynia cordata* showed cytotoxicity against NCI-H 187 cell lines. In contrast, the other crude extracts presented no cytotoxicity.

Table 6 Cytotoxicity of eight crude extracts with ethanol and hexane solvents*

Sample	**Cytotoxicity, IC_{50} (µg/ml)**		
	KB	**NCI-H187**	**Vero cell**
KPE	60.24±1.73	27.22 ±1.04	16.77±1.01
KPH	63.43±1.10	28.54 ± 0.96	20.85±0.53
CSE	Inactive[a]	36.71 ± 0.89	74.72±0.46
CSH	Inactive[a]	43.32±1.52	192.35±1.67
CRE	Inactive[a]	42.47± 2.66	23.10±1.86
CRH	Inactive[a]	36.48 ±0.76	Inactive[a]
HCRE	Inactive[a]	25.12±0.43	138.12±0.98
HCRH	Inactive[a]	27.91±0.75	140.71±1.71
HCE	Inactive[a]	125.76±1.21	Inactive[a]
HCH	Inactive[a]	128.98±1.89	Inactive[a]
MCE	Inactive[a]	Inactive[a]	Inactive[a]
MCH	Inactive[a]	Inactive[a]	Inactive[a]
TLE	Inactive[a]	Inactive[a]	Inactive[a]
TLH	Inactive[a]	Inactive[a]	Inactive[a]
TTE	Inactive[a]	Inactive[a]	Inactive[a]
TTH	Inactive[a]	Inactive[a]	Inactive[a]
Ellipticine	3.32±0.30	1.65±0.12	2.32±0.46
Doxorubicilin	0.62±0.176	0.88±0.09	-

Remark: 1. Data expressed as means ± standard deviations of three independent extractions (n = 3).

2. Inactive[a] at <50% Cytotoxicity

* concentration of crude extracts including 200, 66.67, 22.22, 7.41,2.47, 0.82 µg/ml

Conclusions and Discussion

The study indicated that CRE exhibited the highest level of antioxidant activity, as compared to the other plants. Besides, CRE had catechin and anthocyanin which are phenolic compounds. In addition, CRE showed cytotoxicity against KB cell lines. Moreover, KPE was cytotoxic against KB and NCI-H 187 cell lines. Therefore, it can be concluded that *C. rotundus* and *K. parviflora* are potential sources of natural antioxidants and cytotoxic compounds that can play an important role as therapeutic agents and age associated oxidative stress related degenerative diseases and reduce the risk of chronic diseases, cancer and heart disease.

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