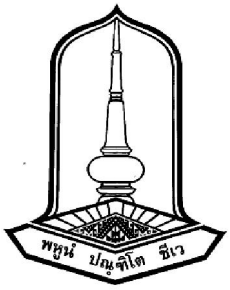

# ISOLATION AND BIOACTIVITY DETERMINATIONS OF PHENOLIC COMPOUNDS FROM *GYNURA PROCUMBENS* LEAVES

**NIWAT KAEWSEEJAN**

**A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Chemistry at Mahasarakham University**

**May 2015**



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**the degree of Doctor of Philosophy in Chemistry**
**at Mahasarakham University**
**May 2015**



The examining committee has unanimously approved this thesis, submitted by Mr. Niwat Kaewseejan, as a partial fulfillment of the requirements for the Doctor of Philosophy degree in Chemistry at Mahasarakham University.

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| | May 19, 2015 |

# ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the Office of the Higher Education Commission, Thailand for supporting via a scholarship under the Human Resource Development in Science Project (Science Achievement Scholarship of Thailand; SAST).

I would like to express my sincere gratitude and great appreciation to my advisors, Asst. Prof. Dr. Vallaya Sutthikhum and Assoc. Prof. Dr. Sirithon Siriamornpun, for their kind excellent supervision, inspiring guidance, encouragement and helpful discussion throughout this thesis.

I would like to acknowledge the Department of Chemistry, Faculty of Science and the Department of Food Technology and Nutrition, Faculty of Technology, Mahasarakham University for partially supports of chemicals, instruments and facilities. I am also wish to thank laboratory equipment center, Mahasarakham University for providing access to the HPLC instrument.

I am grateful to thank Assoc. Prof. Dr. Natthida Weerapreeyakul, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Khon Kaen University, Asst. Prof. Dr. Prapairat Seephonkai and Asst. Prof. Dr. Prasong Srihanam, Department of Chemistry, Faculty of Science, Mahasarakham University for their kind comments and supports as members of my thesis committee.

I would like to thank all of my friends for their help, encouragement, sincerity and impressive friendship.

Most of all, I wish to express my heartfelt gratitude here to my Kaewseejan family for their tender loves, definitely care, encouragement and constant support throughout my study. The usefulness of this thesis, I dedicate to my parents and all the teachers who have taught me since my childhood.

Niwat Kaewseejan

**ชื่อเรื่อง** การแยกและการศึกษาฤทธิ์ทางชีวภาพของสารประกอบฟีนอลจากใบแปะตำปึง
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## บทคัดย่อ

แปะตำปึงเป็นหนึ่งในพืชสมุนไพรที่มีความสำคัญสำหรับนำมาใช้เป็นยาแผนโบราณเพื่อรักษาโรคเรื้อรังต่าง ๆ เช่น เบาหวาน ความดันโลหิตสูง และมะเร็ง เป็นต้น นอกจากนี้ยังมีการนำมารับประทานเป็นอาหารเพื่อประโยชน์ต่อการเสริมสร้างสุขภาพ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการแยกและองค์ประกอบของสารประกอบฟีนอลจากใบแปะตำปึงที่มีศักยภาพในการต้านอนุมูลอิสระและต้านไกลเคชัน โดยเริ่มต้นทำการสกัดสารประกอบฟีนอลจากใบแปะตำปึงด้วยเอทานอล และจากนั้นนำสารสกัดหยาบเอทานอลมาสกัดแยกส่วนเป็นลำดับตามความมีขั้วของตัวทำละลายที่มีขั้วต่างกัน 3 ชนิด คือ คลอโรฟอร์ม เอทิลอะซิเตต และบิวทานอล นำสารสกัดที่ได้มาวิเคราะห์ปริมาณฟีนอลรวมและปริมาณฟลาโวนอยด์รวม และตรวจสอบฤทธิ์ในการต้านอนุมูลอิสระและต้านไกลเคชัน ผลการศึกษาพบว่าสารสกัดชั้นเอทิลอะซิเตต มีปริมาณฟีนอลรวมและปริมาณฟลาโวนอยด์รวมสูงสุด รวมทั้งมีฤทธิ์ในการต้านอนุมูลอิสระและฤทธิ์ต้านไกลเคชันสูงสุด เมื่อเปรียบเทียบกับสารสกัดอื่น ๆ นอกจากนี้สารสกัดชั้นเอทิลอะซิเตตยังมีประสิทธิภาพยับยั้งการเกิดเพอร์ออกซิเดชันของลิพิด และยับยั้งความเสียหายของโปรตีนที่ถูกเหนี่ยวนำด้วยอนุมูลไฮดรอกซิลจากปฏิกิริยาออกซิเดชันที่สูงกว่าสารต้านอนุมูลอิสระสังเคราะห์กรดแอสคอร์บิก จากการวิเคราะห์ชนิดและปริมาณของกรดฟีนอลและฟลาโวนอยด์ในแต่ละสารสกัดด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง พบว่าองค์ประกอบและปริมาณของกรดฟีนอลและฟลาโวนอยด์ในแต่ละสารสกัดแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) โดยเฉพาะอย่างยิ่งกรดฟีนอล ได้แก่ กรดแกลลิก กรด*พารา*-คูมาริก และเฟอรูลิก และฟลาโวนอยด์ ได้แก่ ไมริซิทิน เควอร์ซิทิน และแคมป์เฟอรอล เมื่อทำการแยกบริสุทธิ์สารสกัดชั้นเอทิลอะซิเตตผ่านคอลัมน์ Sephadex LH-20 โครมาโทกราฟี สามารถแยกสารประกอบฟีนอลได้ทั้งหมด 5 แฟรคชันย่อย คือ แฟรคชันย่อยที่ 1 ถึง 5 และทำการตรวจสอบปริมาณสารประกอบฟีนอลและฤทธิ์ในการต้านอนุมูลอิสระของแต่ละแฟรคชันย่อย พบว่าแฟรคชันย่อยที่ 3 มีฤทธิ์ในการต้านอนุมูลอิสระสูงสุด จากการตรวจวัดความสามารถในการรีดิวซ์ด้วยวิธี CUPRAC และ FRAP และความสามารถในการกำจัดอนุมูลอิสระด้วยวิธี DPPH และ ABTS ซึ่งมีประสิทธิภาพยับยั้งอนุมูล DPPH และ $ABTS^{+}$ ที่สูงกว่าสารต้านอนุมูลอิสระสังเคราะห์กรดแอสคอร์บิกและโทรล็อค ด้วยค่า $IC_{50}$ เท่ากับ 19 และ 12 µg/ml ตามลำดับ นอกจากนี้แฟรคชันย่อยที่ 3 ยังมีปริมาณสารฟีนอลรวมและฟลาโวนอยด์รวมสูงสุด เมื่อเปรียบเทียบกับแฟรคชันย่อยอื่น ๆ จากการศึกษาชนิดและปริมาณของกรดฟีนอลและฟลาโวนอยด์ในแฟรคชันย่อยที่ 3 พบว่ามีกรดเฟอรูลิกและกรดไซแนปิกเป็นกรดฟีนอลหลัก และไมริซิทินและแคมป์เฟอรอลเป็นฟลาโวนอยด์หลัก และจากการวิเคราะห์ความสัมพันธ์ระหว่างสารประกอบฟีนอลและฤทธิ์ในการต้านอนุมูลอิสระพบว่า ฤทธิ์ในการต้านอนุมูลอิสระมีความสัมพันธ์เชิงบวกที่สูงกับปริมาณฟลาโวนอยด์รวม แต่ไม่

พบความสัมพันธ์กับปริมาณฟีนอลรวม การศึกษานี้แสดงให้เห็นว่าประสิทธิภาพในการต้านอนุมูลอิสระที่ดีเยี่ยมของใบแปะตำปึงอาจมาจากฟลาโวนอยด์มากกว่ากรดฟีนอล โดยเฉพาะอย่างยิ่ง ไมริซิทินและแคมปเฟอร์รอล ซึ่งข้อมูลนี้อาจมีประโยชน์ในการศึกษาต่อยอดเพื่อพัฒนาเป็นผลิตภัณฑ์เสริมสุขภาพ

**คำสำคัญ:** ไมริซิทิน; แคมป์เฟอรอล; กรดฟีนอล; ฟลาโวนอยด์; การแยกส่วน; อาหารเพื่อสุขภาพ

measuring the radical scavenging activity based on DPPH and $ABTS^{+}$ methods. The $IC_{50}$ values of sub-fraction 3 were 19 and 12 g/ml, which were lower than those of ascorbic acid and trolox for DPPH and ABTS assays, respectively. The total phenolic content and total flavonoid content were also superior for sub-fraction 3 compared to the other sub-fractions. The major phenolic acids identified in sub-fraction 3 were ferulic acid and sinapic acid, whereas predominant flavonoids were myricetin and kaempferol. There was a strong positive correlation between antioxidant activity and total flavonoid content; however, no correlation existed between those and total phenolic content. These findings suggest that the excellent antioxidant capacity in *G. procumbens* leaves came from the flavonoids, especially myricetin and kaempferol, rather than phenolic acids which could be further investigated to develop as functional foods.

**Keywords:** Myricetin; Kaempferol; Phenolic acids; Flavonoids; Fractionation; Functional food

**TITLE** Isolation and Bioactivity Determinations of Phenolic Compounds from *Gynura procumbens* Leaves

**CANDIDATE** Mr. Niwat Kaewseejan

**DEGREE** Doctor of Philosophy degree in Chemistry

**ADVISORS** Asst. Prof. Vallaya Suttikhum, Ph.D.

Assoc. Prof. Sirithon Siriamornpun, Ph.D.

**UNIVERSITY** Mahasarakham University **YEAR** 2015

## ABSTRACT

*Gynura procumbens* is an important medicinal plant for using as traditional medicine to remedy the various chronic diseases, such as diabete, hypertension and cancer. It is also consumed as diet with health promoting benefits. The objectives of present study were to investigate the isolation and composition of phenolic compounds from *G. procumbens* leaves contributing to the potent antioxidant and anti-glycation activities. Firstly, the leaves of *G. procumbens* were extracted with absolute ethanol and then sequentially fractionated according to sovent polarity of three types including chloroform, ethyl acetate and *n*-butanol. The crude ethanolic extract and its fractions were investigated for their contents of phenolic and flavonoid as well as antioxidant and anti-glycation activities. The results showed that the ethyl acetate fraction (EAF) possessed the highest total phenolic and total flavonoid contents, along with the best antioxidant and anti-glycation activities compared to other fractions. Morover, this fraction exhibited superior values for lipid peroxidation inhibition and for protective effect against oxidative protein damage induced by hydroxyl radicals to the ascorbic acid and trolox, which are synthetic antioxidants. The composition and content of phenolic compounds in each fraction as determined using HPLC were significantly different ($p < 0.05$), especially gallic acid, *p*-coumaric acid and ferulic acid for phenolic acids, and myricetin, quercetin and kaempferol for flavonoids. The EAF was further separated into five sub-fractions (sub-fractions 1–5) by Sephadex LH-20 chromatography and their antioxidant activities were investigated. Among all sub-fractions, sub-fraction 3 had the highest antioxidant activities, which were determined by measuring the redecing power based on the CUPRAC and FRAP assays and by

# CONTENTS

**PAGE**



**PAGE**



**PAGE**



**PAGE**

# LIST OF TABLES

**PAGE**



**PAGE**



# LIST OF FIGURES

**PAGE**

**PAGE**



# CHAPTER I

# INTRODUCTION

## 1.1 Background and rationale

In the past few years, interest in the health care of humans has increased significantly, as a result of their lifestyle in the current time have increased the risk of many chronic ailments, such as cancer, diabetes, hypertension and cardiovascular diseases. In addition, therapy with modern medicine sometimes cannot cure such diseases, and also causes unwanted side effects. Thus, natural products from plants have an important role to play in the remedy of diseases, and are widely used in various regions, especially in Asia (Bodeker, 2000). Several studies have reported that consumption of various kinds of vegetables, fruits and medicinal plants provides excellent health benefits and is positively associated with a reduced risk of several diseases described above (Deng *et al.*, 2013; Kaisoon *et al.*, 2011; Khanam *et al.*, 2012; Shahidi and Zhong, 2010). These health benefits are attributed to the antioxidant potency derived from the various types of antioxidant compounds in plants, particularly flavonoids and other phenolics (Deng *et al.*, 2013; Khanam *et al.*, 2012; Pandey and Rizvi, 2009; Shahidi and Chandrasekara, 2013). Thus, interest in the investigation of antioxidant compounds from vegetables, fruits and medicinal plants has increased significantly in recent years, which is due to their health benefits when they are part of the diet as food or when used as pharmaceutical products (Escobedo-Avellaneda *et al.*, 2014; Deng *et al.*, 2013; John and Shahidi, 2010; Kaisoon *et al.*, 2011).

*Gynura procumbens* is an important plant in tropical regions, especially Southeast Asia such as Malaysia, Indonesia and Thailand, which has long been used as a vegetable and a medicinal plant. As a traditional medicine, the leaves of this plant are used for the treatment of many diseases that are caused by oxidative stress, such as inflammation, cancer, diabetes and hypertension as well as other general diseases (Perry, 1980). As part of food, this plant has long been consumed in different ways among various cultures. In Malaysia, the fresh leaves are usually consumed as a raw vegetable with rice and used as an ingredient in dishes, such as salads and ulams

(Hew and Gam, 2011). In Thailand, the leaves of *G. procumbens* are also consumed as a vegetable or used in cooking. It is commonly consumed raw or boiled and eaten with chili paste, or used to cook curries, as well as a garnish or ingredient in salads, soups and entrees. Currently, people in various tropical regions consume an increasing amount of *G. procumbens* leaves due to their believing that consumption of this vegetable can cure illness and diseases. The health benefits of the consuming *G. procumbens* are related to the amount of bioactive compounds found in this plant, such as saponins, flavonoids and terpenoids (Akowuah *et al.*, 2002). However, few studies have focused on the isolation, fractionation and identification of phenolic antioxidants from *G. procumbens* attributes to the excellent biological activities. The composition and content of the phenolic antioxidants are affected by the extraction method, which indicates that the extraction solvent and fractionation-based procedure should be considered when attempting to increase the amount of antioxidant compounds (Jun *et al.*, 2014). For this reason, the aim of the present study was to isolate phenolic antioxidants from *G. procumbens* leaves grown in Thailand, as well as to perform the fractionation and identification of bioactive compounds that contributes to the excellent biological activities. Furthermore, the correlations between the antioxidant and anti-glycation capacities and phenolic compounds were also investigated. There were then compared to those of synthetic antioxidants commonly used in the food industry, including butylated hydroxyanisole (BHA), trolox and ascorbic acid. This information will be useful for consumers who want to use this plant as a medicinal vegetable for health promotion and gain the benefits of reducing the risk of chronic diseases.

**1.2 Objectives of the research**

The objectives of this research can be summarized as follows;

1.2.1 To isolate and fractionate the phenolic compounds from *G. procumbens* leaves

1.2.2 To analyze the phenolic compositions of each fraction derived from *G. procumbens* leaves, as well as determine the contents of total phenolic and total flavonoid

1.2.3 To determine the biological activities including antioxidant and anti-glycation of phenolic compounds in each fraction derived from *G. procumbens* leaves

1.2.4 To analyze the correlation coefficients between the bioactive compounds with biological activities (antioxidant and anti-glycation) of *G. procumbens* leaves

**1.3 Expected results obtained from the research**

This research is expected to obtain results as follows;

1.3.1 The data of phenolic compositions of *G. procumbens* leaves fraction as determined by reverse phase - high performance liquid chromatography (RP-HPLC) analysis, as well as the content of phenolic and flavonoid compounds

1.3.2 The information about the antioxidant activity of phenolic compounds isolated from *G. procumbens* leaves, which was determined by measuring scavenging in $DPPH^{\bullet}$, $ABTS^{+\bullet}$, $^{\bullet}OH$ and $H_2O_2$, as well as linoleic acid peroxidation inhibition and protein damage protecting activities

1.3.3 The effect of anti-glycation activity of phenolic compounds isolated from *G. procumbens* leaves by anti- AGEs formation in BSA-glucose system assay

1.3.4 The information about the correlations between the levels of phenolic compounds with antioxidant and anti-glycation activities of *G. procumbens* leaves

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**1.4 Scopes of the research**

The scopes of this research have been classified into 4 main parts as follows;

1.4.1 Extraction, isolation and fractionation of phenolic compounds from *G. procumbens* leaves and determination of the total phenolic and flavonoid contents, as well as identification and quantification of phenolic compounds by HPLC

1.4.2 Evaluation of antiradical activity of phenolic compounds isolated from *G. procumbens* leaves using $DPPH^{\bullet}$, $ABTS^{+\bullet}$, $^{\bullet}OH$ and $H_2O_2$ scavenging activity assays, and the results were expressed as the percentage of free radicals scavenging and $IC_{50}$ values, including compare to the synthetic standard. In addition, the antioxidant activity

of phenolic compounds isolated from *G. procumbens* leaves was also evaluated using linoleic acid peroxidation inhibition and protein damage protecting activity assays

1.4.3 Evaluation of anti-glycation activity of phenolic compounds isolated from *G. procumbens* leaves using anti- AGEs formation in BSA-glucose system assay, and the results were expressed as percentage of AGEs formation inhibition and the $IC_{50}$ values

1.4.4 Investigation of correlations between the phenolic compounds with the biological activities (antioxidant and anti-gylcation) of *G. procumbens* using statistical option in the Pearson test

**1.5 Definition of terms**

1.5.1 Antioxidant is a molecule that directly or indirectly scavenges, inhibits and prevents free radicals and pro-oxidant, especially reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive sulfur species (RSS) and other substances, which occurred in both the endogenous and exogenous sources.

1.5.2 Advanced glycation end-products (AGEs) are heterogeneous end-products occurred from the process of glycation. AGEs, also known as glycotoxins, are diverse group of highly oxidant compounds with pathogenic significance in diabetic complications and microvascular complications such as nephropathy, arteriosclerosis, retinopathy, neuropathy and cataracts.

1.5.3 Glycation is reaction between the amino group of the body proteins and the aldehyde group of the reducing sugars (glucose and fructose), resulting in the formation of Amadori and AGEs products.

1.5.4 Free radical is a substance that has unpaired electrons and they are highly unstable and reactive with other molecules, especially protein, DNA and RNA. They derive from endogenous and exogenous sources including reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS).

1.5.5 Oxidative stress is an imbalance between the generation of ROS and the scavenging capacity of antioxidants in the reproductive tract.

1.5.6 Phenolic compound is an important substance group of secondary metabolites, which is synthesized by the metabolism in plants such as pentose

phosphate, shikimate and phenylpropanoid pathways. It is of considerable physiological and morphological importance in plants, which not only play an important role in growth and reproduction for plants, but also health benefits for human.

1.5.7 Pro-oxidant is chemical substance that induces oxidative stress, usually through the formation of reactive species or by inhibiting antioxidant systems.

1.5.8 Reactive oxygen species (ROS) are encompassing both free radical species bearing an unpaired electron and their non-radical intermediates. ROS are generated by cytosolic processes, but under normal conditions mitochondria are the principal source. ROS may also play a role in other reproductive organ diseases in human.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 *Gynura* genus

### 2.1.1 Botanical characteristics

*Gynura* is a small genus of plants belongs to the Asteraceae family. It composts of around 50 species of annual or perennial herbs or subshrubs and sometimes scandent shrubs. The plants in this genus are usually distributed in the various regions including Australia, Africa and Asia. The botanical characters of *Gynura* consist of a unique head-like feature of aggregating flowers which is a prominent feature of the Asteraceae. The leaves are alternate. The blades are entire, toothed, pinnatifid or pinnately parted. A solitary too many heads are discoid. Involucre bract is campanulate or cylindrical with several subtending bractlets and flat bracts. Corollas are yellow or orange to red with long, narrow funnel throat and triangular or ovate lobes. Another's are entire or minutely sagittate at the base. The style branceds are slender with long subulate pubescent appendages. Achene is terete with 5-10 ribbed. The pappus is copious white (Dassanayake and Fosberg, 1980).

The *Gynura* genus is distributed in many regions, especially tropical South and East Asia. The most diversity of plants in *Gynura* genus is found in Southeast Asia such as Malaysia, Indonesia and Vietnam, but the plants in this genus have been less understood particular in Thailand (Davies, 1981). In Thailand, all 5 species of *Gynura* are identified and found such as *G. erepidoides* Benth (Ya dok kham in Loei), *G. integrifolia* (Wan chaeng in Nakhon Sawan), *G. procumbens* or *G. sarmentosa* DC. (Pra kham dee khwai in Pattani), *G. pseudochina* (Phakkat kop in Phetchabun) and *G. pseudo-china* var. hispida Thv. (Wan mahakan in central) (Smitinand, 1980).

### 2.1.2 Ethnomedical uses

In general, the plants in the *Gynura* genus have long been commonly used as traditional medicine for treatment of various diseases. Certain species are ornamental due to the violet color of the leaves such as *G. aurantiaca* DC. (Velvet plant), *G. pseudochina* DC. Moreover, the leaves of *G. procumbens* and *G. formosana* have

also been used as vegetables. The ethanomedical uses of the plants in this genus are in Table 2.1. As shows in Table 2.1 demonstrated that the topical remedies for inflammation, pain and allergic conditions are generally recognized among nearly all the mentioned species. *G. procumbens* and *G. pseudochina* are used for medicinal purposes (Perry, 1980). In Thailand, the plants in *Gynura* genus have been used as traditional drugs for treatment of various skin diseases, especially herpes viral infection (Pongboonrod, 1979).

**Table 2.1** Ethnomedical uses of the medicinal plants in the *Gynura* genus.

| Species | Part used | Treatment |
|---|---|---|
| *G. aurantiaca* DC. | Leaf | Poultice for ringworm |
| *G. erepidioides* Benth | Leaf | Decoction for treatment stomach disease |
| | | Lotion for headache |
| *G. divaricata* DC. | Aerial | Decoction internally for bronchitis, pulmonary tuberculosis, pertussis, sore eye, toothache and rheumatic arthralgia |
| | Leaf | Macerate for externally treatment traumatic injury, fracture, wound bleeding, leg ulcer and burns |
| *G. formosana* Kitam | Leaf and root | Juice for externally treatment wound and snake bite |
| *G. sagetum* / | Root | Hemostat, vulnerary for bleeding, bruises |
| *G. pinnatifida* DC. / | | and furunculosis; dried, grated and cook |
| *G. japonica* J. | | with chicken in alcohol as tonic |
| | Aerial | Decocted or crushed in white wine for amenorrhea, epistaxis, hematimesis, |
| | Leaf | hemoptysis mastitis and tramatic injuries |
| | | For treatment of poisonous insect bite |

**Table 2.1** (Continued)

| Species | Part used | Treatment |
|---|---|---|
| *G. procumbens* (Lour.)/ *G. sarmentosa* DC. | Leaf | Rubbed with oil and mashed as a salve for rash<br>Poultice to relief pain, allergic response and inflammation and treatment of poisonous animal bite<br>Gargle for throat inflammation |
| *G. pseudochina* | Leaf | Gargle for throat inflammation |
| | Root | Remedy for uterine hemorrhages, dysentery and inflamed wounds; decocted as antipyretic |
| | Leaf and root | Poultice for inflammation, anti-inflammatory for herpes viral infection |

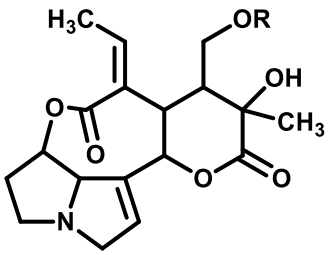
Source: Perry (1980); Pongboonrod (1979); Roeder *et al.* (1996)

2.1.3 Chemical composition and biological activity

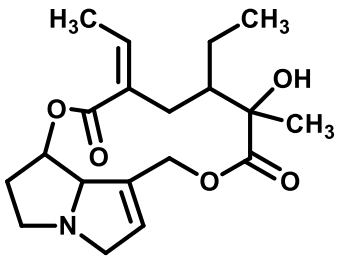
Research on chemical components in the *Gynura* genus has been limited. According to previous studies, the chemical compositions identified in *Gynura* genus have showed the contain of pyrrolizidine (Matheson and Robins, 1992; Roeder *et al.*, 1996), phytosterols (Sadikun *et al*., 1996), terpene-coumarins (Bohlmann and Zdero, 1997), steroids (Takahira *et al*., 1997), chromones and proanthocyanidins (Yoshitama *et al*., 1994). The chemical structures of some compounds are shown in Fig. 2.1-2.4. There are showing the pharmacological and biological activities. For example, *G. integrifolia* possesses the anti-inflammatory and antipyretic activities, while *G. segetum* shows the anesthetic activity (Xueshao and Xizhi, 1987).

Although the chemical studies on the *Gynura* genus are limited however the chemical profile of the Asteraceae family is well reported. Since many plants of this family possess medicinal properties, which is estimated valuable to identify the active compounds from various plants in this family. Recently, there are investigating the

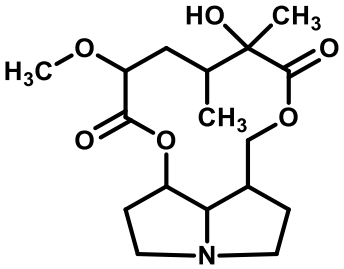
bioactive compounds in many *Gynura* plants and the results showed that these plants contains triterpene monols, diols, polyacetylenes, flavonols, flavones, caffeoyl esters, cyclitols, L-inositol and fatty acid oils. Essential oils and diterpenoids are also widely distributed in these plants. However, the compounds of alkaloids, amides, coumarins, cyanogenic glycosides and various types of phenolic compounds in *Gynura* genus are limited information. Polyacetylenes, sesquiterpene lactones and fructan polysaccharides are regarded as the chemical characters of the *Gynura* genus. The chemical component on the Senecioneae subfamily of the *Gynura* genus shows the presence of pyrrolizidine alkaloids. Previous study reported that *Senecio* species contain many phytochemicals including sitosterol, hydroxycinnamic acids, polyphenols, caffeoylquinic acids and other compounds. In addition, flavonoids such as flavone and flavonol are also commonly found in the Senecioneae subfamily (Heywood *et al.*, 1997).



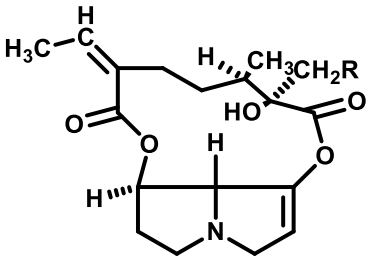

Gynuramine (R = H)
Acetylgynuramine (R = $COCH_3$)
in *Gynura scandenus*




Senecionine in *Gynura segetum*

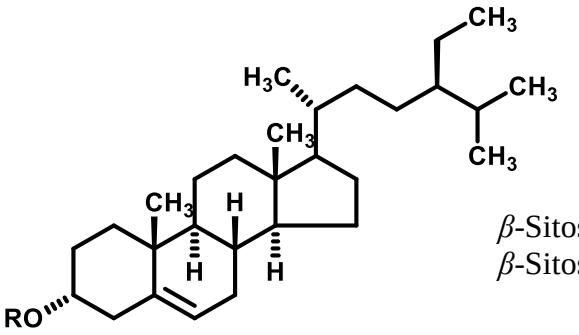



Otosenine in *Gynura sarmentosa*

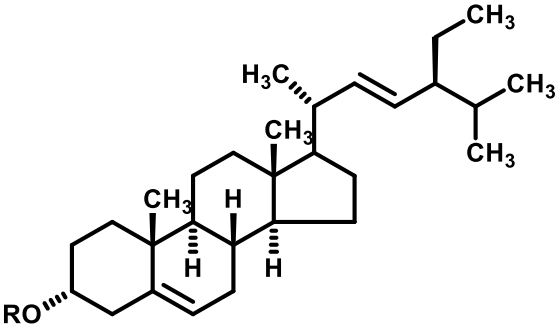



Intergerrimine (R = H)
Usaramine (R = OH)
in *Gynura divaricate*

**Fig. 2.1** Structures of some pyrrolizidine alkaloids found in the *Gynura* genus.

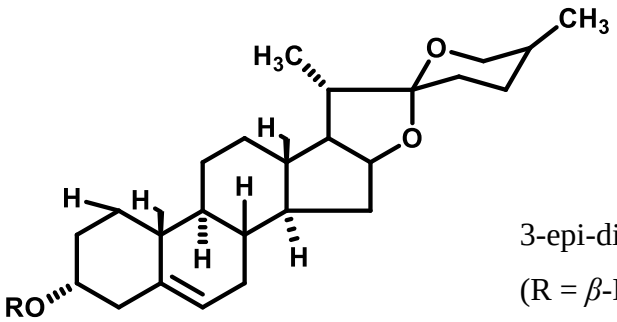


*β*-Sitosteryl glucoside (R = *β*-D-glucose)
*β*-Sitosterol (R= H) in *G. procumbens*

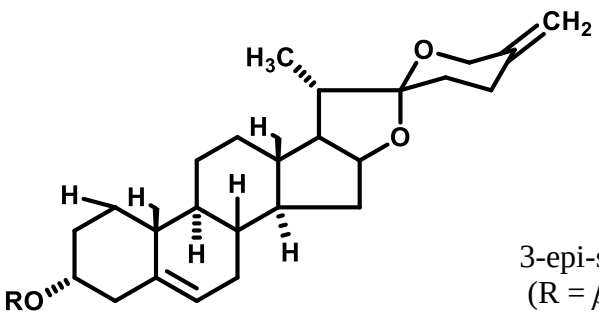


Stigmasteryl glucoside (R = *β*-D-glucose)
Stigmasterol (R= H) in *G. procumbens*

**Fig. 2.2** Structures of some phytosterols isolated in the *Gynura* genus.

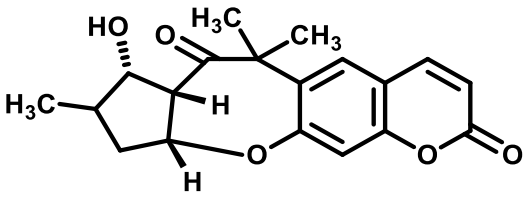


3-epi-diosginin-3-*β*-D-glucopyranoside
(R = *β*-D-glucose) in *G. japonica*

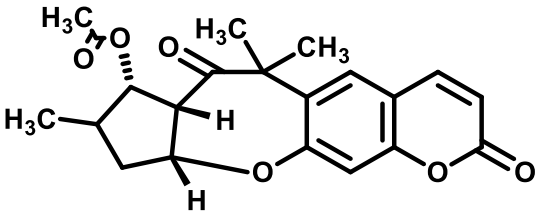


3-epi-sceptrumgenin-3-*β*-D glucopyranoside
(R = *β*-D-glucose) in *G. japonica*

**Fig. 2.3** Structures of some triterpene isolated in the *Gynura* genus.




Gynuron in *Gynura crepioides*




Acetyl gynurone in *Gynura crepioides*

**Fig. 2.4** Structures of some terpene-coumarins isolated in the *Gynura* genus.

## 2.2 *Gynura procumbens*

### 2.2.1 Botanical characteristics

*Gynura* is an important small genus of Asteraceae family and it consists of about 50 species of annual and perennial herbs and subshrubs. The plants are widely distributed in the many regions such as Asia, Africa, and Australia. *G. procumbens* is found in various parts of Asia and widely used in Thailand and Southeast Asia as a traditional medicine (Jarikasem, 2000). In addition, this plant has been also consumed as vegetable with health-promoting properties.

*G. procumbens* (Fig. 2.5) is a decumbent perennial herb; stem angular striate. Leaves spirally arranged; obovate, obovate-oblong or spathulate, 5-8 cm long, 2.5-3.5 cm wide, apex acute or obtuse, base attenuate, margin laxly, irregularly crenate or serrate, venation inconspicuous, pubescent on both surface; petiole about 1 cm long, pubescent. Inflorescences in heads, 2-7 in loose terminal corymbs; heads consists of more than 60 flowers, 1-1.5 cm long, 7-10 mm wide; peduncle short hairy unequal long; involucral bracts arranged in 2 series, linear, about 8 mm long, 1 mm wide, with

translucent margins, during anthesis cohering into a cylindrical tube outer ones 3-5 mm long, linear, free; corolla tubular, orange-yellow, about 8 mm long; stamens 5, style divided into 2 long arms, short hairy; achenes linear, 3-4 mm long, with pappus-hairs (Dassanayake and Fosberg, 1980).

**Fig. 2.5** One year old mature plant of *G. procumbens* (Chan *et al.*, 2009).

2.2.2 Ethnomedical uses

*G. procumbens* was traditionally used as anti-inflammatory remedy for skin rash and inching (Iskander *et al.*, 2002; Pongboonrod, 1979). In Thailand, the aerial part is used as a topical therapy for the treatment of inflammation, rheumatism and viral disease of the skin (Perry, 1980). In Indonesia, the aerial and some other parts were used to treat fevers, skin rashes and as a remedy for ringworm infection (Iskander *et al.*, 2002). In some part of Southeast Asia, the leaves of *G. procumbens* have been used as a remedy for kidney diseases, eruptive fevers, rash, hypertension, diabetes, mellitus and hyperlipidemia (Perry, 1980).

2.2.3 Chemical composition

Recent studies shows that the compounds have been isolated and identified from this plant including caffeoylquinic acids, flavonoids, phytosterols, phytosterol glucosides, glycoglycerolipids and ceramindes are presented in Table 2.2. The leaves of this plant are often consumed in diet for health benefits. Previous work has shown that

the leaves of *G. procumbens* does not have any toxic effects (Rosidah *et al.*, 2009) and the leaves also contain bioactive compounds such as flavonol, unsaturated sterols, terpenoids, polyphenols, essential oil, *β*-sitosterol and stigmasterol, kaempferol-3-*O*-rutinoside and quercetin (Jarikasem, 2000; Rosidah *et al.*, 2008).

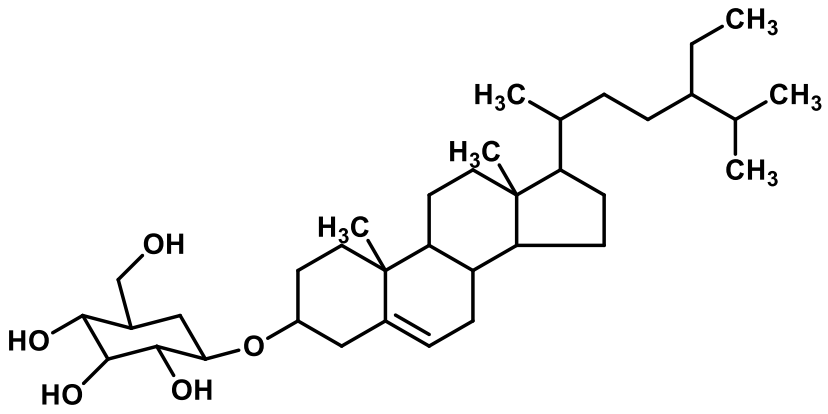
**Table 2.2** Phytochemicals isolated from *G. procumbens*.

| Group | Name |
|---|---|
| Caffeoylquinic acids | 3,5-di-*O*- caffeoylquinic acids |
| | 4,5-di-*O*- caffeoylquinic acids |
| | 5-*O*- caffeoylquinic acids (chlorogenic acid) |
| Flavonoids | Kaemferol-3-*O*-alpha-L-rhamnosyl (1→6) *β*-D-glucopyranoside |
| | Kaemferol-3-*O*-alpha-L-rhamnosyl(1→6)*β*-D-galactopyranoside |
| | 3-*O*-*β*-D-glucopyranosyl-4′,5,7-trihydroxyflovone |
| | 3-*O*-*β*-D-glucopyranosyl-5,7-3′,4′-tetrahydroxyflovone |
| | 3,5,7,4′-tetrahydroxyflovone (kaempferol) |
| Phytosterols | (22E,24S)-24α-ethlcholest-5,22-dien-3*β*-ol (Stigmasterol) |
| | (24S)-24*α*-ethlcholest-5-ene-3*β*-ol (Dihydrostigmasterol) |
| Phytosterol glucosides | 3-*O*-*β*-D-glucopyranosyl-22E,(24S)-24*α*-ethlcholest-5,22-diene |
| | 3-*O*-*β*-D-glucopyranosyl(24S)-24*α*-ethlcholest-5-ene |
| Glucoglycerolipids | 1,2-bis-dodecanoyl-3-*α*-D-glucopyranosyl-*sn*-glycerol |
| Ceramides | 1-(1′,5′dihydroxy-docosanyl)-2″-(2-hydroxy-tetracosanamide)-*sn*-glycerol |

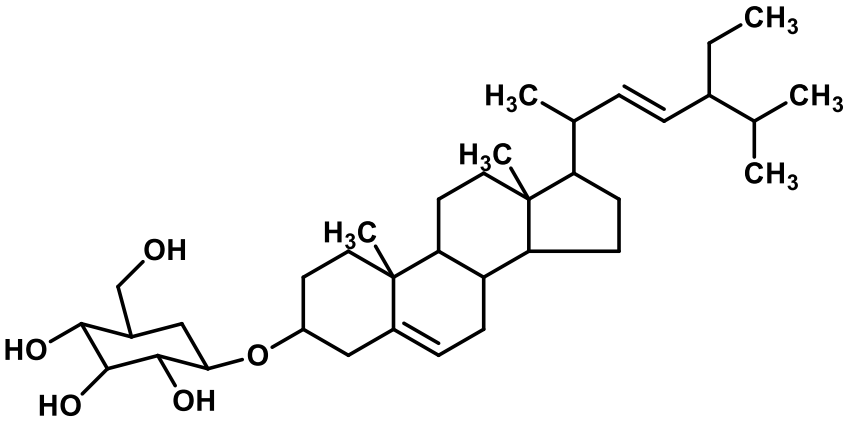
Source: Jarikasem (2000); Rosidah *et al.* (2008)

A 3-*O*-*β*-D-glucopyranosyl-22E,(24S)-24*α*-ethlcholest-5,22-diene and 3-*O*-*β*-D-glucopyranosyl (24S)-24*α*-ethlcholest-5-ene possessed strong antiviral activity. The two compounds differed only in the side chain by one double bond. The structure of stigmasterol-3-*O*-*β*-D-glucopyranoside and sitosteryl-3-*O*-*β*-D-glucopyranoside are shown in Fig. 2.6 (Jarikasem, 2000). The bioactive compounds of *G. procumbens* extracts including polyphenolics (kaempferol-3-*O*-*β*-D-robinobioside, kaempferol-3-*O*-

*β*-D-glucopyranoside), terpenoids (stigmasteryl-3-*O*-*β*-D- glucopyranoside, sitosteryl-3-*O*-*β*-D- glucoside, stigmasterol and *β*-sitosterol) and plant lipids (glycoglycerolipid and ceramide) (Amellal *et al.*, 1985; Ortege *et al.*, 1998; Kimura *et al.*, 1985; Peluso *et al.*, 1995). The bioactive compounds and biological activity are presented in Table 2.3.




3-*O*-*β*-D-glucopyranosyl (24S)-24*α*-ethlcholest-5-ene ($C_{35}H_{60}O_6$)




3-*O*-*β*-D-glucopyranosyl-22E,(24S)-24*α*-ethlcholest-5,22-diene ($C_{35}H_{58}O_6$)

**Fig. 2.6** Structure of stigmasterol-3-*O*-*β*-D-glucopyranoside and sitosteryl-3-*O*-*β*-D-glucopyranoside.

**Table 2.3** Bioactive components and their properties of *Gynura procumbens*.

| Compounds | Biological activities | | |
|---|---|---|---|
| | Anti-inflammatory | Anti-histamine | Anti-HSV |
| *Phenolics* | | | |
| - Chlorogenic acid | AC | AC | NR |
| - A mixture of 3,5- and 4,5-di-*O*-caffeoylquinic acid | AR | AR | AR |
| - Kaempferol-3-*O*-*β*-D-rutinoside | NR | NR | AC |
| - Kaempferol-3-*O*-*β*-D-robinobioside | NR | NR | IN |
| - Kaempferol-3-*O*-*β*-D-glucopyranoside | AC | Active | IN |
| - Kaempferol | AC | Active | IN |
| *Terpenoids* | | | |
| - Stigmasteryl-3-*O*-*β*-D- glucopyranoside | AC | NR | AC |
| - Sitosteryl-3-*O*-*β*-D- glucoside | AC | NR | AC |
| - Stigmasterol | AC | NR | AC |
| - *β*-sitosterol | AC | NR | AC |
| *Plant lipids* | | | |
| - Glycoglycerolipid | NR | NR | AC |
| - Ceramide | NR | NR | IN |

AC, active; IN, inactive; NR, no report

Source: Kimura *et al.* (1985); Ortege *et al.* (1998); Peluso *et al.* (1995)

2.2.4 Biological activities

2.2.4.1 Anti-inflammatory activity

According to previous study, the crude ethanolic extract of aerial part from *G. procumbens* possesses anti-inflammatory activity as determined using an ear inflammation model in mouse induced by croton oil. The administrations of crude ethanolic extract at various doses in mouse showed significantly inhibited by increasing

the ear thickness in response to croton oil. At a dose of 0.75 mg/ear extract, the anti-inflammatory activity (inhibition 65.2%) showed similar to that of hydrocortisone 21-hemisuccinate sodium salt (inhibition 64.8%) at a dose of 6 mg/ear (Iskander *et al.*, 2002; Jarikasem, 2000).

2.2.4.2 Antiviral activity

The crude ethanolic extract of the aerial of *G. procumbens* exhibited the antiviral activity against herpes simplex virus type 1 and herpes simplex virus type 2. A compound of 3,5-di-*O*- caffeoylquinic acids and 4,5-di-*O*- caffeoylquinic acids isolated from this plant possessed the virucidal activity against HSV-2 ($IC_{50}$ = 96.0 µg/ml) and antireplicative activity against HSV-2 ($IC_{50}$ = 61.0 µg/ml). A mixture of *β*-sitosterol and stigmasterol also showed the virucidal activity against HSV-2. In addition, and a mixture between *β*-sitosterol 3-*O*-*β*-D-glucopyranosyl and stigmasterol 3-*O*-*β*-D-glucopyranosyl showed the virucidal activity against HSV-2 ($IC_{50}$ of 50 µg/ml) and also exhibited pretreatment anti-HSV-1 and HSV-2 (Jarikasem, 2000). Recent research reported by Nawawi *et al.* (1999) who demonstrated that the aqueous and methanolic extracts of *G. procumbens* were grown in Indonesia also showed potent anti HSV-1 activity.

2.2.4.3 Antihyperglycaemic and antihyperlipidaemic activities

A previous study has showed that the ethanolic extract of *G. procumbens* leaves at different doses of 50, 150 and 300 mg/kg orally significantly suppressed the elevated serum glucose levels in diabetic rats. There was found to be the optimum dose of hypoglycaemic is 150 mg/kg. However, this extract did not significantly suppress the elevated serum glucose levels in normal rats, unlike glibenclamide. Metformin, but not glibenclamide improved glucose tolerance in the diabetic rats. When the optimum dose was given to diabetic rats for 7 days, the extract significantly reduced serum cholesterol and triglyceride levels in these rats. They results indicate that the leaves of *G. procumbens* may have biguanide-like activity (Zhang *et al.*, 2000).

2.2.4.4 Antihypertensive activity

An aqueous extracts of *G. procumbens* were orally administered to spontaneously hypertensive (SHR) rats for 4 weeks and antihypertensive effects were determined. Oral administration of 500 mg/kg of *G. procumbens* extract (GPE) resulted in significantly lower blood pressure in SHR rats compared with SHR rats not given

GPE. Furthermore, GPE-administered rats had significantly lower serum lactate dehydrogenase, creatine phosphate kinase, and increased nitric oxide (NO), a known vasodilator, compared with the non-GPE-administered SHR group. These results suggest that oral administration of aqueous GPE may be useful for prevention and treatment of hypertension through increasing NO production in blood vessels (Hoe *et al.*, 2006; Kim *et al.*, 2006).

2.2.4.5 Human lymphocyte activity

Sriwanthana *et al.* (2007) reported that an aqueous extract from leaves of *G. procumbens* at the concentrations of 1-100 µg/ml increased human lymphocyte proliferation *in vitro*.

2.2.4.6 Anticarcinogenic activity

The ethanol extract of *G. procumbens* leaves on 4 nitroquinoline 1-oxide (4NQO)-induced rat tongue carcinogenesis. Fifty six 4 week old male Sprague Dawley rats were used in this study and divided into 7 groups. Group 1, 2 and 3 were lingually induced by 4NQO for 8 weeks. In groups 2 and 3 the extract was given simultaneously with or after 4NQO induction finished, each for 10 weeks and 26 weeks, respectively. Groups 4, 5 and 6 were induced by 4NQO for 16 weeks. However, in groups 5 and 6 the extract was given as well simultaneously with or after the 4NQO induction, each for 18 weeks, respectively. Group 7 served as the as untreated control group. The results from microscopically assessment showed that tongue squamous cell carcinomas (SCC) developed in 100% (3/3) of group 1. However, only 33.3% (2/6) and 25% (2/8) of rats in groups 2 and 3, respectively demonstrated tongue SCC. Among groups 4, 5 and 6, no significant difference of tongue SCC incidence was observed. From these results it is apparent that the ethanol extract of *G. procumbens* leaves could inhibit the progression of 4NQO induced rat tongue carcinogenesis in the initiation phase (Agustina *et al.*, 2006).

2.2.4.7 Anti-ulcerogenic activity

*G. procumbens* ethanolic leaf extract (GPELE) was used to investigate its gastroprotective effect in adult Sprague dawley rats which were divided into six groups. The rats were orally pre-treated with carboxymethyl cellulose (CMC) solution (ulcer control groups), omeprazole 20 mg/kg (reference group), 50, 100, 200 and 400 mg/kg of GPELE in CMC solution (experimental groups), one hour before oral administration of

absolute ethanol to generate gastric mucosal injury. After an additional hour, the rats were sacrificed and the ulcer areas of the gastric walls were determined. The ulcer control group exhibited severe mucosal injury, whereas groups pre-treated with GPELE exhibited significant protection of gastric mucosal injury. These findings were also confirmed by histological studies. Acute toxicity study with a higher dose of 5 g/kg did not manifest any toxicological signs in rats. These results suggest that GPELE promotes ulcer protection as ascertained grossly by significant reduction of ulcer area, and histologically by comparatively decreases in ulcer areas, reduction or absence of edema and leucocytes infiltration of submucosal layer compared to ulcer control group (Mahmood *et al.*, 2010).

2.2.4.8 Antidiabetic activity

Bioassay guided fractionation has been carried out to identify the bioactive crude fraction responsible for antidiabetic activity of *G. procumbens*. Both *in vitro* and *in vivo* model study were used to evaluate the antidiabetic properties of this plant. *In vitro* insulin secretion study, glucose uptake study and cytotoxicity were used as primary assay on crude methanolic extract, hexane, ethyl acetate and butanol fractions. Cytotoxicity studies demonstrated that crude methanolic extract have the lowest cytotoxicity when compared with crude fractions *G. procumbens* in BRIN BD11 cell lines. Cytotoxicity study with adipocytes and muscle cell lines showed that the crude methanolic extract of *G. procumbens* have the lowest toxicity when compared with the crude fractions of the plant. Determination of insulin secretion response was done by using BRIN BD11 cell lines and from the result, it showed that crude hexane and ethyl acetate crude fractions have good potential in stimulation of insulin release. Glucose uptake study with adipocytes cell lines (3T3 mouse adipocytes cell lines) indicated that this plant has the dose dependent manner and *G. procumbens* crude hexane fraction indicated the highest activity on stimulating glucose uptake. Effect of crude methanolic extract and crude fractions in the presence of insulin showed moderate glucose uptake activity when compared with *G. procumbens* crude extract/ fractions alone. Glucose uptake study with a mouse L6 muscle cell lines indicated that *G. procumbens* crude methanolic extract has highest reading from all of the crude extracts. When comparing the crude extract and fractions with insulin, all of the results showed

moderate glucose uptake activity and thus expressed that this plant has dose dependent manner (Bohari, 2006).

Hassan *et al.* (2010) have been evaluated the *in vivo* hypoglycemic properties of the water extract of *G. procumbens* following 14 days of treatment and *in vitro* in RIN-5F cells. Glucose absorption from the intestines and its glucose uptake in abdominal skeletal muscle was assessed. The antidiabetic effect of water extract of *G. procumbens* leaves was investigated in streptozotocin-induced diabetic rats. The intraperitoneal glucose tolerance test (IPGTT) was performed in diabetic rats treated with *G. procumbens* water extract for 14 days. In the IPGTT, blood was collected for insulin and blood glucose measurement. After the IPGTT, the pancreases were collected for immunohistochemical study of *β*-cells of the islets of Langerhans. The possible antidiabetic mechanisms of *G. procumbens* were assessed through *in vitro* RIN-5F cell study, intestinal glucose absorption and glucose uptake by muscle. The results showed that *G. procumbens* significantly decreased blood glucose levels after 14 days of treatment and improved outcome of the IPGTT. However, *G. procumbens* did not show a significant effect on insulin level either in the *in vivo* test or the *in vitro* RIN-5F cell culture study. *G. procumbens* also showed minimal effects on *β*-cells of the islets of Langerhans in the pancreas. However, *G. procumbens* only significantly increased glucose uptake by muscle tissues. These results suggested that the water *G. procumbens* extract exerted its hypoglycemic effect by promoting glucose uptake by muscles.

2.2.4.9 Antioxidation activity

The methanol extract of *G. procumbens* was prepared from the dried leaves using a soxhlet apparatus. The methanol extract was then fractionated into chloroform, ethyl acetate, *n*-butanol, and aqueous fractions using a separating funnel. In the current study, the antioxidant potency of *G. procumbens* extract and fractions were investigated, employing various established *in vitro* systems such as trolox equivalent antioxidant capacity, *β*-carotene-linoleic acid model system, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging, reducing power and xanthine oxidase inhibitory activity. Based on the results obtained, the extract and fractions showed different antioxidant potential. Among the fractions, the ethyl acetate fraction displayed higher antioxidant properties. The contents of the potential antioxidant component of the extract and fractions were also determined using high performance thin layer

chromatography (HPTLC) densitometric and spectrophotometric using folin-ciocalteu reagent methods. HPTLC study revealed that the methanol extract and the ethyl acetate and *n*-butanol fractions contain 0.74% and 2.9%, 7.76% and 12.75%, and 4.52% and 0.33% of kaempferol-3-*O*-rutinoside and astragalin, respectively. The relations between the total phenolic content and DPPH scavenging activity and reducing power with correlation coefficients ($R^2$) values of 0.891 and 0.926, respectively. These results suggest that phenolics in these plants provide substantial antioxidant activity (Rosidah *et al.*, 2008).

In addition, previous study has also evaluated the antioxidant properties of solvent extracts of medicinal plant *G. procumbens* by hydroxyl scavenging activity, ferrous ion-chelating activity and inhibition of lipid peroxidation. The results found that the ethanolic *Gynura* extract (EGE) exhibited the highest the antioxidant properties in every assay with $EC_{50}$ of 1.63 mg/ml in hydroxyl scavenging test, $EC_{50}$ of 2.17 mg/ml in chelating activity test and $EC_{50}$ of 2.75 mg/ml in inhibition of lipid peroxidation test (Puangpronpitag *et al.*, 2010).

2.2.4.10 Antiproliferative on human mesangial cell

Aqueous extract of *G. procumbens* inhibits mesangial cell proliferation and the inhibition may be mediated by the suspression of platelet-derived growth factor (PDGF-BB) and transforming growth factor (TGF-$\beta$1) expression and the modulation of cyclin-dependent kinasel and cycllin-dependent kinase 2 expressions (Hoe *et al.*, 2006).

2.2.4.11 Toxicology

A methanol extract from *G. procumbens* leaves at 1000-5000 mg/kg did not produce mortality or significant changes in the general behavior, bodyweight, or organ gross appearance of rats. There were no significant differences in the general condition, growth, organ weights, hematological parameters, clinical chemistry values, or gross and microscopic appearance of the organs from the treatment groups as compared to the control group. Therefore, the no-observed-adverse-effect level (NOAEL) for the *G. procumbens* methanol extract is 500 mg/(kg day) administered orally for 13 weeks (Rosidah *et al.*, 2009).

## 2.3 Free radicals and oxidative stress

### 2.3.1 Free radicals and oxidative stress mechanisms

Free radicals are atoms, molecules or ions with unpaired electrons that are highly unstable and strongly reactive with other molecules. They derive from three elements: oxygen, nitrogen and sulfur, thus creating reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS). ROS include free radicals like the superoxide anion ($O_2^{-\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), nitric oxide (NO), and other species like hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$). RNS derive from NO by reacting with $O_2^{-\bullet}$ and forming $ONOO^-$. RSS are easily formed by the reaction of ROS with thiols (Carocho *et al.*, 2013; Ferreira *et al.*, 2009; Lü *et al.*, 2010).

Oxidative stress is a state where there is disequilibrium between pro-oxidant processes and the antioxidant defense system in favor of the former, and generally occurs as a consequence of increased production of free radicals, or when the antioxidant defense system is inefficient, or a combination of both events. Oxidative stress, regardless of the specific cause, can result in initiation of number of pathophysiological processes leading to cellular toxicity (Carocho *et al.*, 2013).

Free radicals target cellular components indiscriminately, including lipids, proteins, DNA, and carbohydrates. Protein oxidation can lead to loss of sulfhydryl groups (-SH), in addition to modifications of amino acids, leading to the formation of carbonyl moieties. Accumulation of oxidized proteins may result in losses of selected biochemical and physiological functions. Free radicals mediated damage can also affect cellular macromolecules, such as DNA, by destroying pathways critical to the maintenance of normal adenine and pyridine nucleotides status. These alterations can affect the viability of DNA and modify gene expression. Lipids, by virtue of their location in cell membranes, are particularly vulnerable to peroxidation (Carocho *et al.*, 2013; Lü *et al.*, 2010).

### 2.3.2 Chemistry and sources of free radicals

Free radical contains an odd number of electrons, which makes it unstable, short lived and highly reactive. Generally, it reacts with the nearest stable molecule, ‘‘stealing’’ its electron to gain stability. The attacked molecule loses its electron; it

becomes a free radical itself, beginning a chain reaction cascade resulting in disruption of a living cell. Most ROS are generated as by-products during mitochondrial electron transport. In addition they are formed as necessary intermediates of metal catalyzed oxidation reactions. Reactive nitrogen species (RNS) are formed from interactions of NO with $O_2$ or $O_2^{-\bullet}$ resulting in formation of dinitrogen trioxide ($N_2O_3$) and peroxynitrite ($ONOO^-$) (Carocho *et al.*, 2013). Free radicals can be produced from the both endogenous and exogenous substances. Potential endogenous sources include mitochondria, cell metabolism, peroxisomes and inflammatory cell activation (Halliwell *et al.*, 2007). Exogenous sources include environmental agents such as non-genotoxic carcinogens, various xenobiotics, ultrasound, UV light, ozone and microwave radiation. Various free radicals are presented in Table 2.4. They have dual nature, on one hand they are necessary for normal cellular functions but when in excess they can cause cellular damage and can lead to non- communicable chronic diseases such as cancer, hypertension and other degenerative diseases (Valko *et al.*, 2007).

**Table 2.4** Biologically relevant free radicals and reactive.

| Species | Formula | Pathway of origin |
|---|---|---|
| Singlet oxygen | $^1O_2$ | Metabolism of partially reduced oxygen species |
| Ozone | $O_3$ | Atmosphere |
| Nitric oxide | NO | Arginin metabolism |
| Peroxynitrite | $ONOO^-$ | Derives from the reaction between $NO^\bullet$ and $O_2^{-\bullet}$ |
| Thiol radical | $R\text{-}S^\bullet$ | Metabolism of sulphur containing amino acids |
| Hydroxyl radical | $HO^\bullet$ | Derives from previous specie |
| Hydroperoxyl | $HOO^\bullet$ | Derives from previous specie |
| Superoxide anion | $O_2^{-\bullet}$ | Microsomal and mitochondrial electron transport chain, cellular oxydases and phagocytes |
| Alcoxyl | $OR^\bullet$ | Fatty acids from membrane phospholipids, amino acids and carbohydrates |

Source: Valko *et al.* (2007)

2.3.3 Effects of oxidative stress

Free radicals are generated from exogenous and endogenous sources are naturally formed generated by oxygen metabolism in human. However, these free radicals are potentially able to create oxidative damage via interaction with bio-molecules. For instance, lower amounts of free radicals are produced during mitochondrial activity in normal cells to act as the signaling molecules. The point is that level of oxidants and normal biological antioxidants must be in a balance. If the mentioned balance is interrupted then toxic oxidative stress may happen (Fig. 2.7). On cellular level, free radicals have potential to cause DNA mutation, lipid peroxidation, and protein oxidation. On clinical level, free radicals play a role in aging, immunosuppression and carcinogenesis (Potters *et al.*, 2004).

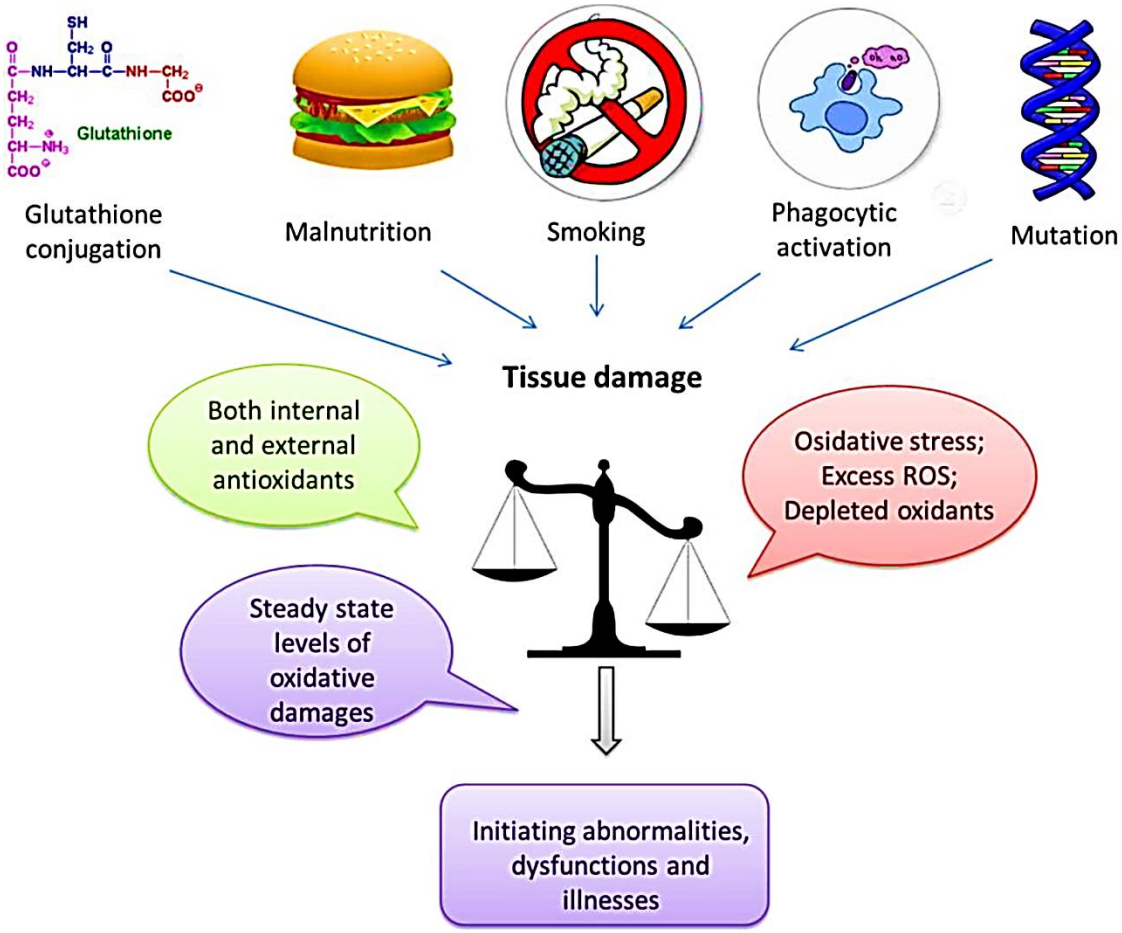



**Fig. 2.7** Induction of oxidative stress and consequent damages leading to oxidative stress related diseases (Potters *et al.*, 2004).

2.3.4 Human disease and ageing from oxidative stress

Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/ reperfusion, other diseases and ageing (Dalle-Donne *et al.*, 2006). These diseases fall into two groups: (i) the first group involves diseases characterised by pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance the so-called "mitochondrial oxidative stress" conditions (cancer and diabetes mellitus); (ii) the second group involves disease characterized by "inflammatory oxidative conditions" leading to atherosclerosis and chronic inflammation or xanthine oxidase-induced formation of ROS implicated in ischemia and reperfusion injury (Fig. 2.8).

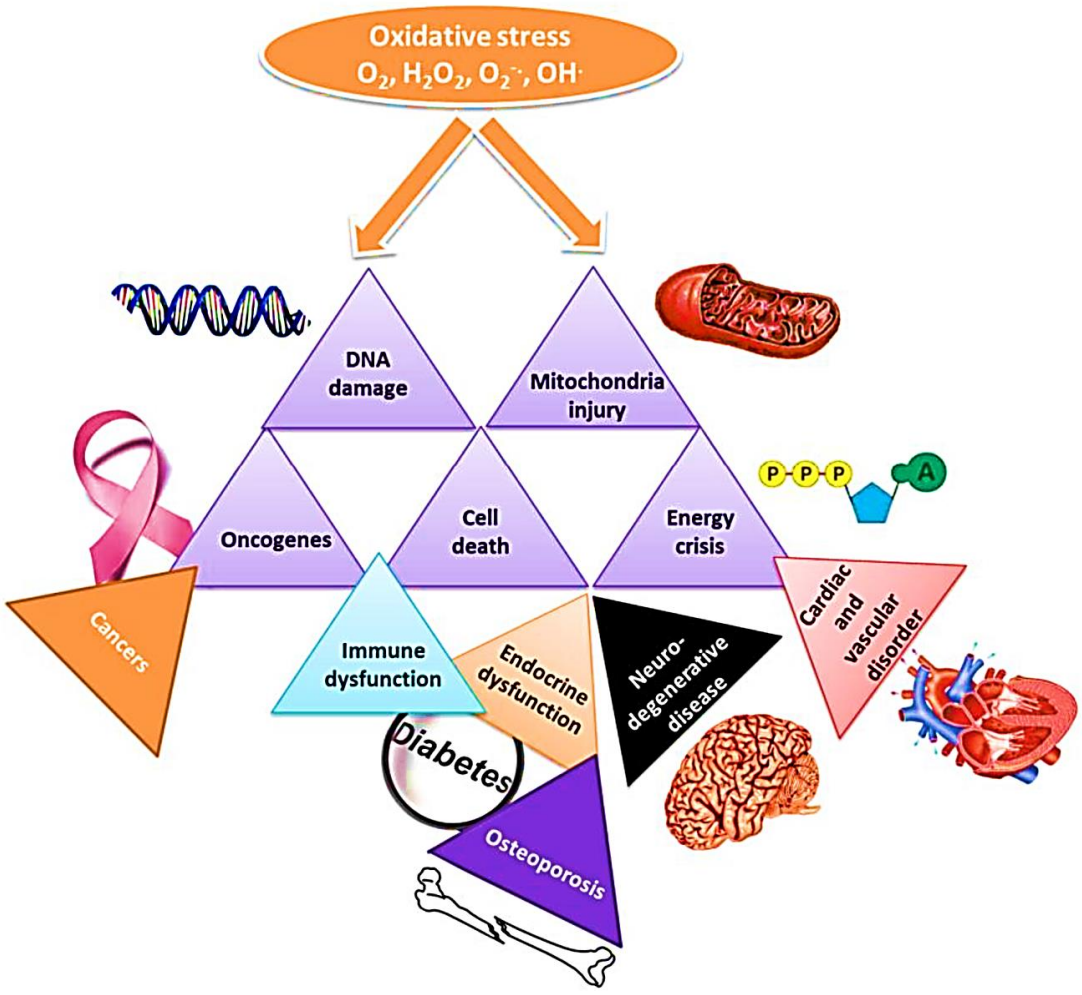



**Fig. 2.8** A simplifying hypothesis describes the relations among oxidative stress and consequent cell death or dysfunctions (Dalle-Donne *et al.*, 2006).

## 2.4 Antioxidants

Antioxidant is any molecule capable of stabilizing or deactivating free radicals before they attack cells (Khlebnikov *et al.*, 2007). Humans have evolved highly complex antioxidant systems, which work synergistically to protect cells and organ systems of the body against free radical damage. These can be endogenous or obtained exogenously. They are abundant in fruits and vegetables as well as in other foods such as nuts, grains, some meats, poultry and fish. They are intimately involved in prevention of cellular damage caused by oxidants, the major mechanism for cancer initiation. An imbalance between antioxidant defense mechanisms and oxidants leads to cell and tissue damage resulting in cancer. Different types of enzymatic and non-enzymatic antioxidants with their antioxidant properties are shown in Table 2.5. Antioxidants can prevent, inhibit or repair damage caused by oxidative stress.

**Table 2.5** Antioxidant compounds and their activities.

| Antioxidant | Activities |
|---|---|
| *Enzymatic antioxidants* | |
| Superoxide dismutase (SOD) | Destroys $O_2^{-\bullet}$; Cu, Zn-SOD catalyzes dismutation of superoxide anion to oxygen and water |
| Catalase | Has high turnover rates; conversion of $H_2O_2$ to water and molecular oxygen |
| Glutathione peroxidise | Selenium-dependent glutathione peroxidise catalyzes; conversion of $H_2O_2$ or organic peroxide to water or alcohol |
| *Non-enzymatic antioxidants* | |
| Vitamin C | Protects membranes against oxidation; decreases degradation of vitamin E; enhances detoxification via cytochrome P450 |

**Table 2.5** (Continued)

| Antioxidants | Activities |
|---|---|
| Vitamin E | Inhibition of free radical formations; inhibition of DNA, RNA and protein synthesis in cancer cells |
| Carotenoids | Quenches singlet oxygen without degradation; prevents damage in lipophilic compartments at low oxygen partial pressure and immune modulation |
| Phenolic compounds | Can scavenge peroxyl radicals and are effective inhibitors of lipid peroxidation; can chelate redox-active metals and thus prevent catalytic breakdown of $H_2O_2$ (Fenton reaction) |
| Flavonoids | Can scavenge peroxyl radicals and are effective inhibitors of lipid peroxidation; can chelate redox-active metals and thus prevent catalytic breakdown of $H_2O_2$ (Fenton reaction) |

Source: Choudhari *et al.* (2013)

2.4.1. Natural antioxidants

During human evolution, endogenous defenses have gradually improved to maintain a balance between free radicals and oxidative stress. The antioxidant activity can be effective through various ways: as inhibitors of free radical oxidation reactions (preventive oxidants) by inhibiting formation of free lipid radicals; by interrupting the propagation of the autoxidation chain reaction (chain breaking antioxidants); as singlet oxygen quenchers; through synergism with other antioxidants; as reducing agents which convert hydroperoxides into stable compounds; as metal chelators that convert metal pro-oxidants (iron and copper derivatives) into stable products; and finally as inhibitors of pro-oxidative enzymes (lipooxygenases) (Kancheva, 2009; Pokorny, 2007).

2.4.1.2 Enzymatic antioxidants

The human antioxidant system is divided into two major groups, enzymatic antioxidants and non-enzymatic oxidants (Fig. 2.9). Regarding enzymatic antioxidants they are divided into primary and secondary enzymatic defenses. With

regard to the primary defense, it is composed of three important enzymes that prevent the formation or neutralize free radicals: glutathione peroxidase, which donates two electrons to reduce peroxides by forming selenoles and also eliminates peroxides as potential substrate for the Fenton reaction; catalase that converts hydrogen peroxide into water and molecular oxygen and has one of the biggest turnover rates known to man, allowing just one molecule of catalase to convert 6 billion molecules of hydrogen peroxide; and finally, superoxide dismutase converts superoxide anions into hydrogen peroxide as a subtract for catalase. The secondary enzymatic defense includes glutathione reductase and glucose-6-phosphate dehydrogenase. Glutathione reductase reduces glutathione (antioxidant) from its oxidized to its reduced form, thus recycling it to continue neutralizing more free radicals. Glucose-6-phosphate regenerates NADPH (nicotinamide adenine dinucleotide phosphate-coenzyme used in anabolic reactions) creating a reducing environment (Ratnam *et al.*, 2006). These two enzymes do not neutralize free radicals directly, but have supporting roles to the other endogenous antioxidants.

2.4.1.2 Non-enzymatic antioxidants

Considering the non-enzymatic endogenous antioxidants (Fig. 2.9), there are quite a number of them, namely vitamins (A), enzyme cofactors (Q10), nitrogen compounds (uric acid) and peptides (glutathione). Vitamins A, C and E of which fruit and vegetables are rich sources are potent dietary antioxidants that can prevent cytotoxicity resulting from free radicals, act as direct scavengers of ROS and up-regulate antioxidant enzyme activity.

Vitamin A or retinol is a carotenoid produced in the liver and results from the breakdown of $\beta$-carotene. There are about a dozen forms of vitamin A that can be isolated. It is known to have beneficial impact on the skin, eyes and internal organs. What confers the antioxidant activity is the ability to combine with peroxyl radicals before they propagate peroxidation to lipids (Jee *et al.*, 2006).

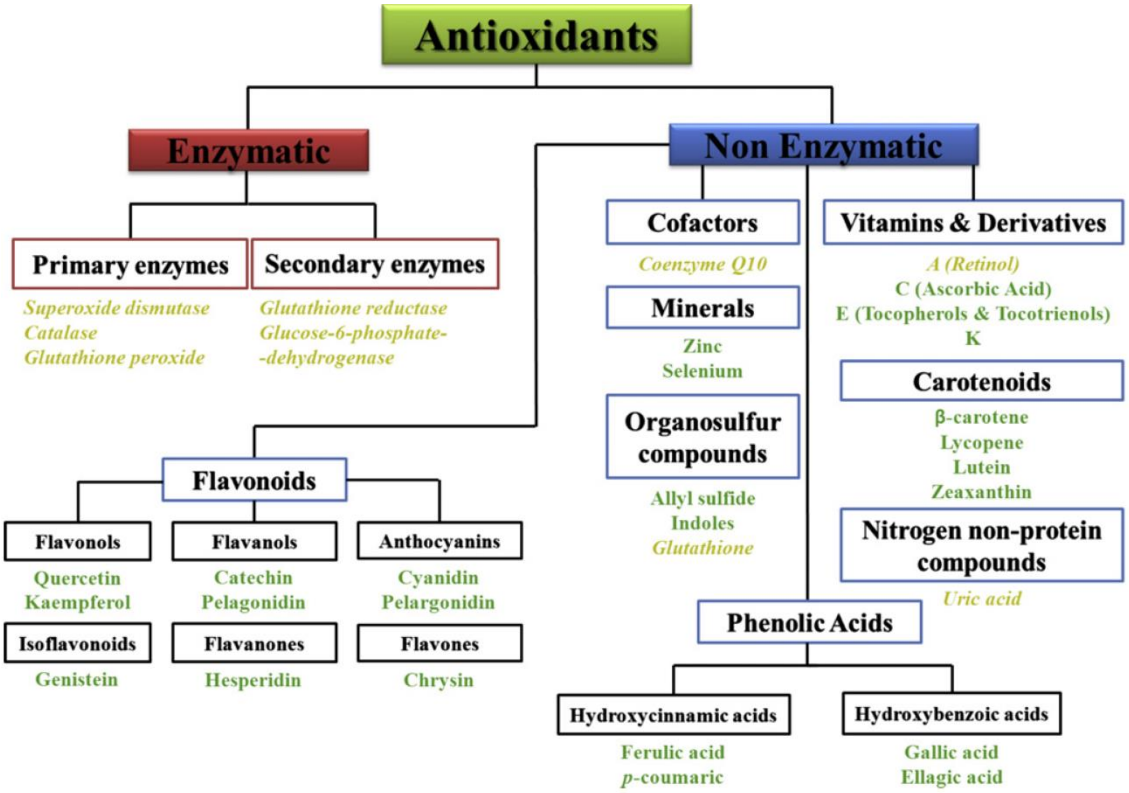



**Fig. 2.9** Classification of natural antioxidants (Ratnam *et al.*, 2006).

Coenzyme Q10 is present in all cells and membranes; it plays an important role in the respiratory chain and in other cellular metabolism. Coenzyme Q10 acts by preventing the formation of lipid peroxyl radicals, although it has been reported that this coenzyme can neutralize these radicals even after their formation. Another important function is the ability to regenerate vitamin E; some authors describe this process to be more likely than regeneration of vitamin E through ascorbate (vitamin C) (Turunen *et al.*, 2004).

Uric acid is the end product of purine nucleotide metabolism in humans and during evolution its concentrations have been rising. After undergoing kidney filtration, 90% of uric acid is reabsorbed by the body, showing that it has important functions within the body. In fact, uric acid is known to prevent the overproduction of oxo-hem oxidants that result from the reaction of hemoglobin with peroxides. On the other hand it also prevents lysis of erythrocytes by peroxidation and is a potent scavenger of singlet oxygen and hydroxyl radicals (Kandár *et al.*, 2006).

Glutathione is an endogenous tripeptide which protects the cells against free radicals either by donating a hydrogen atom or an electron. It is also very important in the regeneration of other antioxidants like ascorbate.

Despite its remarkable efficiency, the endogenous antioxidant system does not suffice and humans depend on various types of antioxidants that are present in the diet to maintain free radical concentrations at low levels.

Vitamins C and E are generic names for ascorbic acid and tocopherols. Ascorbic acid includes two compounds with antioxidant activity: L-ascorbic acid and L-dehydroascorbic acid which are both absorbed through the gastrointestinal tract and can be interchanged enzymatically *in vivo*. Ascorbic acid is effective in scavenging the superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen and reactive nitrogen oxide (Barros *et al.*, 2011). Vitamin E halts lipid peroxidation by donating its phenolic hydrogen to the peroxyl radicals forming tocopheroxyl radicals that, despite also being radicals, are unreactive and unable to continue the oxidative chain reaction. Vitamin E is the only major lipid-soluble, chain breaking antioxidant found in plasma, red cells and tissues, allowing it to protect the integrity of lipid structures, mainly membranes. These two vitamins also display a synergistic behavior with the regeneration of vitamin E through vitamin C from the tocopheroxyl radical to an intermediate form, therefore reinstating its antioxidant potential (Halpner *et al.*, 1998).

Vitamin K is a group of fat-soluble compounds, essential for posttranslational conversion of protein-bound glutamates into $\gamma$-carboxyglutamates in various target proteins. The 1,4-naphthoquinone structure of these vitamins confers the antioxidant protective effect. The two natural isoforms of this vitamin are K1 and K2 (Vervoort *et al.*, 1997).

Flavonoids are an antioxidant group of compounds composed of flavonols, flavanols, anthocyanins, isoflavonoids, flavanones and flavones. All these sub-groups of compounds share the same diphenylpropane ($C_6$–$C_3$–$C_6$) skeleton. Flavanones and flavones are usually found in the same fruits and are connected by specific enzymes, while flavones and flavonols do not share this phenomenon and are rarely found together. Anthocyanins are also absent in flavanone-rich plants. The antioxidant properties are conferred on flavonoids by the phenolic hydroxyl groups

attached to ring structures and they can act as reducing agents, hydrogen donators, singlet oxygen quenchers, superoxide radical scavengers and even as metal chelators. They also activate antioxidant enzymes, reduce $\alpha$-tocopherol radicals (tocopheroxyls), inhibit oxidases, mitigate nitrosative stress, and increase levels of uric acid and low molecular weight molecules. Some of the most important flavonoids are catechin, catechin-gallate, quercetin and kaempferol (Procházková *et al.*, 2011).

Phenolic acids are composed of hydroxycinnamic and hydroxybenzoic acids. They are ubiquitous to plant material and sometimes present as esters and glycosides. They have antioxidant activity as chelators and free radical scavengers with special impact over hydroxyl and peroxyl radicals, superoxide anions and peroxynitrites. One of the most studied and promising compounds in the hydroxybenzoic group is gallic acid which is also the precursor of various tannins, while cinnamic acid is the precursor of all the hydroxycinnamic acids (Krimmel *et al.*, 2010; Terpinc *et al.*, 2011).

Carotenoids are a group of natural pigments that are synthesized by plants and microorganisms but not by animals. They can be separated into two vast groups: the carotenoid hydrocarbons known as the carotenes which contain specific end groups like lycopene and $\beta$-carotene; and the oxygenated carotenoids known as xanthophyls, like zeaxanthin and lutein. The main antioxidant property of carotenoids is due to singlet oxygen quenching which results in excited carotenoids that dissipate the newly acquired energy through a series of rotational and vibrational interactions with the solvent, thus returning to the unexcited state and allowing them to quench more radical species. This can occur while the carotenoids have conjugated double bonds within. The only free radicals that completely destroy these pigments are peroxyl radicals. Carotenoids are relatively unreactive but may also decay and form non-radical compounds that may terminate free radical attacks by binding to these radicals (Paiva and Russell, 1999).

Minerals are only found in trace quantities in animals and are a small proportion of dietary antioxidants, but play important roles in their metabolism. Regarding antioxidant activity, the most important minerals are selenium and zinc. Selenium can be found in both organic (selenocysteine and selenomethionine) and inorganic (selenite and selenite) forms in the human body. It does not act directly on

free radicals but is an indispensable part of most antioxidant enzymes (metalloenzymes, glutathione peroxidase, thioredoxin reductase) that would have no effect without it (Tabassum *et al.*, 2010). Zinc is a mineral that is essential for various pathways in metabolism. Just like selenium, it does not directly attack free radicals but is quite important in the prevention of their formation. Zinc is also an inhibitor of NADPH oxidases which catalyze the production of the singlet oxygen radical from oxygen by using NADPH as an electron donor. It is present in superoxide dismutase, an important antioxidant enzyme that converts the singlet oxygen radical into hydrogen peroxide. Zinc induces the production of metallothionein that is a scavenger of the hydroxyl radical. Finally, zinc also competes with copper for binding to the cell wall, thus decreasing once again the production of hydroxyl radicals (Prasad *et al.*, 2004).

2.4.2 Synthetic antioxidants

Synthetic antioxidants have been developed for use as an additive in various food products, especially fat and oil products to inhibit oxidation and extend shelf-life. In cases of antioxidant assays, these compounds have commonly used as reference compounds to compare with natural antioxidants. Nowadays, the processed food industries have been added the synthetic antioxidants in almost all food products, but some research indicate that non-safety (Carocho and Ferreira, 2013). The usually synthetic antioxidants available in commerce and their applications are shown in Table 2.6.

BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) are the most widely used synthetic antioxidants. Between 2011 and 2012, the European food safety authority (EFSA) re-evaluated all the available information on these two antioxidants, including the apparently contradictory data that have been published. EFSA established revised acceptable daily intakes (ADIs) of 0.25 mg/kg bw/day for BHT and 1.0 mg/kg bw/day for BHA and noted that the exposure of adults and children was unlikely to exceed these intakes. TBHQ (tert-butylhydroquinone) stabilizes and preserves the freshness, nutritive value, flavour and color of animal food products.

**Table 2.6** Chemical structures and applications of the synthetic antioxidants.

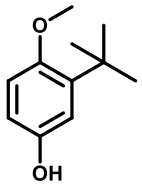
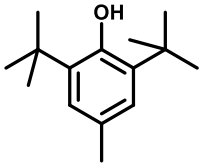
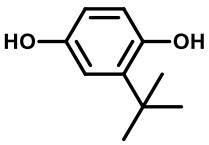
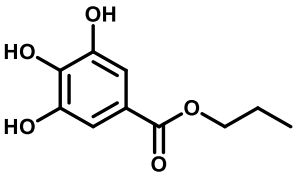
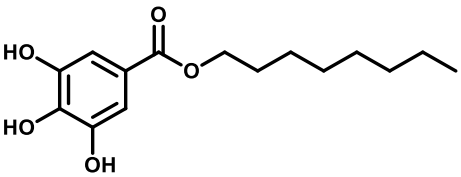
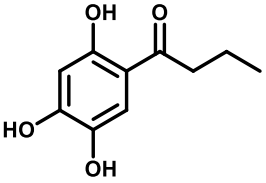
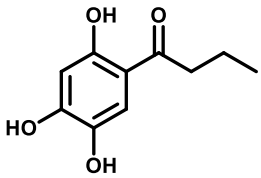
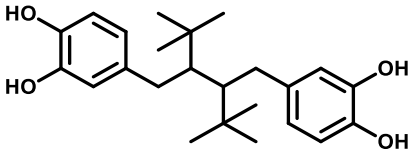
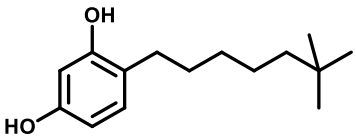
| Compound | Structure | Application |
|---|---|---|
| BHA<br>(butylated hydroxyanisole) |  | Food |
| BHT<br>(butylated hydroxytoluene) |  | Food |
| TBHQ<br>(*tert*-butylhydroquinone) |  | Animal<br>processed food |
| PG (propyl gallate) |  | Food |
| OG (octyl gallate) |  | Food<br>and cosmetic |
| 2,4,5-Trihydroxy<br>butyrophenone |  | Food |

**Table 2.6** (Continued)

| Compound | Structure | Application |
|---|---|---|
| 2,4,5-Trihydroxy butyrophenone |  | Food |
| NDGA (nordihydroguaiaretic acid) |  | Food |
| 4-Hexylresorcinol |  | Prevention of food browning |

Source: Carocho and Ferreira (2013)

Recently, the EFSA published a scientific opinion reviewing the impact of this antioxidant on human health and stated that there was no scientific proof of its carcinogenicity despite previous conflicting data. They pointed out that dogs were the most sensitive species and allocated an ADI of 0-0.7 mg/kg bw/day. Octyl gallate is considered safe to use as a food additive because after consumption it is hydrolysed into gallic acid and octanol, which are found in many plants and do not pose a threat to human health. NDGA (nordihydroguaiaretic acid) despite being a food antioxidant is known to cause renal cystic disease in rodents (EFSA, 2011; 2012).

2.4.3 Phenolic antioxidants-rich in plants

Fortunately, plants are able to generate a broad spectrum of metabolites, many of which are not directly employed by plants in growth and development and often introduced as “secondary metabolites”. It is now understood that even though such metabolites have unknown role inside plant biological system, they might be important

and useful by mediating a vast array of interactions between plants and environment. The second metabolites may act as defensive agents against pathogens and herbivores, causing reproductive advantages to attract pollinators and seed dispersers, as well as protection against different environmental stresses (Saeidnia and Abdollahi, 2013).

It is well reported that plant phenolics are usually characterized as aromatic natural products that possess one or more phenolic hydroxyl groups in their structure and range from relatively simple phenols (like the signaling molecule salicylic acid) to complex polymers (like suberin and lignin). Phenolic class consists of various subgroups, of which hydroxycinnamic acids, flavonoids, anthocyanins and tannins are considerable. In this regard, polyphenols, flavonoids and anthocyanins (Fig. 2.10) are well-known natural antioxidants that increase cell resistance to oxidative stress via scavenging free radicals.

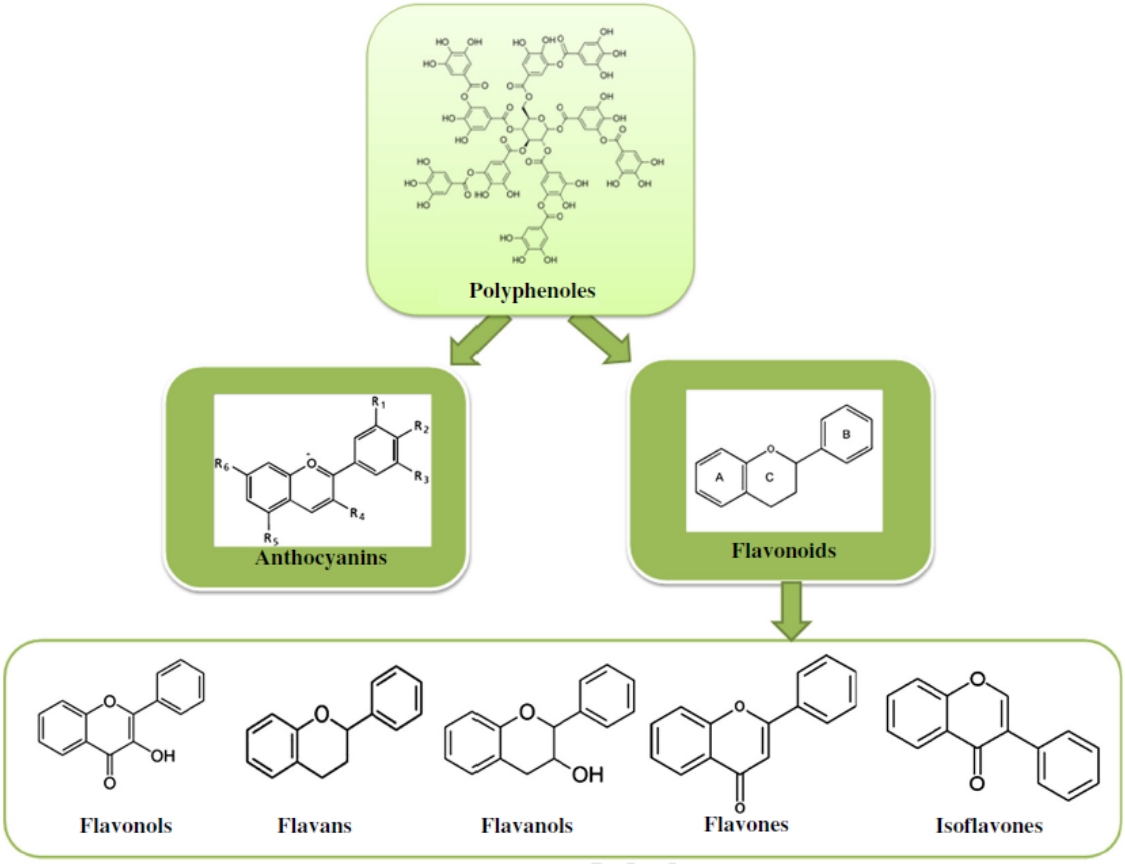



**Fig. 2.10** Diagram of types and chemical structure of natural phenolic compounds (Saeidnia and Abdollahi, 2013).

A recent study revealed that polyphenols possess *in vitro* and *in vivo* activity in reducing the adverse effects of ROS and RNS related to several chronic and stress-related human illnesses. There are sufficient evidences suggesting role of oxidative stress and its consequences in pathology and complications of many clinical disorders (Malekirad *et al.*, 2011; Momtaz and Abdollahi, 2012). Besides phenolics, coumarins, terpenoids like diterpenes, sesquiterpenes and especially tetraterpenes like carotenoids (beta-carotene is a familiar antioxidant), as well as alkaloids have been frequently noted as strong antioxidants (Kahkeshani *et al.*, 2013; Liu *et al.*, 2011; Naseri *et al.*, 2013).

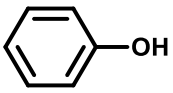
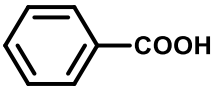
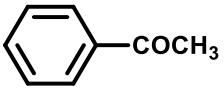
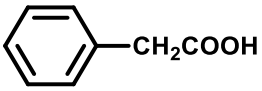
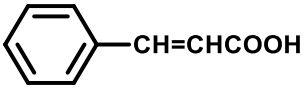
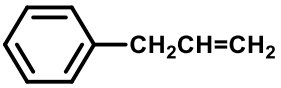
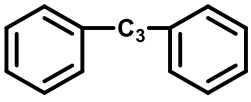
Many studies demonstrated that phenolic compounds including around 30 flavonoids and phenolic acids can exhibit scavenging activity rather than even trolox (an analog of vitamin E) as a standard antioxidant (Rice-Evans *et al.*, 1996), the more details of phenolic compounds can explained in heading 2.5. In addition, previous studies have also showed that flavonoids and hydroxycinnamic acids are commonly more effective than vitamin C in scavenging free radicals. Among the flavonoids tested, quercetin (a flavonol), cyanidin and delphinidin (two anthocyanidins), epicatechin gallate and epigallocatechin gallate (two flavan-3-ol) have been found as the strongest scavengers (Rice-Evans *et al.*, 1996).

## 2.5 Phenolic compounds

### 2.5.1 Chemistry and classification of phenolic compounds

Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerized compounds (Bravo, 1998). Despite this structural diversity, the groups of compounds are often referred to as “polyphenols”. Most naturally occurring phenolic compounds are present as conjugates with mono- and polysaccharides, linked to one or more of the phenolic groups, and may also occur as functional derivatives such as esters and methyl esters (Harborne, 1989; Shahidi and Naczk, 1995). Phenolic compounds that occur in nature can basically be categorized into several classes as shown in Table 2.7 (Harborne, 1989). Of these, phenolic acids, flavonoids and tannins are regarded as the main dietary phenolic compounds (King and Young, 1999).

**Table 2.7** Classes of phenolic compounds in plants.

| Class | | Structure |
|---|---|---|
| Simple phenolics | $C_6$ |  |
| Benzoquinones | $C_6$ |  |
| Hydroxybenzoic acids | $C_6$–$C_1$ |  |
| Acethophenones | $C_6$–$C_2$ |  |
| Phenylacetic acids | $C_6$–$C_2$ |  |
| Hydroxycinnamic acids | $C_6$–$C_3$ |  |
| Phenylpropenes | $C_6$–$C_3$ |  |
| Flavonoids | $C_6$–$C_3$–$C_6$ |  |

Source: Balasundram *et al.* (2006)

Phenolic acids consist of two subgroups, including the hydroxybenzoic acids and hydroxycinnamic acids (Fig. 2.11). Hydroxybenzoic acids include gallic, *p*-

hydroxybenzoic, protocatechuic, vanillic and syringic acids, which in common have the $C_6$–$C_1$ structure. Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain ($C_6$–$C_3$), with caffeic, ferulic, *p*-coumaric and sinapic acids being the most common (Bravo, 1998).

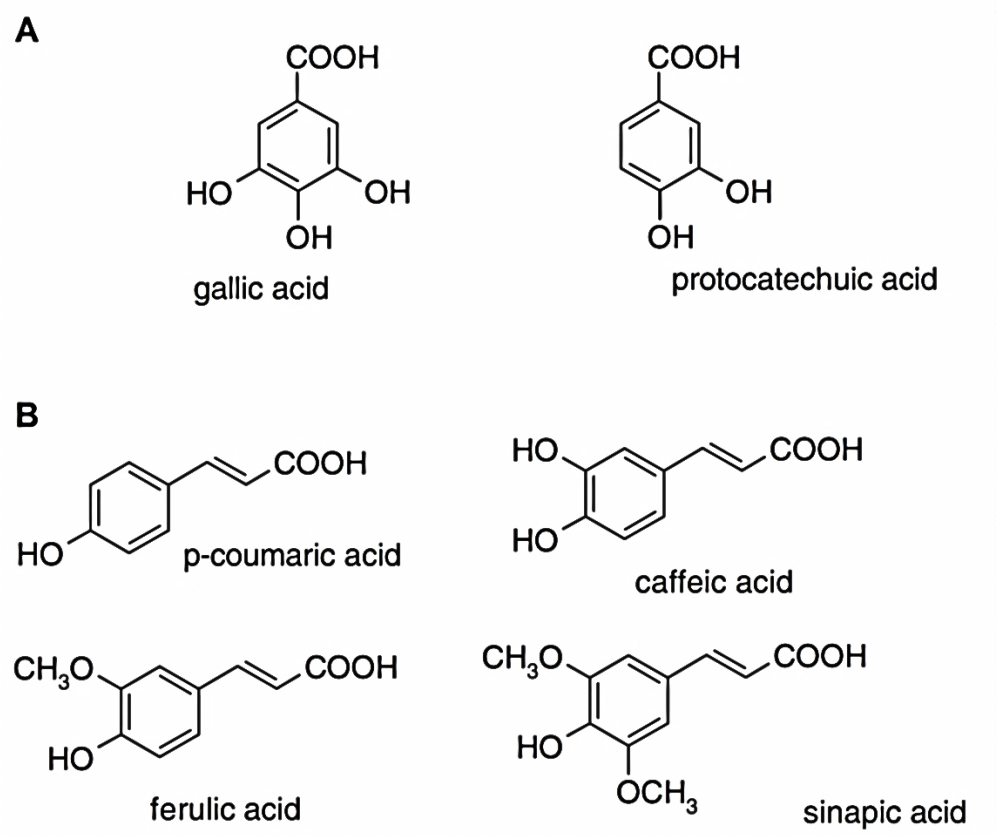



**Fig. 2.11** Chemical structures of some hydroxybenzoic acids (A) and hydroxycinnamic acids (B).

Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Harborne and Baxter, 1999). Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a $C_6$–$C_3$–$C_6$ configuration. Essentially the structure consists of two aromatic rings A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring C (Fig. 2.12). The aromatic ring A is derived from the acetate/malonate pathway, while ring B is derived from phenylalanine through the shikimate pathway (Bohm, 1998). Variations in substitution patterns to ring C result in

the major flavonoid classes, including flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols, and anthocyanidins (Balasundram *et al.*, 2006), of which flavones and flavonols are the most widely occurring and structurally diverse (Harborne and Baxter, 1999). Substitutions to rings A and B give rise to the different compounds within each class of flavonoids. These substitutions may include oxygenation, alkylation, glycosylation, acylation, and sulfation (Bohm, 1998; Hollman and Katan, 1999).

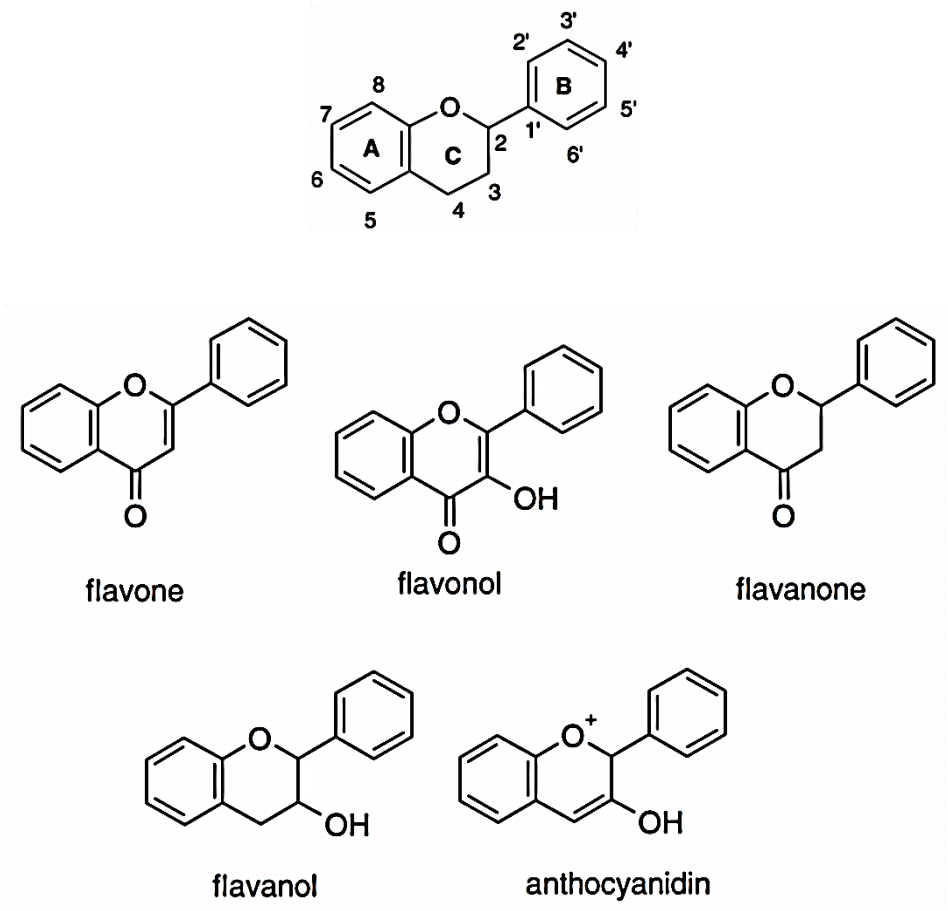



**Fig. 2.12** Generic structure of flavonoids and their major classes.

2.5.2 Biosynthesis of phenolic compounds in plants

Phenolic compounds are aromatic hydroxylated compounds, commonly found in vegetables, fruits and many food sources that form a significant portion of our diet, and which are among the most potent and therapeutically useful bioactive substances. Phenolic derivatives represent the largest group known as ‘secondary plant

products' synthesized by higher plants, probably as a result of antioxidative strategies adapted in evolution by respirative organisms starting from precursors of cyanobacteria. Many of these phenolic compounds are essential to plant life, e.g., by providing defense against microbial attacks and by making food unpalatable to herbivorous predators. Although a precise chemical definition may be given for plant phenolics, it would inevitably include other structurally similar compounds such as the terpenoid sex hormones. Therefore, an operational definition of metabolic origin is preferable, and thus the plant phenols being regarded as those substances derived from the shikimate pathway and phenylpropanoid metabolism, following the phosphoenolpyruvate → phenylalanine → cinnamate → 4-coumarate course, leading to chalcone, flavanone, dihydroflavonol, and anthocyanin (Wang *et al.*, 2013; Kalinowska *et al.*, 2014). The biosynthesis pathway of phenolic compounds in various plants is shown in Fig 2.13.

2.5.3. Biological activities and health benefits of phenolic compounds

Significant antioxidant, antitumor, antiviral and antibiotic activities are frequently reported for plant phenolics. They have often been identified as active principles of numerous folk herbal medicines. In recent years, the regular intake of fruits and vegetables has been highly recommended, because the plant phenolics and polyphenols, especially flavonoids they contain are thought to play important roles in long term health and reduction in the risk of chronic and degenerative diseases. Recognition of the benefits brought by these natural products to human health has encouraged the inclusion in everyday diets of some typical plant-derived food and beverages, among the most preferred examples being olive and vegetable oils, citrus and other fruit juices, chocolate, tea, coffee and wine. Recently, growing interests on phenolic compounds from grapes have focused on their biological activities linking to human health benefits, such as antioxidant, cardioprotective, anticancer, anti-inflammation, antiaging and antimicrobial properties.

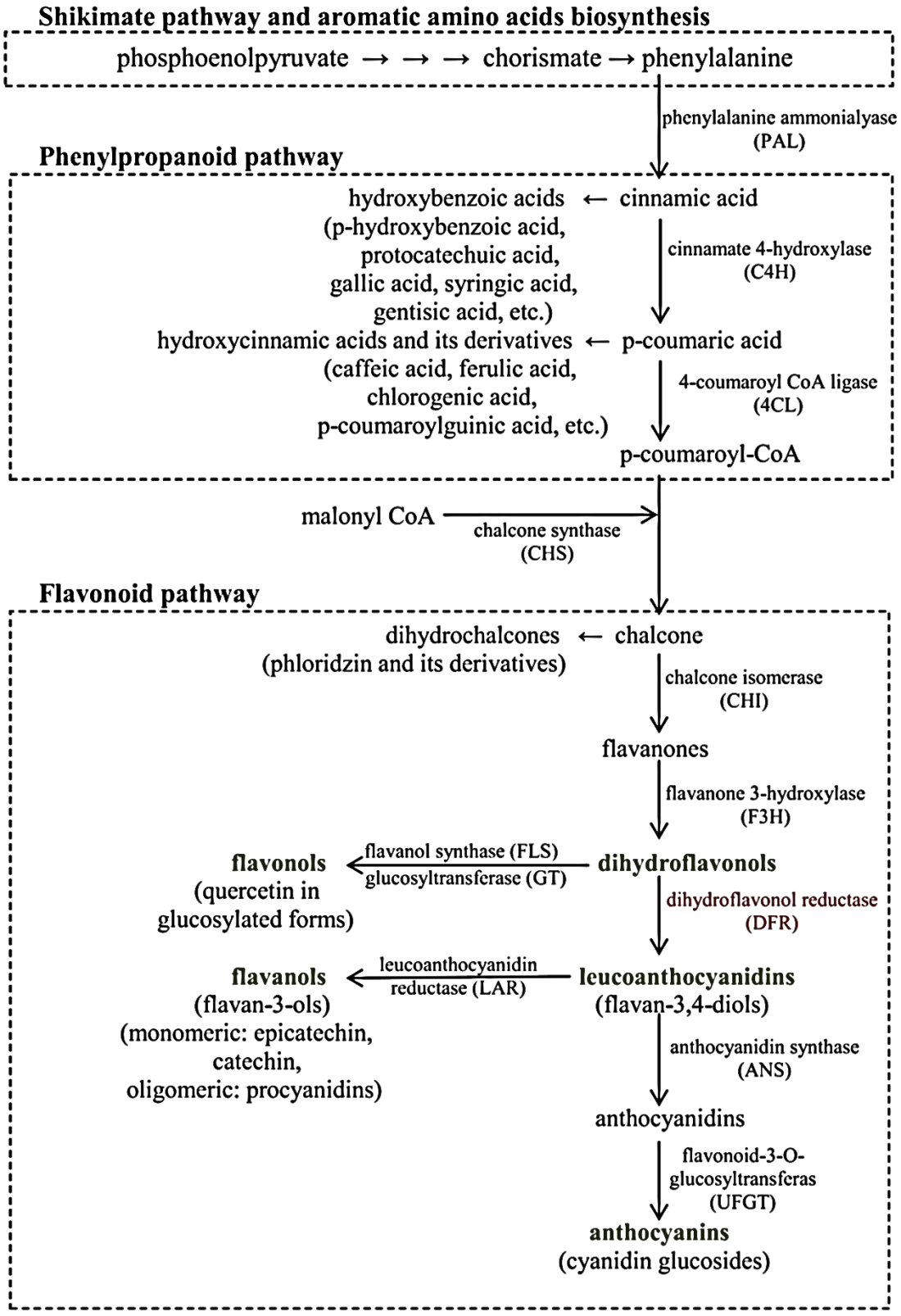



**Fig. 2.13** The biosynthesis pathway of phenolic compounds in plants (Kalinowska *et al.*, 2014).

2.5.3.1 Antioxidant activities

The antioxidant activities of phenolic compounds from various plant, such as fruits, vegetables and medicinal plants have been widely studied in terms of free radicals scavenging, inhibition of lipid oxidation, reduction of hydroperoxide formation, and so on. Several methods were employed to evaluate the antioxidant capacities of phenolic compounds extracted from various grapes or different parts of grapes, such as the 1,1-diphenyl-2-picryhidrazyl (DPPH) method, oxygen radical absorbance capacity (ORAC) assay, crocin bleaching assay (CBA), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, the thiobarbituric acid reactant substances (TBARS), Trolox equivalent antioxidant capacity (TEAC) assay and the ferric reducing antioxidant power (FRAP) assay (Apak *et al.*, 2004). Phenolic compounds are natural antioxidants and most of their pharmacological properties are considered to be due to their antioxidant action. It is considered that the antioxidant activity of phenolic compounds is due to their high redox potentials, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. The antioxidant activity of the phenolics is essentially determined by their structures, in particular the electron delocalization over an aromatic nucleus. Earlier studies on antioxidant effect of caffeic and ferulic acids pertain to radical scavenging, inhibition of lipid peroxidation and protection against LDL oxidation. Caffeic acid and its derivatives are good substrates of polyphenol oxidases, and under certain conditions may undergo oxidation in plant tissues or products of plant origin.

2.5.3.2 Cardioprotective activities

Postprandial hyperlipemia and oxidative stress, a well-defined risk factor for atherosclerosis, could be reduced by grape seed extracts or phenolic-rich grape juice. These oxidative stress factors refer to plasma lipid hydroperoxides, serum lipid peroxidation products, malondialdehyde-modified-LDL (MDA-LDL). The lipid-bound polyphenols increasing in serum were found even two hours after intake of phenolics, and MDA-LDL was detected after six weeks. Phenolic compounds showed protected the rat liver against oxidative damage induced by irradiation *in vivo*, and remained the activities of superoxide dismutase and catalase at normal level. The beneficial effects derived from phenolics, with respect to cardiovascular disease prevention have been attributed to (i) their antioxidant activity, (ii) the prevention of atherosclerosis, and (iii)

the effect on platelet aggregation. Previous studies focused on the protective effects of flavan-3-ols such as catechin against lipid peroxidation and LDLs oxidation. Auclair *et al.* (2009) reported that catechin reduced the mean atherosclerotic lesion area by 32% but had no effect on total cholesterol and triacylglycerol levels in the plasma and the liver. Indeed, the expression of 450 genes was significantly modified by catechin supplementation. Some of the most significantly down-regulated genes included genes coding for adhesion molecules such as CD34 and PSGL-1known to play a key role in leukocyte adhesion to the endothelium. In addition, Bhaskar *et al.* (2011) also reported that quercetin significantly modulates the NF-κB p65 nuclear translocation. The cytokine IL-6 production was significantly increased in ox-LDL treated group and was decreased by quercetin treatment. Anthocyanins from wine and grape possess inhibited phosphodiesterase-5 activity, which reduced the risk of cardiovascular diseases by vasorelaxation.

2.5.3.3 Anticancer activities

Many studies have shown that the phenolic compounds presence in various plants had anticancer activity. Hudson *et al.* (2007) reported that the grape extract induced prostate tumor cell lines apoptosis with high rates. The phenolic compounds from pomace had inhibited activities of matrix metalloproteinases-2 and -9, and expressed a significant antiproliferative effect on human colon adenocarcinoma cells (Caco-2), which implied by-product of wine would help to fight against carcinogenesis (Lazze *et al.*, 2009). Phenolics in grape juice also significantly inhibited carcinogen-induced DNA adducts formation in rat model, and inhibited DNA synthesis in breast cancer cells (God *et al.*, 2007). There are many *in vitro*, *in vivo* and epidemiological studies on cancer preventive properties of phenolic compounds in plants. Mechanism of anticancer activity of apples among others relies on: antimutagenic properties, inhibition of cancer cell proliferation, modulation of phase 1 and 2 of carcinogen metabolism, antioxidant properties, induction of tumour suppressor gene expression, inhibition of cell growth, induction of apoptosis, modulation of signal transduction pathways, and enhancement of immune system (Fig. 2.14).

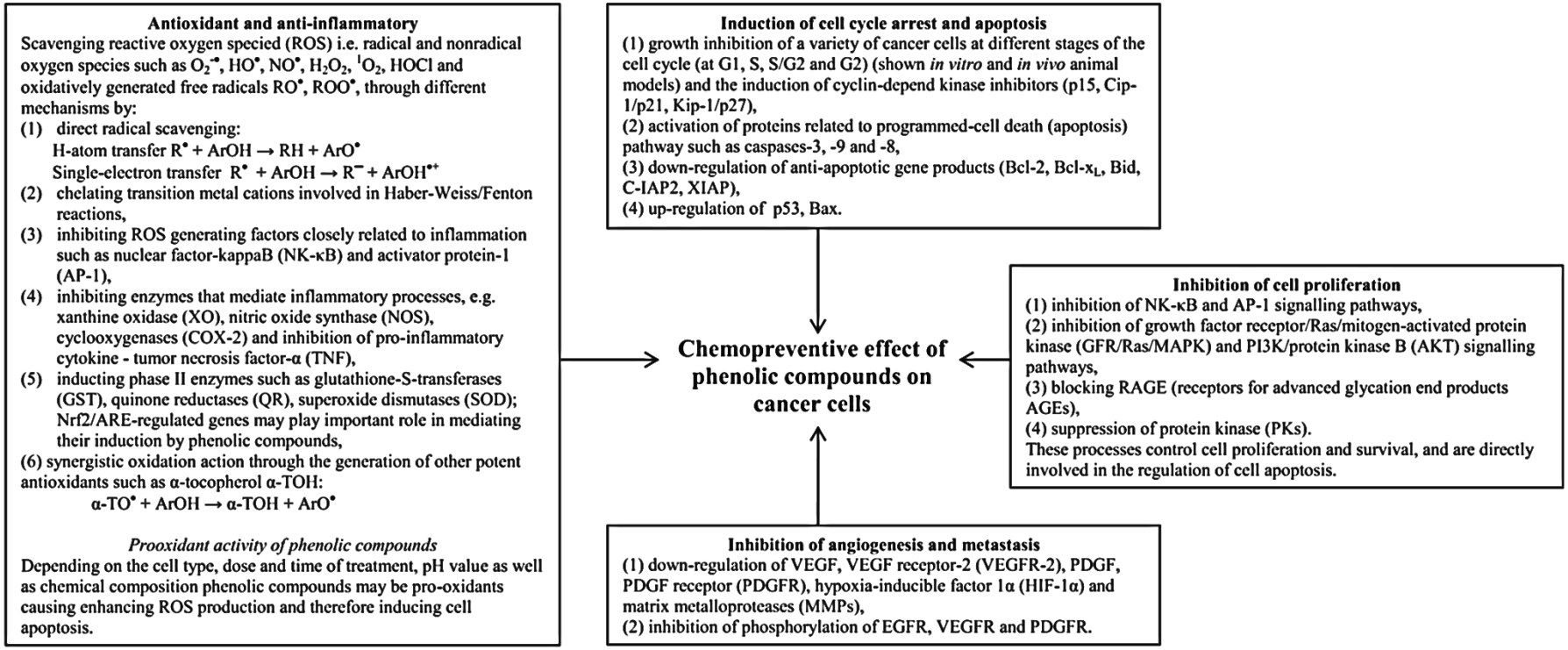



**Fig. 2.14** Summarized selected mechanisms of cancer preventive properties of phenolic compounds (Kalinowska *et al.*, 2014).

2.5.3.4 Antimicrobial activities

Plant polyphenols have been demonstrated potential antibacterial, antifungal and antiviral activities. Rodriguez-Vaquero *et al.* (2007) have showed that phenolics from grape wine inhibited microbial, especially *Escherichia coli* growth, and the inhibition increased as the polyphenol concentration increased, and clarified wines were inactive against all bacteria tested. The extracts of alcohol-free red and white wine exhibited antimicrobial activity to some pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. The results suggested that polyphenolic compounds contained in red wines were responsible for the antimicrobial effects. Some studies reported phenolic compounds inhibited other food-borne species such as *Salmonella typhimurium* and *Listerial monocytogenes* (Sivarooban *et al.*, 2008).

2.5.3.5 Anti-inflammatory activities

Phenolic compounds in plants, especially flavonols, flavanols and procyanidins have showed significant anti-inflammation effects on rats, mice and human. Mahat *et al.* (2010) demonstrated that polyphenol as kaempferol modulates the cyclooxygenase pathway via inhibition of nitric oxide production, which in turn contributes to its anti-inflammatory activity. Studies carried out *in vitro* have shown that quercetin exhibits, besides other activities, pronounced anti-inflammatory property, an effect that seems to be associated with its ability to block some inflammatory mediators, adhesion molecules expression, inducible enzymes and nuclear transcription factor activation (Sergent *et al.*, 2010).

# CHAPTER III

# RESEARCH METHODOLOGY

The present study was divided into 4 experiments as follows:

3.1 Experiment I: Extraction and isolation of phenolic compounds using solvent polarity and evaluation of their biological activities.

3.2 Experiment II: Analysis of phenolic compounds in crude ethanolic extract and it fractions.

3.3 Experiment III: Fractionation of phenolic compounds using column chromatography and evaluation of their antioxidant activities.

3.4 Experiment IV: Identification and quantification of phenolic compounds in each fraction after Sephadex LH-20 column fractionation.

## 3.1 Experiment I: Extraction and isolation of phenolic compounds using solvent polarity and evaluation of their biological activities

In this experiment, we focused on the extraction and isolation of phenolic compounds present in *G. procumbens* leaves using solvent extraction. Additionally, the biological activities including antioxidant and anti-glycation of crude ethanolic extract and it solvent fractions were evaluated. The correlation between phenolic content and biological activities was also investigated in the present study. A diagram for the whole protocols in experimental I is shown in Fig. 3.1.

3.1.1 Chemicals and reagents

Folin–Ciocalteu reagent, 2,4,6-tripiridyl-S-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylenebenzothiozoline-6-sulphonic acid) (ABTS), catechin and gallic acid were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Butylated hydroxyanisole (BHA), 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (trolox), ascorbic acid and ethyl acetate were obtained from Fluka (Buchs, Switzerland). The methanol, ethanol, *n*-butanol and chloroform were purchased from Merck (Darmstadt, Germany). All chemicals and reagents used in this study were of analytical grade.

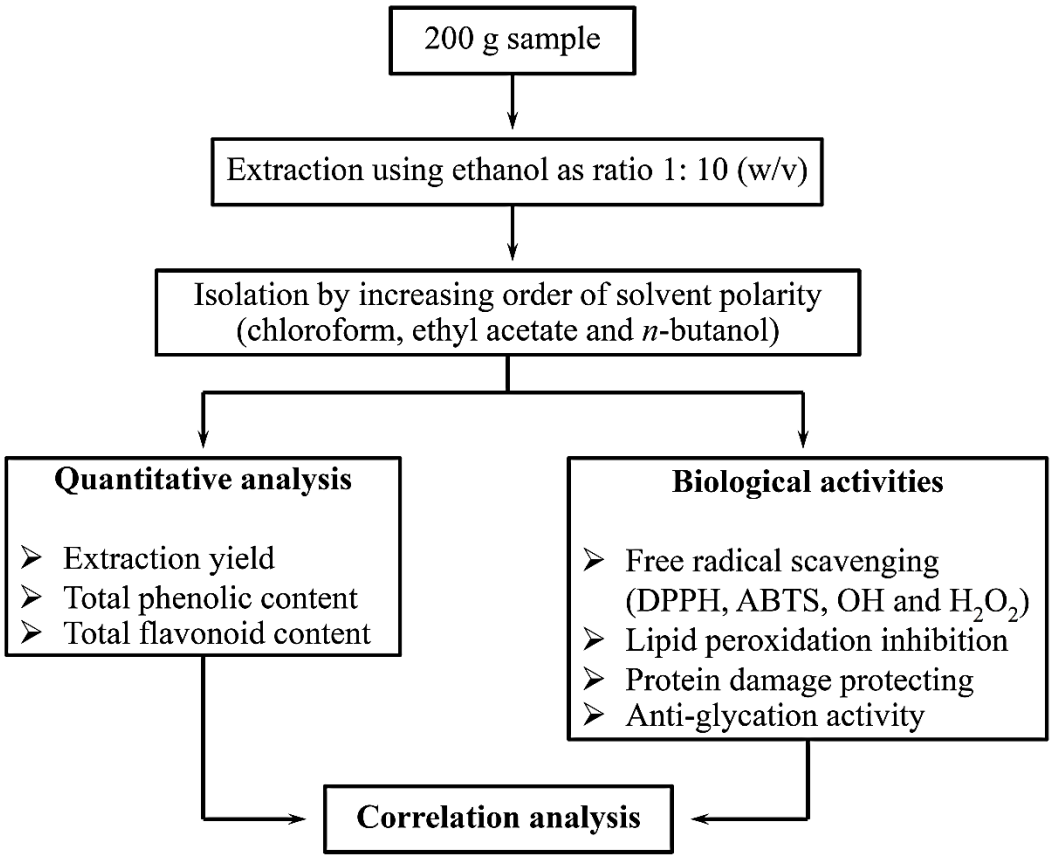



**Fig. 3.1** Diagram of the whole protocols in experiment I.

3.1.2 Plant materials and sample preparation

The fresh leaves of *G. procumbens* were collected in March 2013 from Khon Kaen Province, Thailand. Fresh leaves (5 kg) were washed to remove surface pollutants and dried under hot air at 60 °C for 48 h, as according to our pre-experiments, in which the temperature range of 30 to 90 °C was considered and we found that the contents of total phenolics and flavonoids increased up to a maximum at 60 °C and decreased at temperatures higher than 60 °C during the drying process. After drying, the sample was ground into a fine powder, passed through a 60 mesh sieve to get a particle size of approximately 210 μm (moisture content of 7% dry basis) and then stored at room temperature in desiccators until further analysis within 24 h.

3.1.3 Extraction and isolation of phenolic compounds

Phenolic compounds were extracted from *G. procumbens* leaves and then fractionated using three steps. Hot air-dried *G. procumbens* leaves (200 g) were

extracted with 2 l of ethanol and stirred for 3 h at room temperature. After centrifugation at 12,000 g for 30 min to remove the residue, the supernatant was filtered through filter paper (Whatman No. 1). This procedure was performed in triplicate, and then the filtrates were pooled and concentrated using a rotary evaporator under vacuum at 40 °C. The crude ethanolic extract (CEE) was obtained with a yield of 27.83% (w/w, on a dry weight basis). The crude ethanolic extract was further fractionated according to solvent polarity. The crude ethanolic extract (50.10 g) was dissolved in distilled water and stirred for 3 h followed by fractionation three times sequentially with chloroform, ethyl acetate and *n*-butanol. These three fractions were centrifuged, filtered and concentrated as described earlier. The yields of the chloroform, ethyl acetate and *n*-butanol fractions were 1.68, 6.40 and 8.64% (w/w, on a dry weight basis), respectively. All obtained fractions, including CEE were evaluated bioactive properties and determined bioactive components.

3.1.4 Determination of total phenolic content (TPC)

The TPC of the CEE and its fractions of *G. procumbens* were determined using the Folin-Ciocalteau method (Bakar *et al.*, 2009), with some modifications. Briefly, 200 µl of each extract solution was mixed with 1 ml of 10% (v/v) Folin-Ciocalteau reagent. After 5 min, 800 µl of 7.5% (w/v) sodium carbonate was added and the mixture was left to stand for 30 min at room temperature. The absorbance of the resulting blue colored solution was measured at 765 nm using a spectrophotometer (GENESYSTM 10, Thermo Scientific, USA). The TPC in samples was calculated based on the linear regression equation of the gallic acid standard curve ($y = 0.001x$; $R^2 = 0.998$). Results were expressed as mg gallic acid equivalents per g dry weight (mg GAE/g DW).

3.1.5 Determination of total flavonoid content (TFC)

The TFC of each fraction was determined using a modified colorimetric method as described previously (Bakar *et al.*, 2009). Each extract solution (250 µl) was mixed with 1.25 ml of distilled water and 75 µl of 5% $NaNO_2$ solution. The mixture was allowed to stand for 6 min and then 150 µl of 10% $AlCl_3$ was added to the solution. After 5 min, 500 µl of 1M NaOH was added to the mixture and made up to a total volume of 2.5 ml with distilled water. The absorbance of the solutions was measured at 510 nm using a spectrophotometer. The TFC in samples was calculated based on the

linear regression equation of the gallic acid standard curve ($y$ = 0.001$x$; $R^2$ = 0.999). Results were expressed as mg catechin equivalent per g dry weight (mg CE/g DW).

3.1.6 DPPH$^{\bullet}$ scavenging activity

The scavenging activity of the DPPH$^{\bullet}$ of the CEE and its fractions were measured according to a previously published method (Brand-Williams *et al.*, 1995), with some modifications. One half milliliter of each extract solution containing different concentrations of compounds was added to 0.5 ml of a freshly prepared 0.1 mM DPPH solution dissolved in 95% methanol. The mixture was shaken and left to stand at room temperature for 30 min in the dark. Absorbance was detected at 517 nm using a spectrophotometer (GENESYSTM 10, Thermo Scientific, USA) against a control. A decrease in the absorbance indicates an increase in the DPPH$^{\bullet}$ scavenging activity. Results were represented as $IC_{50}$ value, which is the concentration of the extract that scavenges 50% of the DPPH$^{\bullet}$.

3.1.7 ABTS$^{+\bullet}$ scavenging activity

The ABTS$^{+\bullet}$ decolorization activity of each extract was determined using a modified method as described previously (Re *et al.*, 1999). The ABTS$^{+\bullet}$ was generated by oxidation of 7 mM ABTS solution with 2.45 mM potassium persulfate at the ratio 1:1, and the mixture was left to stand in the dark at room temperature for 12-16 h before use. The resulting blue-green colored ABTS$^{+\bullet}$ solution was adjusted to an absorbance of 0.700 ± 0.020 at 734 nm. Briefly, 200 µl of each extract solution was introduced into the test tubes after the addition of 800 µl of the ABTS$^{+\bullet}$ solution. The mixture was incubated at room temperature for 6 min and the absorbance was measured at 734 nm against a control. Data were expressed as an $IC_{50}$ value, which is the concentration of the extract that scavenges 50% of the ABTS$^{+\bullet}$.

3.1.8 Hydroxyl radical scavenging activity

The $^{\bullet}$OH scavenging assay was carried out according to a method described previously (Zhang *et al.*, 2011), with some modifications. The hydroxyl radical was produced by the Fenton reaction between $FeSO_4$ and $H_2O_2$. Briefly, 60 µl of 0.75 mM 1,10-phenanthroline, 40 µl of 0.75 mM $FeSO_4$ and 600 µl of 50 mM phosphate buffer at pH 7.4 were introduced into the test tubes and mixed thoroughly. Then 100 µl of 0.5% $H_2O_2$ and 200 µl of each extract were added. The mixture was incubated at 37 °C for 60 min and the absorbance was measured at 536 nm using a spectrophotometer

(GENESYSTM 10, Thermo Scientific, USA). The results were expressed as $IC_{50}$ value, which is the concentration of the extract that scavenges 50% of the $^{\bullet}OH$.

3.1.9 Hydrogen peroxide scavenging activity

The hydrogen peroxide ($H_2O_2$) scavenging assay was carried out following the procedure of Gülçin *et al.* (2010) with some modifications. The principle of this method is that there is a decrease in the absorbance of $H_2O_2$ upon oxidation of the $H_2O_2$. A solution of 40 mM $H_2O_2$ was prepared in 50 mM phosphate buffer at pH 7.4. Then, 200 µl of each extract solution containing different concentrations was added into 2.2 ml of 50 mM phosphate buffer at pH 7.4 in the test tubes. After that, 600 µl of 40 mM $H_2O_2$ solution was added and incubated in a water bath at 25 °C for 30 min. The absorbance of the mixture was recorded at 230 nm using a UV-Vis spectrophotometer ($T80^+$, PG Instruments, UK). Data were expressed as $IC_{50}$ value, which is the concentration of the extract that scavenges 50% of the $H_2O_2$.

3.1.10 Linoleic acid peroxidation inhibition activity

The linoleic acid peroxidation inhibition activity of the CEE and its fractions were measured by the ferric thiocyanate method (FTC) as described previously (Ismail *et al.*, 2010). Briefly, 2 ml of each extract, 2 ml of 2.51% linoleic acid solution dissolved in 95% ethanol, 4 ml of 50 mM phosphate buffer at pH 7.0 and 2 ml of distilled water were mixed in a 10 ml vial with a screw cap. Then, the mixture was kept at 40 °C in the dark for several days; the accelerated oxidation of the linoleic acid was measured after 8 days of thermal treatment. The determination of the oxidation degree (as peroxide formation) was performed according to the FTC method. Thirty micro-litters of the reaction mixture was added to 2.91 ml of 75% ethanol, 30 µl of 30% ammonium thiocyanate and 30 µl of 20 mM $FeCl_2$ dissolved in 3.5% hydrochloric acid. After 3 min incubation, the degree of color development, which represents linoleic acid peroxidation, was measured at an absorbance of 500 nm using a UV-Vis spectrophotometer ($T80^+$, PG Instruments, UK). The absorbance of the mixture was measured every 24 h until a constant absorbance value was reached.

3.1.11 Protein damage protecting activity

The *in vitro* protein damage protecting activity of the CEE and its fractions were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The oxidative protein damage was induced by hydroxyl radicals generated in

the Fenton reaction between $CuSO_4$ and $H_2O_2$. Briefly, 100 µl of 5 mg/ml BSA, 20 µl of 10 mM $CuSO_4$, 20 µl of 0.2 M $H_2O_2$ and 160 µl of each fraction were mixed in an eppendorf tube, the reaction was started by the addition of $H_2O_2$. The mixture was incubated in a water bath at 37 °C for 20 min and then 120 µl of 5x loading dye was added to the mixture. This mixture was shaken and heated to 100 °C for 5 min. After cooling, 10 µl of each sample solution was loaded into a 12% SDS-PAGE separating gel. The gel was electrophoresed at a voltage of 180 volts in a running buffer at pH 8.3 for 45 min and stained with coomassie brilliant blue G-250 to determine the protein bands. After destaining, the gel was photographed and the intensity of the original-BSA band and damaged-BSA band were calculated using the 1D gel analysis software (ImageQuant TL version 7.0, GE Healthcare Bio-Sciences, Tokyo, Japan). The percentage of oxidative protein damage prevention activity was calculated using the following equation:

$$\text{Protein damage protecting activity (\%)} = [(P_s - P_0) / (P_1 - P_0)] \times 100$$

where $P_s$ is the peak of the highest protein band containing plant extracts; $P_0$ is the peak of the highest protein band containing $CuSO_4$ and $H_2O_2$ without plant extracts; and $P_1$ is the peak of the highest protein band without plant extracts, $CuSO_4$ and $H_2O_2$.

3.1.12 Evaluation of anti-AGEs formation activity

The anti-AGEs formation capacities of the CEE and its fractions were determined according to the method of Vinson and Howard (1996) with some modifications. A 2.5 ml total volume of glycation reaction solution including 500 µl of each plant extract, 500 µl of 20 mg/ml BSA in phosphate buffer, 500 µl of 0.5 M glucose in phosphate buffer and 1 ml of 0.1 M phosphate buffer at pH 7.4 containing 0.02% (w/v) sodium azide was prepared. The mixture was incubated in the dark at 37 °C for 5 days and then the amount of total fluorescent AGEs formed in the BSA-glucose system was determined using a fluorescent spectrometer (LS 50B, Perkin Elmer, USA) with an excitation wavelength of 330 nm and emission wavelength of 410 nm. The percentage of AGEs formation inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - (FI_{sample} - FI_{sample\ blank})/(FI_{control} - FI_{control\ blank})] \times 100$$

where $FI_{sample} - FI_{sample\ blank}$ was the difference between the fluorescent intensity of a sample incubated with glucose and that of one incubated without, and $FI_{control} - FI_{control\ blank}$ was the difference between the fluorescent intensity of the distillated water control with glucose and that of the control without glucose.

3.1.13 Correlation analysis

The correlations between different assays including TPC, TFC, antioxidant activity against $DPPH^{\bullet}$, $ABTS^{+\bullet}$, $^{\bullet}OH$ and $H_2O_2$ and anti-glycation assay were calculated using the correlation coefficient statistical option in the Pearson's test by the SPSS statistical software (SPSS 11.5 for Windows; SPSS Inc., Chicago, IL, USA).

3.1.14 Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was carried out to determine any significant differences in measurements using SPSS statistical software (SPSS 11.5 for Windows; SPSS Inc., Chicago, IL, USA). The significance of the differences between the means was determined using Duncan's test and the differences were considered to be significant at $p < 0.05$.

## 3.2 Experiment II: Analysis of phenolic compounds in crude ethanolic extract and it fractions

In this experiment, the crude ethanolic extract (CEE) and it fractions obtained from experiment I were further investigated with regard to identify and quantify the phenolic compounds using HPLC method. The phenolic compounds identified in our investigation can be classified into three groups according to their structures: flavonoids (rutin, myricetin, quercetine, apigenin and kaempferol), hydroxybenzoic acids (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic and syringic acids) and hydroxycinnamic acids (chorogenic, caffeic, *p*-coumaric, ferulic and sinapic acids). A diagram for the whole protocols in experiment II is shown in Fig. 3.2.

3.2.1 Chemicals and reagents

Standards of phenolic compounds, namely gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanilic acid, chorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid and sinapic acid, and standards of flavonoids such as

catechin, rutin, myricetin, quercetin, apigenin and kaempferol were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). HPLC grade methanol, ethanol, acetonitrile and acetic acid were purchased from purchased from Fisher Scientific (Leicester, UK).

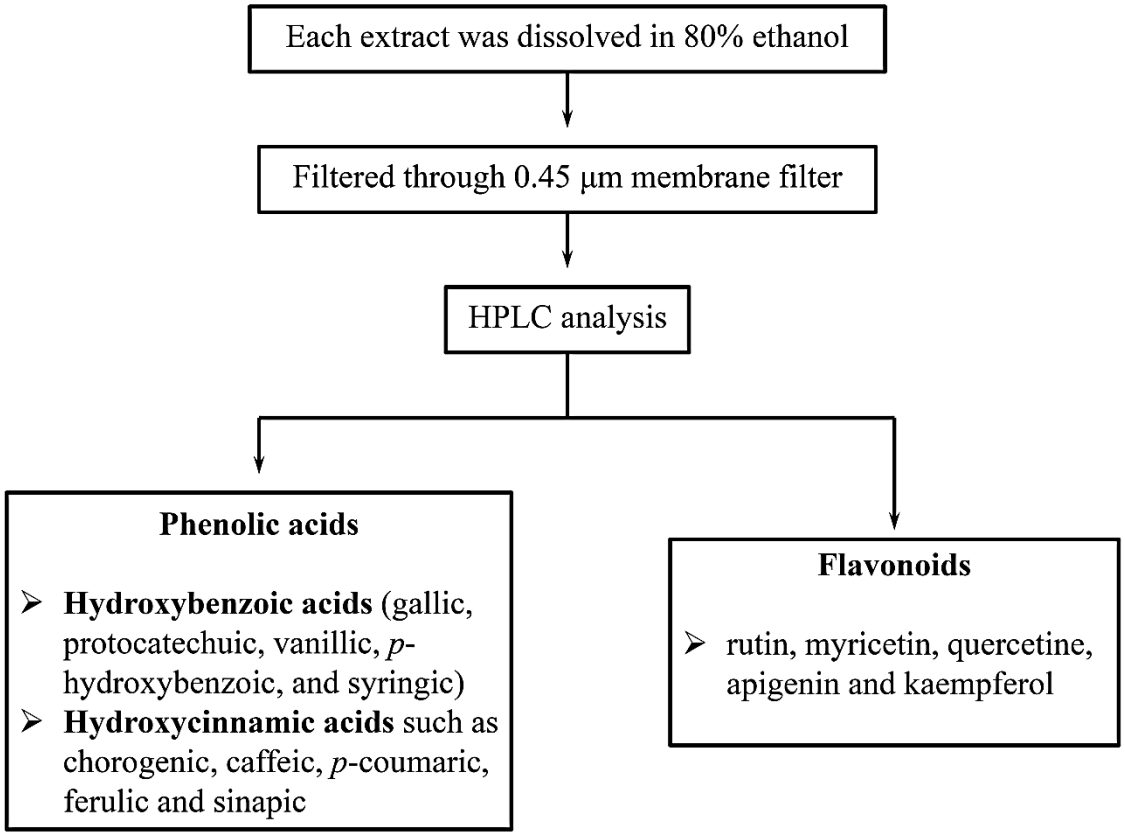



**Fig. 3.2** Diagram of the whole protocols in experiment II.

3.2.2 Preparations of samples and standard solutions

The CEE and it fractions were dissolved in 80% ethanol and then filtered through 0.45 μm membrane filters (VertiClean™ Nylon Syringe filter). The samples were diluted 10 or 20 fold using 80% ethanol before injection onto HPLC.

Stock standard solutions were prepared by dissolving accurately weighed standards in absolute ethanol to provide the concentration of 1 mg/ml. The standard solutions were prepared using serial dilution of the stock solutions with ethanol to the concentrations ranged between 6.25 and 100 μg/ml. Before injection onto HPLC, all of the standard solutions were filtered through 0.45 μm membrane filters.

3.2.3 Analysis of phenolic acids and flavonoids using HPLC

HPLC analysis was performed using Shimadzu LC-20AC pumps, SPD-M20A diode array detection and chromatographic separations were performed on a column Inetsil ODS-3, C18 (4.6 mm × 250 mm, 5 µm) (Hichrom Limited, Berks, UK). The composition of solvents and gradient elution conditions were described previously by Butsat *et al.* (2010) with some modifications. The mobile phase consisted of 1% acetic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 ml/min. Gradient elution was performed as follows: from 0 to 5 min, linear gradient from 5% to 9% solvent B; from 5 to 15 min, 9% solvent B; from15 to 22 min, linear gradient from 9% to 11% solvent B; from 22 to 38 min, linear gradient from 11% to 18% solvent B; from 38 to 43 min, linear gradient from 18% to 23% solvent B; from 43 to 44 min, linear gradient from 23% to 90% solvent B; from 44 to 45 min, linear gradient from 90% to 80% solvent B; from 45 to 55 min, isocratic at 80% solvent B; from 55 to 60 min, linear gradient from 80% to 5% solvent B; and a re-equilibration period of 5 min with 5% solvent B used between individual runs. Operating conditions were as follows: column temperature 38 °C, injection volume 20 µl and UV–diode array detection at 280 nm (phenolic acids) and 370 nm (flavonoids).

Phenolic compounds in the extracts were identified by a direct comparison of their retention times and their UV spectrum with those of authentic compounds. The contents of phenolic acids and flavonoids were calculated based on the linear regression equations ($y = ax + b$) from the external calibration curves of the standard. The calibration curves were performed by plotting the peak area (*y* axis) at 280 nm for hydroxybenzoic acids, 320 nm for hydroxycinnamic acids and 370 nm for flavonoids against concentrations of standard compounds (*x* axis) that ranged from 6.25-100 ppm, and the linear responses with the correlation coefficients ($R^2$) higher than 0.99 were obtained.

3.2.4 Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was carried out to determine any significant differences in measurements using SPSS statistical software (SPSS 11.5 for Windows; SPSS Inc., Chicago, IL, USA). The significance of the

differences between the means was determined using Duncan's test and the differences were considered to be significant at $p < 0.05$.

### 3.3 Experiment III: Fractionation of phenolic compounds using column chromatography and evaluation of their antioxidant activities

Since the ethyl acetate fraction (from experiment I & II) contained the highest phenolic content and showed the strongest antioxidant activity when compared to other the fractions. Thus, it was collected to further investigate in terms of fractionation and purification of phenolic antioxidants. A scheme for the all protocols in experiment III is shown in Fig. 3.3.

3.3.1 Fractionation of phenolic compounds

The ethyl acetate fraction was further sub-fractionated using Sephadex LH-20 column chromatography as follows (Amarowicz *et al.*, 2000). The ethyl acetate fraction (500 mg) was dissolved in 5 ml of 80% ethanol, filtered through a 25 mm syringe filter (0.45 µm Nylon filter, Whatman) and then loaded onto a 60 cm × 4.5 cm i.d. glass column packed with Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The sample was eluted with 85% methanol at a flow rate of 1.0 ml/min and fractions of 10 ml/tube were collected continuously. The absorbance of each tube was measured at 280 nm using a UV–Vis spectrophotometer ($T80^+$, PG Instruments, Leicestershire, UK) to identify each ethyl acetate fraction. Sub-fractions that had similar absorbance at 280 nm were pooled to major fractions and concentrated as described earlier in 3.1.3. The yields of sub-fraction 1 (collection tubes 9-24), sub-fraction 2 (collection tubes 25-43), sub-fraction 3 (collection tubes 44-64), sub-fraction 4 (collection tubes 65-87) and sub-fraction 5 (collection tubes 88-150) from the ethyl acetate fraction were 14.50, 37.80, 13.70, 7.28 and 6.65% (w/w, on a dry weight basis), respectively. All sub-fractions were determined the contents of total phenolic and total flavonoid as well as evaluated the antioxidant activity.

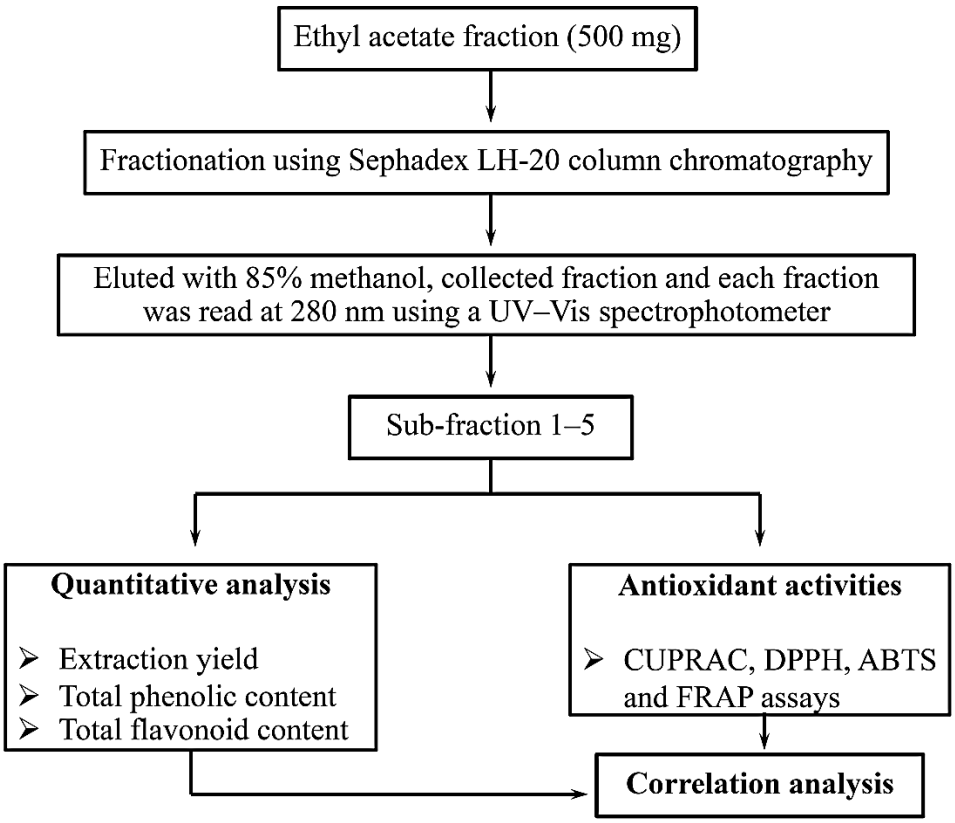



**Fig. 3.3** Scheme of the whole protocols in experiment III.

3.3.2 Determination of total phenolic content (TPC)

The TPC was determined using the Folin-Ciocalteau method (Bakar *et al.*, 2009). The more details of this method as described earlier in a section 3.1 under the heading "determination of total phenolic content" (see 3.1.4).

3.3.3 Determination of total flavonoid content (TFC)

The TFC was determined using a modified colorimetric method as described previously by Bakar *et al.* (2009), with some modifications. The more details of this protocol as described earlier in a section 3.1 (see 3.1.5).

3.3.4 Total antioxidant capacity assay

The total antioxidant capacity was determined using the cupric reducing antioxidant capacity (CUPRAC) method as described previously (Apak *et al.*, 2004). Briefly, 1 ml of 10 mM $CuCl_2$, 7.5 mM neocuproine and 1 M $NH_4Ac$ buffer pH 7.0 solutions were added to a test tube, followed by the addition of 0.1 ml of the extract and then the total volume was adjusted to 4.1 ml with distilled water. The mixture was left

to stand for 30 min at room temperature and the absorbance was measured at 450 nm using a spectrophotometer. Results were expressed as mg trolox equivalents antioxidant capacity (TEAC) per g dry weight (mg TEAC/g DW).

3.3.5 $DPPH^{\bullet}$ scavenging activity assay

$DPPH^{\bullet}$ scavenging activity was measured according to a previously published method (Brand-Williams *et al.*, 2008) as described earlier in a section 3.1.6.

3.3.6 $ABTS^{\bullet+}$ scavenging activity assay

$ABTS^{\bullet+}$ scavenging activity was measured using a modified method as described previously (Re *et al.*, 1999). The more details of this protocol as described earlier in sections 3.1.7.

3.3.7 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted as described by Benzie and Strain (1996). The FRAP reagent was freshly prepared by mixing 100 ml of 0.3 M acetate buffer pH 3.6, 10 ml of 10 mM TPTZ solution in 40 mM HCl and 10 ml of 20 mM $FeCl_3$ in a ratio of 10:1:1 and 12 ml of distilled water, at 37 °C. In brief, 60 µl of the extract, 180 µl of distilled water and 1.8 ml of FRAP reagent were added to the same test tube and thoroughly mixed. After incubation at 37 °C for 4 min, the absorbance was measured at 593 nm against a control. Data were calculated according to the following equation that was obtained with $FeSO_4$ from a calibration curve and then expressed as mmol Fe(II) per g dry weight (mmol Fe(II)/g DW).

3.3.8 Correlation analysis

The correlations between different assays including TPC, TFC and antioxidant activity against $DPPH^{\bullet}$, $ABTS^{+\bullet}$, CUPRAC and FRAP assays were calculated using the correlation coefficient statistical option in the Pearson's test by the SPSS statistical software (SPSS 11.5 for Windows; SPSS Inc., Chicago, IL, USA).

3.3.9 Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was carried out to determine any significant differences in measurements using SPSS statistical software (SPSS 11.5 for Windows; SPSS Inc., Chicago, IL, USA). The significance of the differences between the means was determined using Duncan's test and the differences were considered to be significant at $p < 0.05$.

### 3.4 Experiment IV: Identification and quantification of phenolic compounds in each fraction after Sephadex LH-20 column fractionation

In experiment IV, we investigated the phenolic components in all obtained sub-fractions after through column chromatographic purification to provide the possible bioactive compounds contributing to the antioxidant activity. The diagram for the whole protocol of this experiment is shown in Fig. 3.4.

3.4.1 Chemicals and reagents

All chemicals and reagents were used for analysis of phenolic compounds by using HPLC as described earlier in a section 3.2 (see 3.2.1).

3.4.2 Sample and standard solution preparations

The protocol of preparations of samples and standard solutions for analysis individual phenolic compounds by HPLC as described earlier in a section 3.2.2.

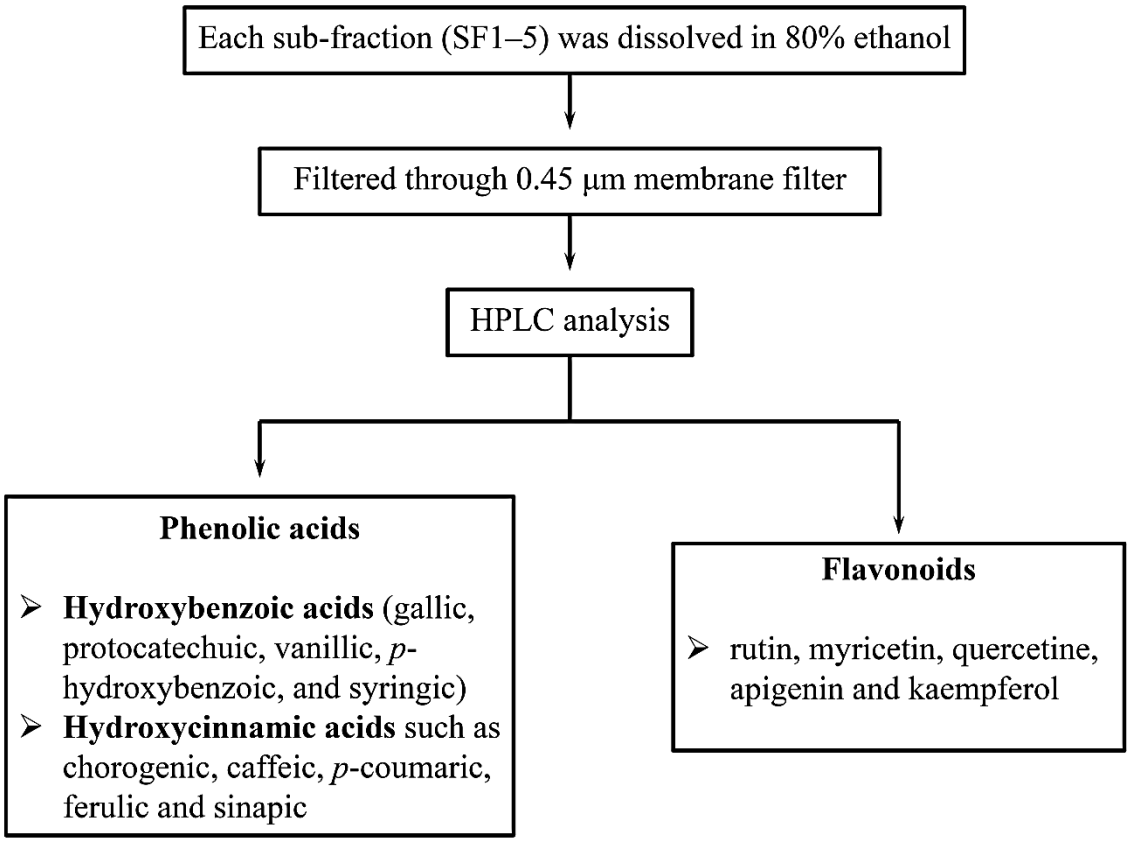



**Fig. 3.4** Diagram of the whole protocols in experiment IV.

3.4.3 Analysis of phenolic acids and flavonoids by HPLC

HPLC analysis was performed using Shimadzu LC-20AC pumps, SPD-M20A diode array detection and chromatographic separations on a column Inetsil ODS-3, C18 (4.6 mm × 250 mm, 5 µm) (Hichrom Limited, Berks, UK). The compositions of the mobile phase and gradient elution conditions were described previously by Butsat and Siriamornpun (2010). The mobile phase used were acetic acid pH 2.74 (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 ml/min. The gradient elution was performed as follows: from 0 to 5 min, linear gradient from 5 to 9% solvent B; from 5 to 15 min, 9% solvent B; from15 to 22 min, linear gradient from 9 to 11% solvent B; from 22 to 38 min, linear gradient from 11 to 18% solvent B; from 38 to 43 min, linear gradient from 18 to 23% solvent B; from 43 to 44 min, linear gradient from 23 to 90% solvent B; from 44 to 45 min, linear gradient from 90 to 80% solvent B; from 45 to 55 min, isocratic at 80% solvent B; from 55 to 60 min, linear gradient from 80 to 5% solvent B; and a re-equilibration period of 5 min with 5% solvent B used between individual runs. The working conditions were as follows: column temperature 38 °C, injection volume 20 µl and UV-diode array detection at 280 nm for phenolic acids or 370 nm for flavonoids. The methods of identification and quantification of phenolic compounds in the samples as described earlier in a section 3.2.3.

3.4.4 Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was carried out to determine any significant differences in measurements using SPSS statistical software (SPSS 11.5 for Windows; SPSS Inc., Chicago, IL, USA). The significance of the differences between the means was determined using Duncan's test and the differences were considered to be significant at $p < 0.05$.

# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Experiment I: Extraction and isolation of phenolic compounds using solvent polarity and evaluation of their biological activities

### 4.1.1 Extraction yield, TPC and TFC

The percentage yield of the CEE of *G. procumbens* leaves and its different fractions are shown in Table 4.1. The extraction yield of these samples varied from 1.68% to 27.83% (w/w) with a descending order of CEE > BF > EAF > CF ($p < 0.05$). These results indicated that extraction may give significantly different compositions and ratios in its constituents. Extraction is the first step in the recovery of bioactive compounds especially phenolic compounds from plant materials, which is an important process and various techniques have been studied. The yield, composition and purity of phenolic compounds recovered from plant materials is dependent on the chemical nature, sample size, extraction method and conditions, as well as the presence of interfering substances (Cheng *et al.*, 2012).

Phenolic compounds have received increasing attention because of their biological activities and health benefits due to their potent antioxidants (Shahidi and Naczk, 2004). Table 4.1 summarizes the total phenolic content (TPC) in the CEE and its fractions determined through a linear gallic acid standard curve ($y = 0.001x$; $R^2 = 0.999$) and expressed as gallic acid equivalents (GAE). The TPC of these fractions varied from 0.77 to 24.36 mg GAE/g DW. The highest TPC was detected in the EAF (24.36 mg GAE/g DW), whereas the lowest TPC was measured in the CF (0.77 mg GAE/g DW). The TPC of each fraction was arranged in the following descending order: EAF > CEE > BF > CF ($p < 0.05$). These results indicated an increased concentration of phenolics, with probably more pronounced antioxidative attributes, in the EAF. The extractability of a compound is strongly influenced by the medium, conditions and nature of the extractant (Cho *et al.*, 2011). Our findings are in good agreement with some previous studies that reported the total phenolic content of EAF was higher than other solvent fractions for *Allium cepa* (Singh *et al.*, 2009). This suggests that EAF might be the part

that is rich in phenolic compounds in many plants. Several studies have revealed that the phenolic content in the plants is associated with their antioxidant activities, probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Ismail *et al.*, 2010).

**Table 4.1** Extraction yield, TPC and TFC of CEE and its solvent fractions isolated from *G. procumbens* leaves.

| Extracts | Yield (%) | TPC (mg GAE/g DW) | TFC (mg CE/g DW) | TFC/TPC (%) |
|---|---|---|---|---|
| CEE | $27.83 \pm 1.11^{a}$ | $16.08 \pm 0.38^{b}$ | $10.33 \pm 0.88^{b}$ | 64.24 |
| CF | $1.68 \pm 0.70^{d}$ | $0.77 \pm 0.20^{d}$ | $0.08 \pm 0.00^{d}$ | 10.38 |
| EAF | $6.40 \pm 0.93^{c}$ | $24.36 \pm 1.11^{a}$ | $17.33 \pm 1.39^{a}$ | 71.14 |
| BF | $8.64 \pm 0.09^{b}$ | $5.92 \pm 1.61^{c}$ | $4.92 \pm 0.29^{c}$ | 83.11 |

Values are expressed as mean ± SD of triplicate measurements. Means with different letters in the same column represent significant differences at $p < 0.05$.

Flavonoids are the most common and widely distributed group of plant phenolic compounds, and they are usually very effective antioxidants (Gülçin *et al.*, 2010). In this study, the total phenolic content (TFC) of the solvent fractions from *G. procumbens* was evaluated by the aluminum colorimetric assay. Catechin was used as a standard ($y = 0.001x$; $R^2 = 0.999$) and results were expressed as catechin equivalents (CE). The TFC of each solvent fraction varied considerably from 0.08 to 17.33 mg CE/g DW. The data presented in Table 4.1 indicates that the highest TFC of 17.33 mg CE/g DW was observed in the EAF and the lowest content was observed in the CF (0.08 mg CE/g DW). The TFC of each solvent fraction was arranged in the following order: EAF > CEE > BF > CF ($p < 0.05$). These amounts were comparable to the results described in the literature for other extracts form *Camellia sinensis*, *Ficus bengalensis* and *Ficus racemosa* (Manian *et al.*, 2008). Flavonoid rich plants could be good sources of antioxidants that would help to increase the overall antioxidant capacity of an organism (Sharififar *et al.*, 2009). The relationships between the TPC and TFC were represented as percentages of TPC/TFC. The ratios of TPC to the TFC ranged

from 10.38% to 83.11% (Table 4.1). From these results it can be understood that in the EAF, CEE and BF of *G. procumbens* more than 28.28%, 35.76% and 16.89% of the TPC, respectively, were flavonoid compounds rather than other phenolic compounds.

4.1.2 Free radical scavenging activity

The CEE and its fractions of *G. procumbens* leaves showed concentration dependent free radical scavenging activities as assayed by $DPPH^{\bullet}$, $ABTS^{+\bullet}$, $^{\bullet}OH$ and hydrogen peroxide ($H_2O_2$) (data not shown). The antioxidant activities of each extract were expressed as $IC_{50}$ value (Table 4.2).

DPPH has been extensively used as a free radical to determine the antioxidant substances that reduce $DPPH^{\bullet}$ by donating a hydrogen radical to form the DPPH non-radical (Cho *et al.*, 2011). The $DPPH^{\bullet}$ scavenging activity of all the fractions from *G. procumbens* was expressed as $IC_{50}$ values (Table 4.2). The $IC_{50}$ values of all the tested samples as determined from their $DPPH^{\bullet}$ scavenging activity were in the range 0.22 to 3.33 mg/ml. The $DPPH^{\bullet}$ scavenging activity showed the highest value in the EAF ($IC_{50}$ = 0.22 mg/ml), whereas the CF showed the lowest value ($IC_{50}$ = 3.33 mg/ml). $DPPH^{\bullet}$ scavenging activity was arranged in the following descending order: BHA ≈ ascorbic acid ≈ trolox > EAF > CEE > BF > CF ($p < 0.05$). The observed differential scavenging activities of the fractions against the $DPPH^{\bullet}$ system could be due to the presence of different compounds in the fraction. The $DPPH^{\bullet}$ scavenging activities of all fractions were less than ($p < 0.05$) those of the standard antioxidants tested (BHA, ascorbic acid and trolox). However, the $DPPH^{\bullet}$ scavenging activity of EAF from *G. procumbens* was considerably higher than the capacity of ethyl acetate fractions of other plants reported from previous studies such as *Nigella sativa* (Mariod *et al.*, 2009). The results indicate that the EAF and other fractions have free radical scavenger or inhibitors, acting possibly as primary antioxidants.

Meanwhile, $ABTS^{+\bullet}$ scavenging activity of the CEE and other fractions showed $IC_{50}$ values in the ranging 0.06 to 0.90 mg/ml (Table 4.2). Among all tested fractions, the EAF exhibited the strongest $ABTS^{+\bullet}$ scavenging activity ($IC_{50}$ = 0.06 mg/ml), whereas the CF showed the lowest antiradical activity ($IC_{50}$ = 0.90 mg/ml). The $ABTS^{+\bullet}$ scavenging activity of all the tested samples was in the following descending order: BHA ≈ trolox > ascorbic acid ≈ EAF > CEE > BF > CF ($p < 0.05$). This finding was in good agreement with that of Singh et al. (2007) who reported that

the extraction with ethyl acetate gave the highest antiradical activity. Moreover, the $ABTS^{+\bullet}$ scavenging activity of the EAF form *G. procumbens* was the most similar to ascorbic acid ($IC_{50}$ = 0.06 mg/ml). Deetae *et al.* (2012) investigated the antioxidant activity of herbal teas from different plants and verified that the phenolic compounds were important scavengers of $ABTS^{+\bullet}$. This may be due to the presence of a high phenolic content in the EAF, as phenolic compounds play a vital role as antioxidants in living systems.

In this study, the Fenton reaction was used to generated $^{\bullet}OH$ and investigate the scavenging activity of the CEE and its fractions toward $^{\bullet}OH$. The $^{\bullet}OH$ scavenging activities of all tested samples are in the following descending order: BHA ≈ trolox ≈ EAF > ascorbic acid > CEE > BF > CF. The results shown in Table 4.2 indicate that the highest $^{\bullet}OH$ scavenging activity with an $IC_{50}$ value of 0.01 mg/ml was detected in the EAF and the lowest antiradical activity was observed in the CF ($IC_{50}$ = 0.51 mg/ml). Moreover, the $^{\bullet}OH$ scavenging activity of the EAF was higher than that of ascorbic acid as a standard reference ($IC_{50}$ = 0.10 mg/ml) and also exhibited the strongest capacity that was similar to the standard synthetic antioxidants (BHA and trolox). The results suggested that the $^{\bullet}OH$ scavenging activity of the EAF obtained from the CEE of *G. procumbens* was comparable to or even more effective than those of ascorbic acid as a standard antioxidant. Our study indicated that the EAF exhibited a scavenging effect on $^{\bullet}OH$ that could help to prevent oxidative damage in the human body.

The $H_2O_2$ scavenging abilities of CEE and its fractions are shown in Table 4.2, and compared to those of commercial synthetic antioxidants (BHA, ascorbic acid and trolox), which were reference compounds. The $IC_{50}$ values of the $H_2O_2$ scavenging activity of all fractions ranged from 0.03 to 2.31 mg/ml. Among all the tested fractions, EAF exhibited the strongest $H_2O_2$ scavenging activity ($IC_{50}$ = 0.03 mg/ml), whereas CF showed the lowest $H_2O_2$ scavenging activity ($IC_{50}$ = 2.31 mg/ml). The $H_2O_2$ scavenging activity was arranged in the following descending order: ascorbic acid ≈ BHA ≈ trolox ≈ EAF > CEE > BF > CF ($p < 0.05$). These results suggested that the EAF exhibited potential to be a $H_2O_2$ scavenger, which was similar to those of the synthetic antioxidants tested (ascorbic acid, BHA and trolox). Since $H_2O_2$ itself is not very reactive, it can sometimes be toxic to cells because it may give rise to $^{\bullet}OH$ within the cells. The toxicity of $H_2O_2$ derives from its conversion to an $^{\bullet}OH$ formation. Giving

$H_2O_2$ to cells in culture can lead to transition metal ion-dependent $^{\bullet}OH$ mediating oxidative DNA damage. Levels of $H_2O_2$ at or below about 20-50 µg/cell have limited cytotoxicity to many cell types. Thus, removing hydrogen peroxide as well as the superoxide anion is very important for protection of pharmaceuticals and food systems (Chai *et al.*, 2003). Our findings demonstrated the EAF showed the highest antioxidant activity through all *in vitro* assays (DPPH$^{\bullet}$, $ABTS^{+\bullet}$, $^{\bullet}OH$ and $H_2O_2$), possibly because the EAF contains the highest amounts of phenolics and flavonoids. The antioxidant activity of a plant extract depends on the type and polarity of the solvent, the isolation procedures and the purity of the active compounds as well as the test system used to evaluate the activity (Cheng *et al.*, 2012).

In summary, the EAF demonstrated the TPC and TFC, accompanied by the highest antioxidant activity, compared to other fractions through all assays. Ethyl acetate seems to be the solvent that concentrates the best antioxidant substances of intermediated polarity. This is in accordance with previous findings in *Smilax sebeana* Miq (Ao *et al.*, 2011) and *Allium cepa* (Singh *et al.*, 2009). The antioxidant activity of a plant extract depends on the type and polarity of the solvent, the isolation procedures and the purity of the active compounds as well as the test system used to evaluate the activity (Chan *et al.*, 2014; Cheng *et al.*, 2012).

**Table 4.2** Free radical scavenging activities of CEE and its fractions isolated from *G. procumbens* against DPPH, $ABTS^{+}$, OH and $H_2O_2$ radicals.

| Extracts | $DPPH^{\bullet}$ scavenging | $ABTS^{+\bullet}$ scavenging | $^{\bullet}OH$ scavenging | $H_2O_2$ scavenging |
|---|---|---|---|---|
| | $IC_{50}$ [a] (mg/ml) | $IC_{50}$ [a] (mg/ml) | $IC_{50}$ [a] (mg/ml) | $IC_{50}$ [a] (mg/ml) |
| CEE | $0.47 \pm 0.02^{c}$ | $0.22 \pm 0.00^{c}$ | $0.12 \pm 0.01^{c}$ | $0.27 \pm 0.00^{c}$ |
| CF | $3.33 \pm 0.06^{a}$ | $0.90 \pm 0.01^{a}$ | $0.51 \pm 0.01^{a}$ | $2.31 \pm 0.03^{a}$ |
| EAF | $0.22 \pm 0.01^{d}$ | $0.06 \pm 0.00^{d}$ | $0.01 \pm 0.00^{e}$ | $0.03 \pm 0.00^{d}$ |
| BF | $0.71 \pm 0.03^{b}$ | $0.49 \pm 0.01^{b}$ | $0.23 \pm 0.00^{b}$ | $0.88 \pm 0.02^{b}$ |
| Trolox [b] | $0.04 \pm 0.00^{e}$ | $0.01 \pm 0.00^{e}$ | $0.01 \pm 0.00^{e}$ | $0.02 \pm 0.00^{d}$ |
| BHA [b] | $0.02 \pm 0.00^{e}$ | $0.01 \pm 0.00^{e}$ | $0.01 \pm 0.00^{e}$ | $0.02 \pm 0.00^{d}$ |
| Ascorbic acid [b] | $0.03 \pm 0.00^{e}$ | $0.06 \pm 0.00^{d}$ | $0.10 \pm 0.00^{d}$ | $0.01 \pm 0.00^{d}$ |

[a] The concentration of the plant extract that scavenges 50% of free radical. Lower $IC_{50}$ values indicate higher radical scavenging activity.

[b] Standard synthetic antioxidants were used as a reference for radical scavenging activity.

Results are expressed as mean ± SD of triplicate measurements. Means with different letters in the same column represent significant differences at $p < 0.05$.

4.1.3 Inhibition of linoleic acid peroxidation

The ferric thiocyanate (FTC) method measures the amount of peroxide produced during the initial stages of oxidation, which are the primary products of oxidation. Fig. 4.1 shows the linoleic acid peroxidation inhibitory activity of the CEE and its fractions from *G. procumbens*, as well as commercial synthetic antioxidants as obtained from the FTC assay. As shown in Fig. 4.1, all tested fractions significantly retarded the formation of hydroperoxides in the linoleic acid emulsion system throughout the incubation times as compared to the control sample ($p < 0.05$). From the third day onwards, the absorbance value of the control was higher than the other samples and reached its maximum absorbance on the eighth day of incubation. The overall inhibitory activity of all samples against hydroperoxides formation can be established in the following descending order: trolox > BHA > EAF > ascorbic acid > CEE > BF > CF > control ($p < 0.05$). EAF exhibited a higher lipid peroxidation inhibitory activity than the other fractions, including ascorbic acid. The lipid peroxidation inhibitory activity of EAF was 73.44% at the eighth day, which was higher than that of ascorbic acid (62.48%). These findings clearly indicate that EAF had an effective and potent antioxidant activity in the FTC assay. Consequently, this result suggests that EAF might contain primary antioxidant compounds, which are able to react aggressively with free radicals, particularly hydroxyl radicals, thereby terminating the radical-chain reaction and retarding the formation of hydroperoxides (Shahidi and Wanasundara, 1992).

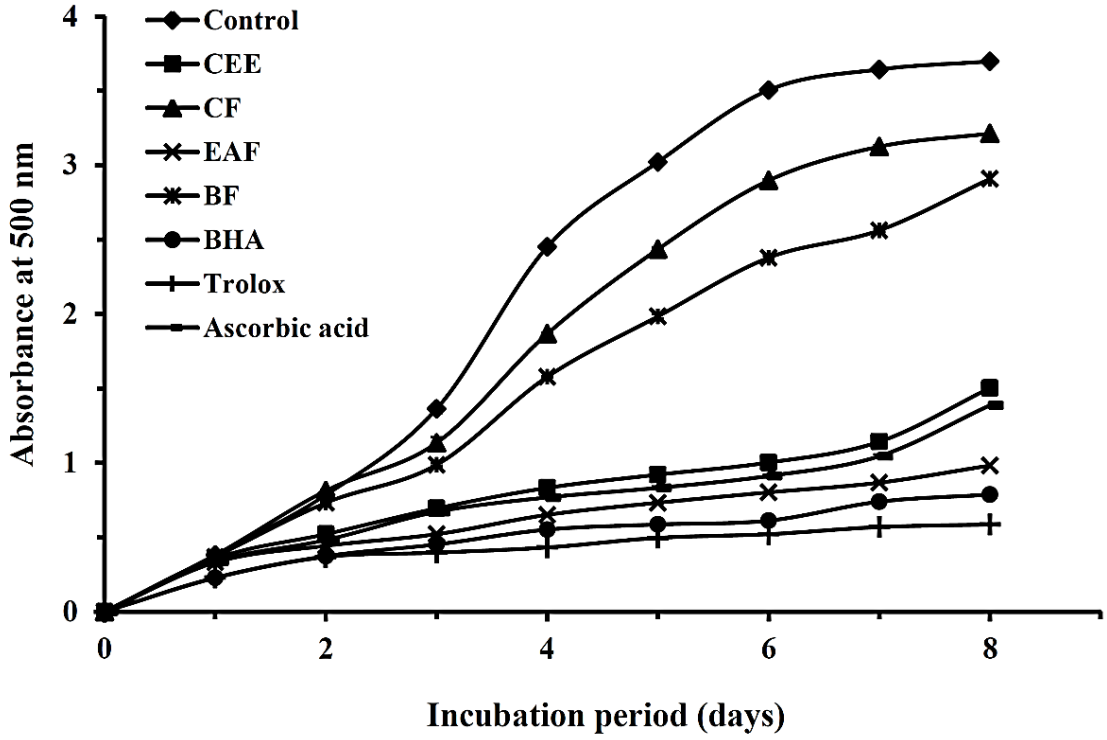



**Fig. 4.1** Linoleic acid peroxidation inhibitory activity of the CEE and its solvent fractions from *G. procumbens* leaves and standards through the ferric thiocyanate test.

4.1.4 Oxidative protein damage protecting activity

To study the oxidative protein damage protecting effect of CEE and it fractions on hydroxyl radical-mediated BSA protein, the incubation of BSA protein with Fenton reagent ($CuSO_4$ and $H_2O_2$) for 20 min in a water bath resulted in the production of $^{\bullet}OH$, leading to protein damage induced by $^{\bullet}OH$ generated in the Fenton reaction. The gel patterns of the BSA protein exposed to Fenton reagent in the presence and absence of plant extracts are presented in Fig. 4.2. As shown in Fig. 4.2, all tested fractions possessed significant protective effects ($p < 0.05$) by restoring the band intensity of the BSA protein when compared to the negative control. The percentage of oxidative protein damage protection was calculated using the intensity of BSA protein from the 1D gel analysis software as shown in Table 4.3. The protein damage protecting activity of all the tested samples is as follows: BHA > EAF > trolox > ascorbic acid ≈ BF ≈ CEE > CF ($p < 0.05$). The inhibition activity of the protein damage for all samples

increased with an increase in fraction concentration. At a concentration of 80 µg/ml, the EAF showed the highest protein damage protection activity (68.89%), which was superior to ascorbic acid (23.07%) and trolox (48.72%). These results indicated that the EAF possesses strong efficiency and potency when protecting against oxidative protein damage induced by $^{\bullet}OH$ generated in the Fenton reaction. This is the first report to date on the protective effect of *G. procumbens* leaves against protein damage induced by hydroxyl radicals generated in the Fenton reaction and it may have a positive role in inhibiting several stress or toxicity induced-protein oxidations.

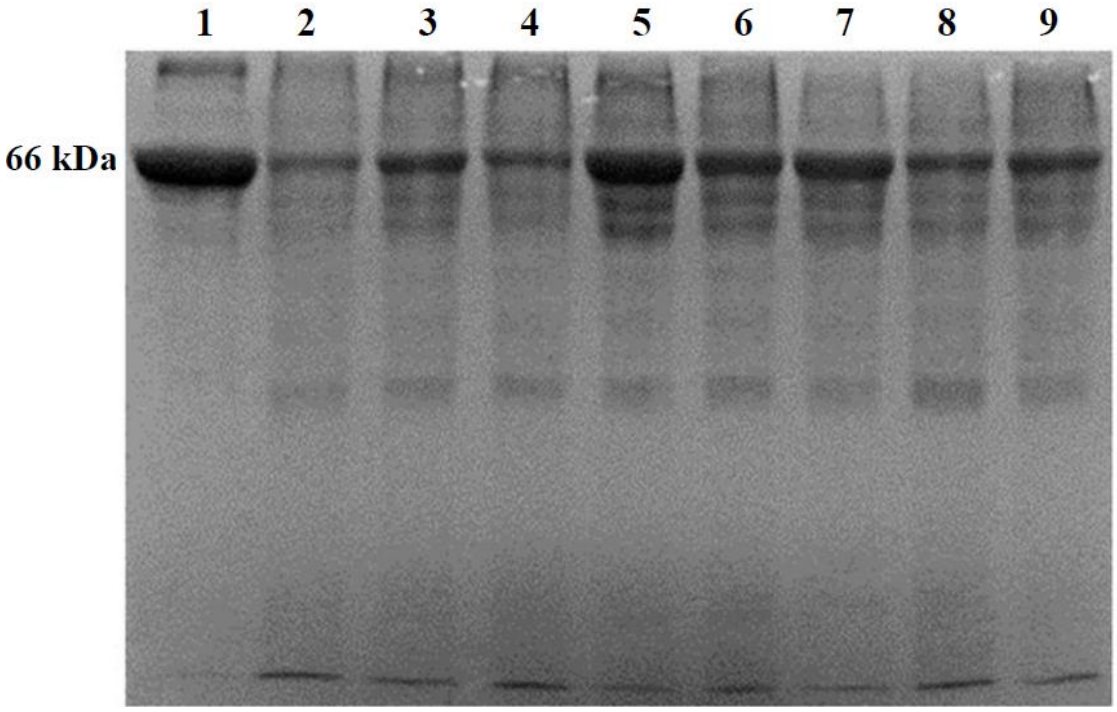



**Fig. 4.2** SDS-PAGE profile of CEE and its fractions and synthetic antioxidants on oxidative protein damage protecting activity induced with the Fenton reagent. Lane 1; BSA marker (positive control), Lane 2; BSA + Fenton reagent (negative control) and Lanes 3-9; BSA + Fenton reagent in the presence of CEE, CF, EAF, BF, BHA, ascorbic acid and trolox at a concentration of 80 µg/ml, respectively.

**Table 4.3** Inhibitory activity of CEE and its solvent fractions against oxidative protein damage induced by hydroxyl radicals generated in the Fenton reaction.

| Extract | Protein damage protecting activity (%) | | |
|---|---|---|---|
| | Concentration (µg/ml) | | |
| | 20 | 40 | 80 |
| CEE | 11.67 ± 0.37[c] | 15.97 ± 1.87[c] | 21.35 ± 1.21[d] |
| CF | ND | ND | 5.02 ± 1.62[e] |
| EAF | 48.18 ± 1.88[b] | 66.25 ± 0.81[a] | 68.89 ± 1.13[b] |
| BF | 13.23 ± 1.85[c] | 21.37 ± 1.55[b] | 22.11 ± 1.60[d] |
| Trolox [a] | 14.67 ± 2.86[c] | 16.34 ± 3.39[c] | 48.72 ± 1.06[c] |
| BHA [a] | 61.80 ± 0.59[a] | 65.42 ± 1.77[a] | 71.60 ± 1.68[a] |
| Ascorbic acid [a] | 7.61 ± 1.83[d] | 13.72 ± 1.30[c] | 23.07 ± 1.20[d] |

[a] Standard synthetic antioxidants were used as a references. ND = not detected.
Values are expressed as mean ± SD of triplicate measurements. Means with different letters in the same column represent significant differences at $p < 0.05$.

4.1.5 Anti-advances glycation end-products (AGEs) formation activity

Glycation of protein alters the biological activity of the protein and initiates its degradation and conversion to AGEs. Endogenous AGEs formation is known to contribute to the progression of pathogenesis under conditions associated with diabetic complications, Alzheimer's disease and aging (Mosihuzzman *et al.*, 2013). We focused on the potential benefits of exploiting *G. procumbens* as a medicinal plant having anti-glycation properties. To confirm the potent inhibitory effect of this plant, we first evaluated its ability to inhibit the formation of AGEs in a glucose-mediated protein glycation system. The results presented in Table 4.4 show that the anti-glycation activity of all the fractions was arranged in the following descending order: EAF ≈ CEE > BF > CF ($p < 0.05$). The inhibition activity of protein glycation of all the samples increased with an increase in fraction concentration. At a concentration of 0.20 mg/ml, EAF exhibited the highest anti-glycation activity (99.86%), followed by CEE (96.27%), BF (87.24%) and CF (66.02%). These findings are in good agreement with Sun *et al.*

(2012) who reported that the ethyl acetate fractions of *Chlorella pyrenoidosa* and *Nitzschia laevis* had the highest anti-AGEs formation activities (86.35% and 91.68%, respectively). The concentrations of the fractions that inhibit 50% of the formation of AGEs in the BSA-glucose system ($IC_{50}$ values) ranged from 41.91 to 145.86 µg/ml (Table 4.4). Previous studies reported the anti-glycation activity of standard anti-glycation agents, catechin (81%) at the concentration of 0.5 mg/ml (Wang *et al.*, 2011) and rutin (82.5%) at the concentration of 3mM or 1.83 mg/ml (Mosihuzzman *et al.*, 2013). In this study, we revealed that the EAF, CEE and BF of *G. procumbens* showed stronger anti-AGEs formation capacity than that of the standards catechin and rutin. Consequently, this result suggested that EAF, CEE and BF had strong efficiency and potency to inhibit the formation of AGEs in the glucose-mediated protein glycation system. The evidence provided in this study is useful for designing further studies and to investigate these anti-glycation agents for the management of late diabetic complications *in vivo*.

**Table 4.4** Inhibition activity of CEE and its solvent fractions on the fluorescent AGEs formation in a BSA-glucose system.

| Extracts | Inhibition of AGEs formation (%) | | | | $IC_{50}$[a] (µg/ml) |
|---|---|---|---|---|---|
| | Concentration (µg/ml) | | | | |
| | 40 | 80 | 160 | 200 | |
| CEE | 46.61 ± 6.59[a] | 61.33 ± 3.12[b] | 87.74 ± 2.42[b] | 96.27 ± 0.45[a] | 51.71 |
| CF | 14.76 ± 4.34[c] | 36.73 ± 4.23[c] | 52.56 ± 3.31[d] | 66.02 ± 3.60[c] | 145.86 |
| EAF | 50.28 ± 2.64[a] | 73.35 ± 2.86[a] | 94.48 ± 1.69[a] | 99.86 ± 0.15[a] | 41.91 |
| BF | 37.96 ± 1.11[b] | 56.62 ± 4.09[b] | 77.72 ± 3.31[c] | 87.24 ± 2.04[b] | 62.71 |

[a] The concentration of the plant extract that inhibits 50% of AGEs formation. Lower $IC_{50}$ values indicate higher anti-AGEs formation activity. Values are expressed as mean ± SD of triplicate measurements. Means with different letters in the same column represent significant differences at $p < 0.05$.

4.1.6 Correlation analysis

The correlation coefficients between the mean values obtained from each assay were analyzed by performing a Pearson test (Table 4.5). The TPC and TFC were strongly positively correlated with the radical scavenging assays against DPPH$^{\bullet}$ (r = 0.986 and r = 0.956, respectively), ABTS$^{+\bullet}$ (r = 0.918 and r = 0.984, respectively), $^{\bullet}$OH (r = 0.819 and r = 0.931, respectively), $H_2O_2$ (r = 0.858 and r = 0.937, respectively) and anti-AGEs formation capacity (r = 0.856 and r = 0.940, respectively) ($p < 0.01$). The amount of phenolics, especially flavonoids, present in the leaves of *G. procumbens* was the key determinant for their capacities of primary antioxidants and anti-AGEs formation. These results are in good agreement with previous studies that reported a strongly positive correlation between phenolic content, antioxidant and anti-AGEs formation (Deetae *et al.*, 2012).

**Table 4.5** Correlation coefficients (r) for relationships between TPC, TFC, different antioxidant assays and anti-AGEs formation.

| | (1) | (2) | (3) | (4) | (5) | (6) | (7) |
|---|---|---|---|---|---|---|---|
| (1) TPC | 1 | 0.944** | 0.986** | 0.918** | 0.819** | 0.858** | 0.856** |
| (2) TFC | - | 1 | 0.956** | 0.984** | 0.931** | 0.937** | 0.940** |
| (3) DPPH$^{\bullet}$ | - | - | 1 | 0.940** | 0.818** | 0.840** | 0.887** |
| (4) ABTS$^{+\bullet}$ | - | - | - | 1 | 0.905** | 0.915** | 0.922** |
| (5) $^{\bullet}$OH | - | - | - | - | 1 | 0.896** | 0.948** |
| (6) $H_2O_2$ | - | - | - | - | - | 1 | 0.852** |
| (7) Anti- AGEs | - | - | - | - | - | - | 1 |

**Asterisks indicate a significant correlation between two variants ($p < 0.01$).

The anti-AGEs formation capacities were strongly positively correlated to DPPH$^{\bullet}$, ABTS$^{+\bullet}$, $^{\bullet}$OH and $H_2O_2$ radicals' scavenging activities with values of r = 0.887, r = 0.922, r = 0.948 and r = 0.852, respectively (Table 4.5). These results indicated that the efficiency of anti-AGEs formation depended on the capacities of the antioxidants. Our results are also in good agreement with Deetae *et al.* (2012) who found a strongly

positive correlation between antioxidant and anti-AGEs formation in different herbal teas. Wu and Yen (2005) demonstrated that phenolic compounds inhibited AGEs formation through scavenging free radicals and antioxidant capacities. These results demonstrated that phenolic compounds may be responsible for antioxidant and anti-glycation potentials.

## 4.2 Experiment II: Analysis of phenolic compounds in crude ethanolic extract and it fractions

### 4.2.1 Composition and content of phenolic acids in CEE and it fractions

The CEE and its derived fractions had their phenolic compositions determined by HPLC to evaluate the presence of hydroxybenzoic acids (HBA) and hydroxycinnamic acid (HCA); in addition, the total phenolic acid (TPA) was also investigated in the present study. The HPLC chromatogram of phenolic acid standard and phenolic acid identified in CEE and it ethyl acetate fraction are shown in Fig. 4.3. The individual phenolic acids in samples were identified by a direct comparison of their retention times with those of authentic compounds. The contents of flavonoids in each sample were calculated based on the external calibration curves of the standard and the data shown in Table 4.6. For HBA, four phenolic compounds were identified, namely gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid and vanillic acid, which were detected in CEE and EAF. However, vanillic acid was not detected in CF and BF, whereas protocatechuic acid and *p*-hydroxybenzoic acid were found in all fractions except for CF. Meanwhile, five phenolic compounds of HCA (caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid and sinapic acid) were detected in CEE, EAF and BF. Chlorogenic acid was not identified in all the fractions, whereas syringic acid and *p*-coumaric acid were not found in CF. The major phenolic acids in all fractions were HCA bearing *ortho*-dihydroxyl or 4-hydroxy-3-methoxyl groups, which contributed about 65-90 % to the total amount. Previous studies have reported that HCA shows higher antioxidant activity than HBA. The presence of a CH=CHCOOH group in the HCA derivatives that is more active than the COOH group in HBA derivatives leads to the greater antioxidant activity (Eom *et al.*, 2012; Sánchez-Maldonado *et al.*, 2011). Hence, the results suggested that the HCA derivatives have better antioxidant activity

than HBA. CEE had the TPA (HBA+HCA) amount of 2086 µg/g dry weight (Table 5). Interestingly, fractionation with ethyl acetate enhanced the TPA of CEE from 2086 µg/g dry weight to 4398 µg/g dry weight in EAF. The TPA of EAF was 2, 3 and 11 fold higher than that of the CEE, BF and CF, respectively, and it was found to be the richest source of phenolic acids especially, HCA derivatives, in comparison to other fractions. Gallic acid, protocatechuic acid, caffeic acid, syringic acid, *p*-coumaric acid and ferulic acid were determined to be the most dominant phenolic acids in the EAF, whereas *p*-hydroxybenzoic acid and sinapic acid dominated in the CEE. Our studies found that the EAF exhibited the highest antioxidant and anti-AGEs formation activities, indicating that EAF is consisted of various individual phenolic acids that provide significant activities. Eom *et al.* (2012) suggested that the number and position of the hydroxyl (OH) groups available on the structure of the phenolic compounds influenced the antioxidant activity, with an increase in the OH groups on the aromatic ring indicating higher activities, and this also indicates that the presence of OH groups in the *ortho* or *para* position also increased the antioxidant activity. In addition, the presence of methoxy ($OCH_3$) substituent groups in the structure also increased the antioxidant activity. Since, the major dominant phenolic acids that are present in the EAF, especially gallic acid, protocatechuic acid and caffeic acid, have more OH groups in their structure than *p*-hydroxybenzoic acid and sinapic acid, which are dominant in the CEE, they may tend to show increased antioxidant and anti-AGEs formation activities, as well as a higher content of TPA that may also influence the stronger activities. These results suggest that the differences in activities are due to differences in structure, the type of substituent groups and number or position of OH groups on the structure of the phenolic compounds. Sánchez-Moreno *et al.* (1998) reported that the efficiency of phenolic compounds as antioxidants depends on their chemical structures as well as the position and number of OH groups attached to the aromatic ring. These results are in agreement with the findings of Jun et al. (2014) who found that the ethyl acetate fractionation of the ethanolic extract of *Perilla frutescens* var. *acuta* leaves increased total phenolic content and antioxidant activities. Our findings suggest that phenolic compounds of *G. procumbens* could be responsible for antioxidant and anti-glycation activities in which the potential of bioactivities depending on their chemical structures, especially the position and number of OH groups.

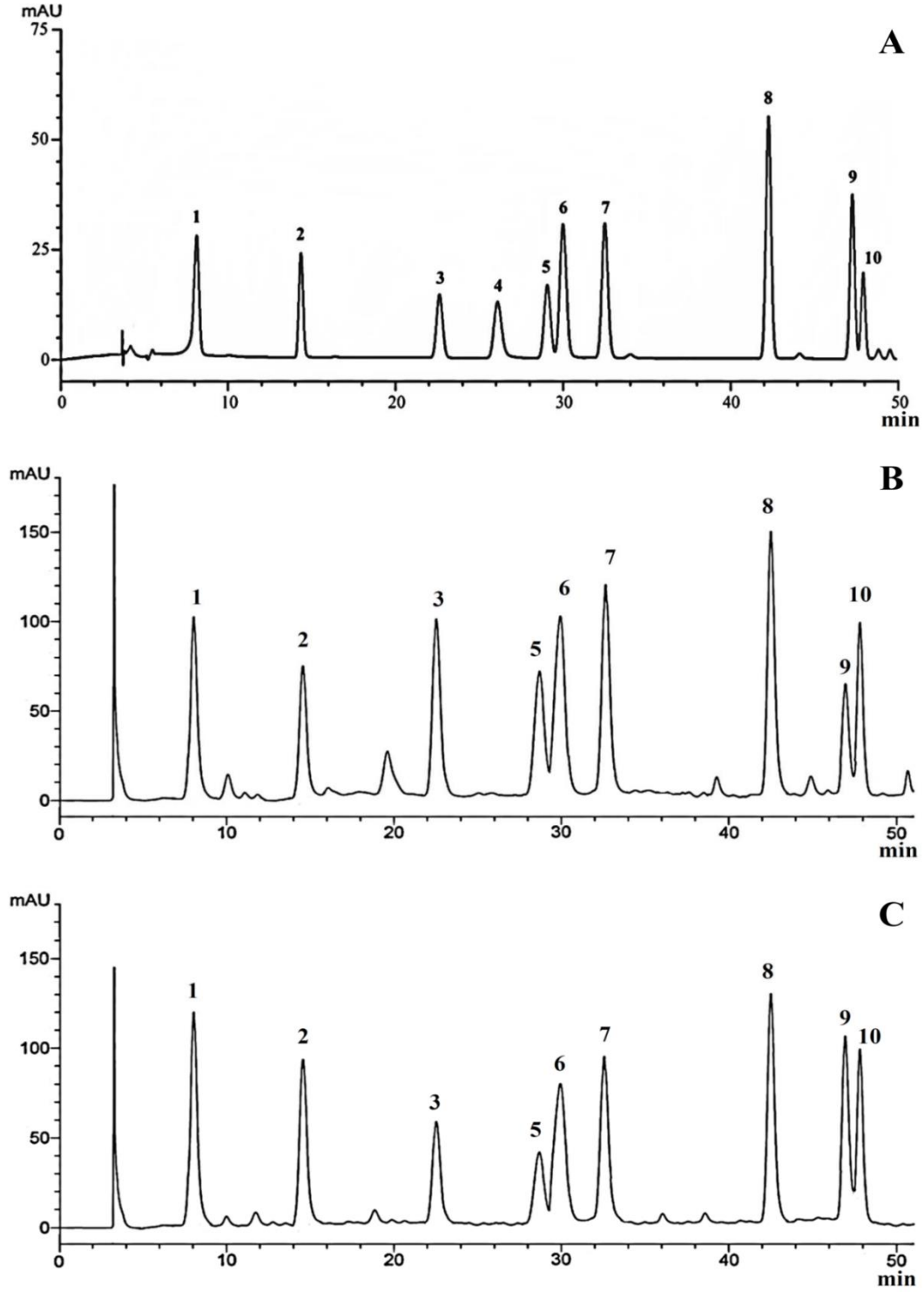



**Fig. 4.3** The HPLC chromatogram at wavelength 280 nm of phenolic acid standards (A), crude ethanolic extract (B) and ethyl acetate fraction (C). Peaks: 1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, chorogenic acid; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, *p*-coumaric acid; 9, ferulic acid; 10, sinapic acid.

**Table 4.6** Composition and content of phenolic acids (µg/g dry weight) of ethanolic extract and it soluble fractions from *G. procumbens*.

| Phenolic compounds | | Extracts | | | |
|---|---|---|---|---|---|
| | | CEE | CF | EAF | BF |
| Hydroxybenzoic acids | Gallic acid | 88.00 ± 2.27$^{b}$ | 39.20 ± 1.35$^{d}$ | 501.91 ± 1.28$^{a}$ | 53.45 ± 0.98$^{c}$ |
| | Protocatechuic acid | 83.71 ± 3.78$^{b}$ | ND | 209.24 ± 3.81$^{a}$ | 45.79 ± 2.15$^{c}$ |
| | *p*-Hydroxybenzoic acid | 292.47 ± 2.51$^{a}$ | ND | 132.40 ± 1.10$^{b}$ | 20.11 ± 3.10$^{c}$ |
| | Vanilic acid | 76.77 ± 4.23$^{a}$ | ND | 77.15 ± 0.70$^{a}$ | ND |
| | Total | 540.78 ± 4.87 | 39.20 ± 1.35 | 920.70 ± 4.69 | 119.35 ± 3.80 |
| Hydroxycinnamic acids | Chlorogenic acid | ND | ND | ND | ND |
| | Caffeic acid | 111.72 ± 0.86$^{c}$ | 123.02 ± 0.55$^{b}$ | 136.34 ± 0.20$^{a}$ | 109.72 ± 0.46$^{d}$ |
| | Syringic acid | 120.55 ± 1.68$^{b}$ | ND | 169.20 ± 1.40$^{a}$ | 86.82 ± 2.64$^{c}$ |
| | *p*-Coumaric acid | 826.15 ± 4.22$^{c}$ | ND | 2701.75 ± 8.64$^{a}$ | 844.13 ± 4.09$^{b}$ |
| | Ferulic acid | 99.08 ± 0.36$^{d}$ | 113.16 ± 0.72$^{b}$ | 280.95 ± 0.50$^{a}$ | 106.79 ± 0.57$^{c}$ |
| | Sinapic acid | 387.99 ± 3.65$^{a}$ | 113.31 ± 1.00$^{d}$ | 188.85 ± 2.56$^{c}$ | 228.38 ± 3.32$^{b}$ |
| | Total | 1545.49 ± 2.32 | 349.49 ± 3.28 | 3477.09 ± 8.75 | 1375.84 ± 1.52 |

Values are expressed as mean ± SD of triplicate measurements. Means with different letters in the same raw represent significant differences at $p < 0.05$.

ND = not detected.

4.2.2 Composition and content of flavonoids in CEE and it fractions

The flavonoids' composition and content in the CEE and its derived fractions were performed using HPLC. In all the analyzed fractions, it was possible to identify five flavonoids: rutin, myricetin, quercetin, apigenin and kaempferol. The HPLC chromatograms of standard flavonoids and flavonoid identified in CEE and ethyl acetate fraction (EAF) are presented in Fig 4.4. The order of the elution and the retention time in minutes were as follows: rutin, 53.26; myricetin, 55.91; quercetin, 57.04; apigenin, 58.19; and kaempferol, 61.50. Flavonoids in samples were identified by a direct comparison of their retention times with those of authentic compounds. The contents of flavonoids in each sample were calculated based on the linear regression equations from the external calibration curves of the standard and the results are shown in Table 4.7. As shown in Table 4.7, rutin, quercetin and kaempferol were found in all fractions, whereas myricetin and apigenin were not detected in CF and BF. All individual flavonoids were found to be dominant in the EAF, except for kaempferol, which was found to be the most predominant flavonoid in the CEE. Kim *et al.* (2011) found that kaempferol is the dominant flavonoid present in the crude extract of *G. procumbens* grown in Korea, which agrees with the present result. Interestingly, we found that after fractionation with ethyl acetate the content of rutin and apigenin were 2 fold higher than that of CEE and the amounts of myricetin and quercetin were slightly increased, whereas the content of kaempferol decreased by up to 2.4 fold from CEE. In addition, fractionation with ethyl acetate also enhanced the antioxidant and anti-glycation activities, indicating that the activities could be composed of different individual flavonoids, especially rutin, myricetin, quercetin and apigenin that have significant activities. This may indicate that besides kaempferol other flavonoids could be potential active compounds in the leaves of this plant (Table 4.7). Rutin and quercetin are the major flavonoids found in plants that have various biological activities that are beneficial to human health, such as antioxidant effects (Cartea *et al.*, 2011). These results demonstrated that differences in the antioxidant and anti-AGEs formation activities of the derived fractions are due to differences in the individual phenolic acids and flavonoids, as well as to the chemical structures and the type or number of substituents, especially OH groups on the aromatic ring of their compounds.

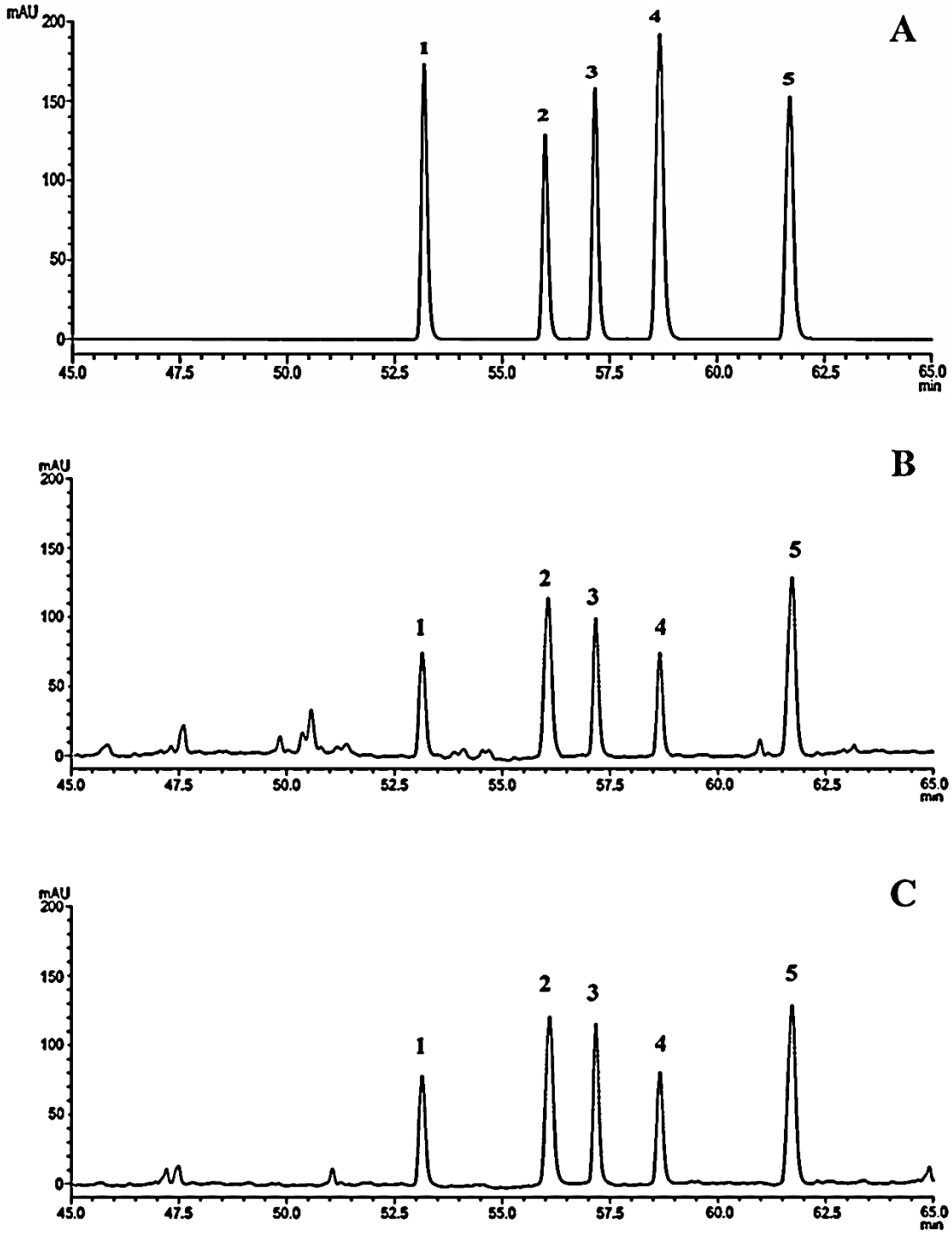



**Fig. 4.4** HPLC chromatogram at wavelength 370 nm of flavonoid standard (A), crude ethanolic extract (B) and ethyl acetate fraction (C) from *G. procumbens*. Peak identifications: 1, rutin; 2, myricetin; 3, quercetin; 4, apigenin; 5, kaempferol.

**Table 4.7** Composition and content of flavonoids (µg/g dry weight) of ethanolic extract and it soluble fractions from *G. procumbens*.

| Flavonoids | Extracts | | | |
|---|---|---|---|---|
| | CEE | CF | EAF | BF |
| Rutin | 42.56 ± 0.36$^{c}$ | 36.88 ± 0.69$^{d}$ | 84.38 ± 0.24$^{a}$ | 53.26 ± 3.65$^{b}$ |
| Myricetin | 251.10 ± 3.67$^{b}$ | ND | 261.18 ± 1.65$^{a}$ | ND |
| Quercetin | 135.87 ± 0.40$^{b}$ | 122.79 ± 0.59$^{d}$ | 193.22 ± 1.47$^{a}$ | 129.78 ± 1.31$^{c}$ |
| Apigenin | 49.92 ± 0.73$^{b}$ | ND | 85.92 ± 1.45$^{a}$ | ND |
| Kaempferol | 464.53 ± 1.81$^{a}$ | 240.27 ± 0.63$^{b}$ | 192.60 ± 0.67$^{d}$ | 232.34 ± 1.80$^{c}$ |
| Total | 943.98 ± 9.91 | 399.94 ± 3.66 | 817.30 ± 6.69 | 415.38 ± 8.17 |

Values are expressed as mean ± SD of triplicate measurements. Means with different letters in the same raw represent significant differences at $p < 0.05$.
ND = not detected.

## 4.3 Experiment III: Fractionation of phenolic compounds using column chromatography and evaluation of their antioxidant activities

### 4.3.1 Fractionation of phenolic antioxidants with Sephadex LH-20

The crude ethanolic extract (CEE) was partitioned sequentially extracted according to solvent polarity with chloroform, ethyl acetate and *n*-butanol. Among all the solvent partitioned fractions, the ethyl acetated fraction (EAF) showed the highest total phenolic content (TPC) and total flavonoid content (TFC) as well as antioxidant activities as determined using different assays (see section 3.1). Our findings were in agreement with previous studies that reported the amount of active compounds was dependent on the extraction solvent used and its polarity (Cho *et al.*, 2011). Less polar compounds like aglycone flavonoids may be dissolved in chloroform, while ethyl acetate was used to extract medium polar flavonoids and glycosides. Polar compounds such as glucosides and sugars were extracted by *n*-butanol. These results indicated that active compounds with the strongest antioxidant activity were more soluble in a medium polarity solvent as ethyl acetate. Thus, the EAF was further fractionated by

column chromatography on a Sephadex LH-20, and five sub-fractions, namely sub-fraction 1 (SF1), sub-fraction 2 (SF2), sub-fraction 3 (SF3), sub-fraction 4 (SF4) and sub-fraction 5 (SF5) were obtained (Fig. 4.5). Each fraction was pooled, concentrated and then antioxidant compounds and activities were measured. Interestingly, SF2 showed the highest UV absorbance value at 280 nm for phenolics detection but had the lowest antioxidant activity, whereas SF3 showed the highest antioxidant activity, which indicates that phenolic compounds may not be the only components in sub-fractions that could possess strong antioxidant potency.

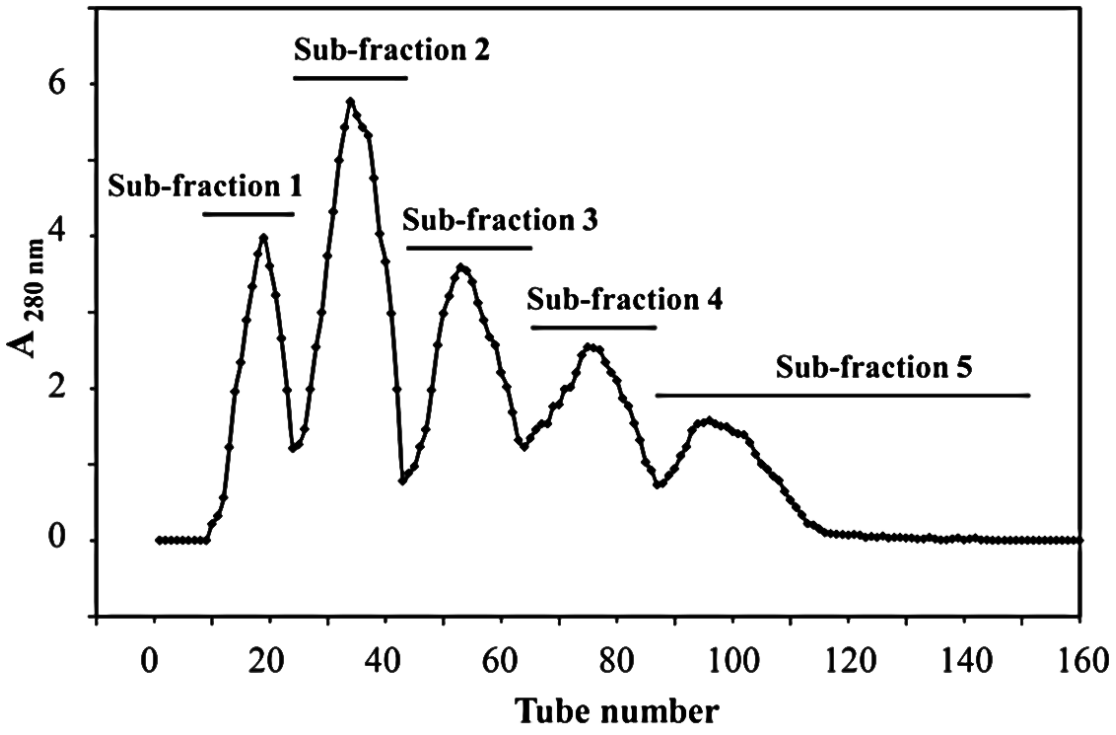



**Fig. 4.5** Sephadex LH-20 column chromatogram of sub-fractions from *G. procumbens* leaf extracts.

4.3.2 Total phenolic content (TPC) and total flavonoid content (TFC)

The results of the TPC and TFC of the *G. procumbens* leaf extracts, including the crude ethanolic extract (CEE) as well as the ethyl acetated fraction (EAF) and various sub-fractions are shown in Table 4.8. The comparisons of the different solvent partitioned fractions found that the ethyl acetate fraction exhibited the highest

TPC (24.4 mg GAE/g DW) and TFC (17.3 mg CE/g DW) levels, whereas the chloroform fraction had the lowest values (data not shown). Thus, ethyl acetate fraction may be responsible for higher effectiveness in scavenging radicals and antioxidant activities. For sub-fractions (SF1-SF5), the TPCs ranged from 62.0 to 95.9 mg GAE/g DW, whereas the TFCs were in the range of 18.1 to 94.1 mg CE/g DW (Table 1). When comparing all sub-fractions, SF3 showed the highest TPC (95.9 mg GAE/g DW) and TFC (94.1 mg CE/g DW) along with the highest total antioxidant activity. These results indicated that SF3 could be consisted of different individual phenolics or flavonoids that provide significant antioxidant activity. Specifically, the TPC and TFC of SF3 were 6.0 and 9.0 fold as well as 4.0 and 5.4 fold higher than those of the crude extract and ethyl acetate fraction, respectively. These clearly a highlight the Sephadex LH-20 chromatography increased the TPC and TFC as well as concentrated the phenolic antioxidants from this plant extracts. Phenolic compounds are associated with a decreased risk of several diseases, such as cancer, cardiovascular disease and diabetes (Deng *et al.*, 2013; Pandey and Rizvi, 2009). Therefore, the consumption of this plant that is rich in phenolic compounds could reduce the risk of diseases due to oxidative stress and provide health benefits.

4.3.3 Total antioxidant capacity

The total antioxidant capacity of all extracts was determined using the CUPRAC method, which is based on the reduction of Cu(II) to Cu(I) by antioxidants. The CUPRAC values of the extracts, including the crude extract as well as ethyl acetate fraction and sub-fractions are shown in Table 4.8. When comparing solvent partitioned fractions, the ethyl acetate fraction showed the highest CUPRAC value (3.2 mg TEAC/g DW), which was 2.2 fold higher than those of the starting crude extract (1.4 mg TEAC/g DW). Of the ethyl acetate sub-fractions, SF3 had the highest total antioxidant capacity with a CUPRAC value of 11.7 mg TEAC/g DW, whereas SF2 showed the lowest antioxidant capacity (3.3 mg TEAC/g DW), which may be due to the fact that SF3 contains the highest total flavonoid content. The order of effectiveness in cupric reducing power was: SF3 > SF4 > SF5 > SF1 > SF2 > ethyl acetate fraction > crude ethanolic extract ($p < 0.05$). Our findings suggest that SF3 consisted of various individual compounds that have potential reducing power. As far as we know, this is the first report regarding cupric reducing activity in derived fractions from *G. procumbens*

leaves. The antioxidant activity of plant materials or extracts is known to be associated with polyphenols and their chemical structures that contain hydroxyl groups acting as the primary antioxidant (Jun *et al.*, 2014).

4.3.4 DPPH• scavenging activity

DPPH• has been widely used to evaluate the antioxidant power of many plant extracts on the basis of their hydrogen donating ability (Cho *et al.*, 2011). The DPPH• scavenging activities of different fractions obtained from fractionation using three steps were expressed as $IC_{50}$ values and the antioxidant levels of the extracts were compared with ascorbic acid, trolox and BHA as synthetic antioxidants (Table 4.9). Among all solvent partitioned fractions, the ethyl acetate fraction showed the most potent activity with the $IC_{50}$ value of 220.6 µg/ml (lower than crude extract 2.2 times), which indicates that compounds with the strongest DPPH• scavenging activity were concentrated by this solvent of medium polarity. The SF3 showed the lowest $IC_{50}$ value (19 µg/ml) for the DPPH• assay when compared to other sub-fractions, indicating that SF3 has the strongest antioxidant activity. The $IC_{50}$ values of SF3 were 3.2, 4.8, 2.0 and 2.8 fold lower than those of SF1, SF2, SF4 and SF5, respectively. Moreover, the levels of DPPH• scavenging activities of these fractions were 1.3 and 1.9 times higher than those of ascorbic acid and trolox, used as synthetic antioxidants, respectively. Thus, SF3 showed the most potent activity, indicating that compounds with the strongest DPPH radical scavenging activity were selectively concentrated in this sub-fraction during the fractionation with Sephadex LH-20. Previous studies demonstrated that phenolic compounds have potent hydrogen donors, which indicates a high DPPH• scavenging activity (Hatamnia *et al.*, 2014; Maqsood and Benjakul, 2010).

**Table 4.8** Total phenolic content, total flavonoid content and total antioxidant activity of different fractions derived from *G. procumbens* leaves.

| Fractions | | Total phenolic content (mg GAE/g DW) | Total flavonoid content (mg CE/g DW) | Total antioxidant capacity (mg TEAC/g DW) |
|---|---|---|---|---|
| Crude ethanolic extract [A] | | 16.08 ± 0.38[g] | 10.33 ± 0.88[g] | 1.45 ± 0.01[g] |
| Ethyl acetate fraction | | 24.36 ± 1.11[f] | 17.33 ± 1.39a[f] | 3.16 ± 0.01[f] |
| Sub-fractions | SF1 | 91.26 ± 0.01[b] | 40.97 ± 0.02[d] | 4.65 ± 0.02[d] |
| | SF2 | 89.28 ± 0.03[c] | 18.15 ± 0.01[e] | 3.28 ± 0.01[e] |
| | SF3 | 95.93 ± 0.06[a] | 94.08 ± 0.02[a] | 11.71 ± 0.02[a] |
| | SF4 | 61.99 ± 0.01[e] | 72.75 ± 0.01[b] | 6.43 ± 0.02[b] |
| | SF5 | 78.36 ± 0.04[d] | 64.28 ± 0.01[c] | 5.07 ± 0.01[c] |

Results are expressed as mean ± SD (n = 3). Values with different letters in the same column represent significant differences at $p < 0.05$.

[A] Yield of the starting crude ethanolic extract (27.8% w/w, on a dry weight basis).

*Abbreviations:* GAE, gallic acid equivalents; CE, catechin equivalents; TEAC, trolox equivalents antioxidant capacity; DW, dry weight.

4.3.5 $ABTS^{\bullet+}$ scavenging activity

The $ABTS^{\bullet+}$ scavenging abilities of the crude extract as well as the ethyl acetate fraction and its sub-fractions are presented in Table 4.9. For the solvent partitioned fraction, a similar trend to the $DPPH^{\bullet}$ scavenging results was observed in the $ABTS^{\bullet+}$ scavenging activity, with the highest activity being found in the ethyl acetate fraction ($IC_{50}$ = 61.0 µg/ml), which was 3.7 times lower than those of the crude ethanolic extract ($IC_{50}$ = 223.2 µg/ml). The SF3 showed the highest $ABTS^{\bullet+}$ scavenging activity ($IC_{50}$ = 12 µg/ml) among all sub-fractions tested, followed by SF4, SF5, SF1 and SF2. The comparisons of the different sub-fractions showed that SF3 was 3.3, 4.6, 2.0 and 2.6 fold more active than SF1, SF2, SF4 and SF5, respectively. The observed differential scavenging activities of the sub-fractions against $ABTS^{\bullet+}$ could be due to the presence of different compounds in the sub-fractions. In addition, the radical scavenging activities of SF3 against $ABTS^{\bullet+}$ were 17.5 and 4.8 times higher than those of the crude extract and ethyl acetate fraction, respectively. The present study is in good agreement with Jun *et al.* (2014) who found that fractionation of antioxidant compounds using the Sephadex LH-20 chromatography can increase the purity and concentrate active compounds with the most potent free radical scavenging activities. The levels of $ABTS^{\bullet+}$ scavenging ability of SF3 were 1.1 and 4.4 times greater than that of the trolox and ascorbic acid, respectively. We demonstrated that SF3 exhibited the strongest radical scavenging effects when compared to the other fractions, which was also greater than that of the commercial synthetic antioxidants.

4.3.6 Ferric reducing antioxidant power (FRAP)

The FRAP assay is a simple, rapid and inexpensive method of measuring the reductive ability of an antioxidant, and it is evaluated by the reduction of Fe(III) to Fe(II), as a measure of reducing antioxidant power (Benzie and Strain, 1996). The FRAP values of the crude extract as well as ethyl acetate and its five sub-fractions were expressed as mmol Fe(II) per g dry extract, and the results are shown in Table 4.9. Among the crude extract and solvent partitioned fractions, the highest amount of reducing power was observed in the ethyl acetate fraction with a FRAP value of 3.4 mmol Fe(II)/g DW. Of the ethyl acetate sub-fractions, the FRAP values of the five sub-fractions (SF1-SF5) ranged from 2.6 to 10.2 mmol Fe(II)/g DW, with a descending order of SF3 > SF4 > SF5 > SF1 > SF2 ($p < 0.05$), which indicates that SF3 had the

highest antioxidant activity. These considerable differences in the FRAP values of the various sub-fractions could be due to differences in the chemical compositions of each sub-fraction. Moreover, the FRAP value of SF3 was 3.2 times higher than that of the ascorbic acid as a synthetic antioxidant. This result was in agreement with both the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity results in which the SF3 was also the most efficient radical scavenger. Thus, SF3 appears to have good potential as a source of natural antioxidants for use in the reduction of oxidative damage in the human body and for providing health protection.

4.3.7 Correlation analysis

Correlations between TPC, TFC and antioxidant activity were analyzed and the results are presented in Table 4.10. No correlation was observed between the TPC and antioxidant activities in any of the assays, in which the r-values were lower than 0.228, which indicates that the phenolic compounds are not the only components in the extracts that could possess antioxidant activity. The results obtained in this work suggest that antioxidant activity may be related to other antioxidant compounds besides phenolics. These results were in good agreement with previous ones reported by López-Mejía *et al.* (2014) who reported that the antioxidant activity of the extracts from *Amaranthus hypochondriacus* L. was not correlated with their TPC. However, the r-values between the TFC and antioxidant activities ranged from 0.855 to 0.987, which indicated a high positive correlation between TFC and antioxidant activity. Thus, this indicates that flavonoids are the most important contributors to antioxidant activity in *G. procumbens*. Bakar *et al.* (2009) reported that antioxidant activities were strongly positively correlated with TFC. Flavonoids are excellent antioxidant compounds because they are highly reactive as hydrogen and electron donors (Cotelle, 2001). Furthermore, high positive correlations between antioxidant activities (for example, $DPPH^{\bullet}$ assay versus FRAP assay) were also observed in the present study in which the r-values were higher than 0.937. Our finding suggests that the content and composition of flavonoids in this plant could play an important role in antioxidant activity.

**Table 4.9** Antioxidant activity of the crude extract as well as the ethyl acetate fraction and its sub-fractions from *G. procumbens* leaves.

| Fractions | | DPPH$^{\bullet}$ | ABTS$^{\bullet+}$ | FRAP |
|---|---|---|---|---|
| | | $IC_{50}$ [A] (µg/ml) | $IC_{50}$ [A] (µg/ml) | mmol Fe(II)/g DW |
| Crude ethanolic extract | | 473.70 ± 0.01$^{a}$ | 223.18 ± 0.01$^{a}$ | 1.64 ± 0.02$^{i}$ |
| Ethyl acetate fraction | | 220.58 ± 0.01$^{b}$ | 60.99 ± 0.01$^{b}$ | 3.40 ± 0.03$^{g}$ |
| Sub-fractions | SF1 | 61.68 ± 0.17$^{d}$ | 41.76 ± 0.17$^{e}$ | 3.51 ± 0.01$^{g}$ |
| | SF2 | 90.65 ± 2.04$^{c}$ | 58.66 ± 0.23$^{c}$ | 2.63 ± 0.01$^{h}$ |
| | SF3 | 19.08 ± 0.08$^{i}$ | 12.47 ± 0.71$^{i}$ | 10.18 ± 0.01$^{c}$ |
| | SF4 | 38.32 ± 0.04$^{f}$ | 25.59 ± 1.64$^{g}$ | 6.48 ± 0.01$^{d}$ |
| | SF5 | 52.60 ± 0.60$^{e}$ | 32.53 ± 0.05$^{f}$ | 5.17 ± 0.01$^{f}$ |
| Comparisons [B] | Ascorbic acid | 25.29 ± 0.32$^{h}$ | 55.48 ± 0.81$^{d}$ | 6.14 ± 0.10$^{e}$ |
| | Trolox | 35.80 ± 0.31$^{g}$ | 13.88 ± 0.16$^{h}$ | 10.50 ± 0.06$^{b}$ |
| | BHA | 20.14 ± 0.07$^{i}$ | 7.18 ± 0.03$^{j}$ | 15.15 ± 0.22$^{a}$ |

Data are expressed as mean ± SD (n = 3). Means with different letters in the same column represent significant differences at $p < 0.05$.

[A] The concentration of the test samples that scavenges 50% of free radical. Lower $IC_{50}$ values indicate higher radical scavenging activity.

[B] Standard synthetic antioxidants were used as a comparison for antioxidant activity.

**Table 4.10** Correlation coefficients (r) for relationships between TPC, TFC and different antioxidant assays.

| | (1) | (2) | (3) | (4) | (5) | (6) |
|---|---|---|---|---|---|---|
| (1) TPC [A] | 1 | -0.166 | 0.228 | 0.225 | 0.219 | -0.033 |
| (2) TFC [B] | - | 1 | 0.883* | 0.877* | 0.885* | 0.987* |
| (3) CUPRAC [C] | - | - | 1 | 0.998** | 0.998** | 0.943* |
| (4) DPPH$^{\bullet}$ | - | - | - | 1 | 0.999** | 0.937* |
| (5) ABTS$^{\bullet+}$ | - | - | - | - | 1 | 0.941* |
| (6) FRAP | - | - | - | - | - | 1 |

[A] TPC; total phenolic content. [B] TFC; total flavonoid content.

[C] CUPRAC; cupric reducing antioxidant capacity.

** Significant different at $p < 0.01$.

* Significant different at $p < 0.05$.

## 4.4 Experiment IV: Identification and quantification of phenolic compounds in each fraction after Sephadex LH-20 column fractionation

### 4.4.1 Analysis of phenolic-antioxidant compounds

Phenolic compounds are important for human health due to their many potential health benefits such as antioxidant, anticarcinogenic, anti-inflammatory and vasodilatory actions (Shahidi and Naczk, 1995, 2004). In the present study, ten phenolic acids, namely gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, chorogenic acid, vanilic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid and sinapic acid, were identified using RP-HPLC analysis. The composition and contents of individual phenolic acids in the crude extract, ethyl acetate fraction and sub-fractions are presented in Table 4.11. All phenolic acids except for chlorogenic acid were detected in the crude extract and ethyl acetate fraction but were not detected in the sub-fractions. For the sub-fractions, SF2 showed the highest content of total phenolic acids, followed by SF1, SF3, SF5 and SF4 in descending order of content. Remarkably, SF2 exhibited the lowest antioxidant activity, whereas SF3 showed the highest antioxidant activity. Gallic acid,

protocatechuic acid, vanilic acid and caffeic acid were determined to be the most dominant phenolic acids in SF2, whereas ferulic acid and sinapic acid dominated in SF3. This result indicates that the type of phenolic acid as well as their chemical structure could be responsible for antioxidant activity. The antioxidant activities of ferulic acid and sinapic acid may be considerably stronger than those of other phenolic acids as determined in the present study, due to the presence of methoxy ($OCH_3$) substituent groups in their structures. Eom *et al.* (2012) suggested that the presence of the $OCH_3$ substituent group in the structure of phenolics increased the antioxidant activity. Many studies reported that natural plant materials rich in phenolic compounds, especially phenolic acids and flavonoids, possess the most potent antioxidants (Jun *et al.*, 2014; Kaisoon *et al.*, 2011; Kubola and Siriamornpun, 2011; Trabelsi *et al.*, 2012). However, no correlation between antioxidant activity and TPC was observed in this present study, which indicates that phenolic acids are not the only components in the extracts that could present antioxidant activity.

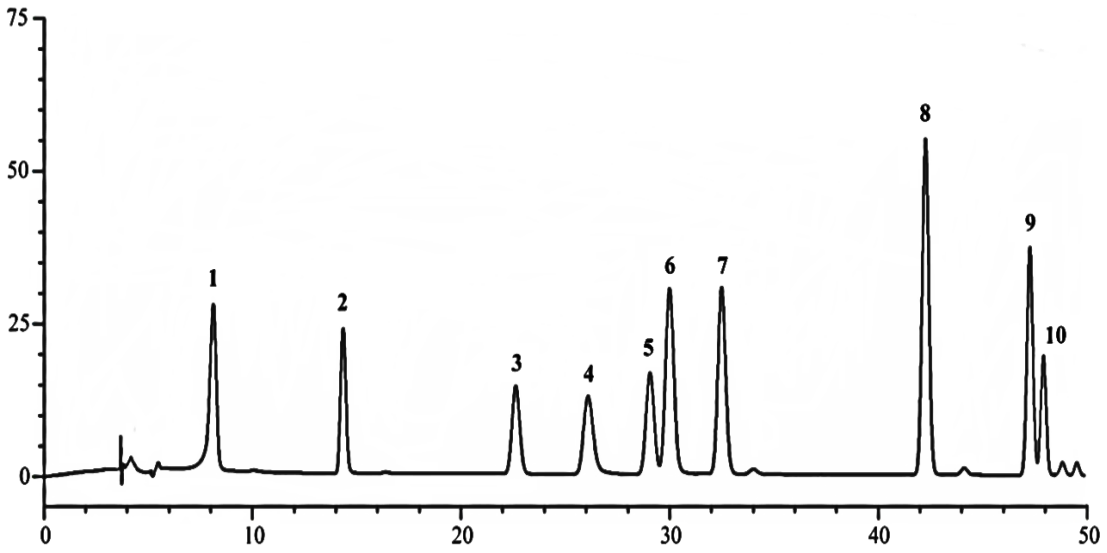



**Fig. 4.6** HPLC chromatogram of standard phenolic acids at wavelength 280 nm. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, p-hydroxybenzoic acid; 4, chorogenic acid; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, p-coumaric acid; 9, ferulic acid; 10, sinapic acid.

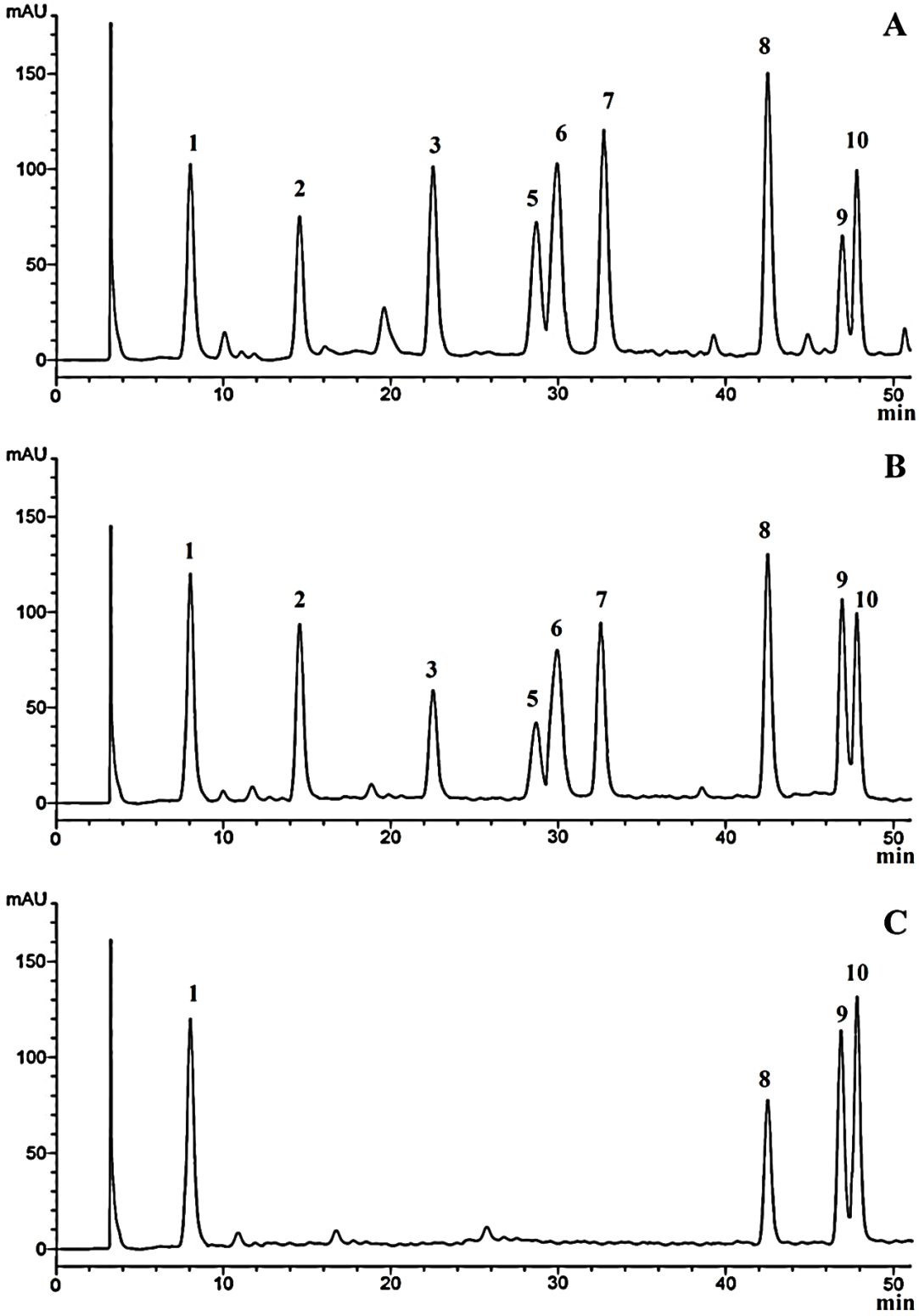



**Fig. 4.7** Chromatogram of phenolic acids in ethanolic extract (A), ethyl acetate fraction (B) and sub-fraction 3 (C). Peak number was identified as described earlier in Fig. 4.6.

**Table 4.11** The composition and content of phenolic acids in different fractions derived from *G. pocumbens* leaves.

| Phenolic acids | Individual phenolic acid content in extract/fraction (µg/g DW) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Crude ethanolic extract | Ethyl acetate fraction | Sub-fractions | | | | |
| | | | SF1 | SF2 | SF3 | SF4 | SF5 |
| Hydroxybenzoic acids | | | | | | | |
| Gallic acid | 88.00±2.27$^{g}$ | 501.91±1.28$^{f}$ | 1063.28±0.04$^{d}$ | 2405.08±2.43$^{a}$ | 1071.69±0.03$^{c}$ | 655.51±2.42$^{e}$ | 2239.88±8.91$^{b}$ |
| Protocatechuic acid | 83.71±3.78$^{d}$ | 209.24±3.81$^{c}$ | ND | 699.56±1.66$^{a}$ | ND | ND | 331.95±0.98$^{b}$ |
| *p*-Hydroxybenzoic acid | 292.47±2.51$^{b}$ | 132.40±1.10$^{c}$ | 838.22±0.01$^{a}$ | ND | ND | ND | ND |
| Vanilic acid | 76.77±4.23$^{b}$ | 77.15±0.70$^{b}$ | ND | 148.59±0.38$^{a}$ | ND | ND | ND |
| Syringic acid | 120.55±1.68$^{e}$ | 169.20±1.40$^{d}$ | 698.92±0.01$^{a}$ | 346.52±1.23$^{b}$ | ND | ND | 290.72±0.01$^{c}$ |
| Hydroxycinnamic acids | | | | | | | |
| Chlorogenic acid | ND | ND | ND | ND | ND | ND | ND |
| Caffeic acid | 111.72±0.68$^{c}$ | 136.34 ±0.20$^{b}$ | ND | 283.43±0.03$^{a}$ | ND | ND | ND |
| *p*-Coumaric acid | 826.15±4.22$^{b}$ | 2701.75±8.64$^{a}$ | 665.94±0.02$^{c}$ | 243.27±1.15$^{g}$ | 384.32±0.01$^{d}$ | 334.23±0.39$^{e}$ | 281.37±0.80$^{f}$ |
| Ferulic acid | 99.08±0.36$^{f}$ | 280.95±0.50$^{d}$ | 517.44±0.05$^{b}$ | 317.64±0.02$^{c}$ | 778.32±0.02$^{a}$ | 229.22±0.30$^{e}$ | ND |
| Sinapic acid | 387.99±3.65$^{d}$ | 188.85±2.56$^{e}$ | 604.58±0.02$^{b}$ | 77.14±0.04$^{f}$ | 1426.79±0.07$^{a}$ | 514.48±0.25$^{c}$ | ND |
| Total | 2086.44±2.34$^{f}$ | 4397.79±2.02$^{c}$ | 4388.38±0.06$^{b}$ | 4521.23±4.39$^{a}$ | 3661.12±0.12$^{d}$ | 1733.44±2.52$^{g}$ | 3143.92±9.16$^{e}$ |

Results are expressed as mean ± SD (n = 3). Means with different letters in the same raw represent significant differences at $p < 0.05$.

ND: not detected.

4.4.2 Analysis of flavonoid-antioxidant compounds

Flavonoids are the most potent scavengers of free radicals and are potentially useful in the prevention of many diseases due to oxidative damage, such as cancer, diabetes and other degenerative diseases (Zainol *et al.*, 2003). Five flavonoid compounds (rutin, myricetin, quercetine, apigenin and kaempferol) were identified in the present study by comparing their retention times against those of standards. The content and composition of the individual flavonoids in the crude extract as well as the ethyl acetate fraction and its sub-fractions (SF1-SF5) are listed in Table 4.12. Four flavonoids, namely rutin, myricetin, quercetine and kaempferol were detected in all extracts from *G. procumbens* leaves. Apigenin was detected in the crude extract and ethyl acetate fraction but was not detected in the sub-fractions except for SF5, indicating that apigenin is not critical for the antioxidant activity of flavonoids. These results support the data previously reported by Zhou *et al.* (2014) who found that apigenin isolated from perilla showed little antioxidant activity. In particular, SF3 had the highest concentration of flavonoids (about 60.2% of the total sub-fractions) and showed the strongest antioxidant activities, suggesting that fractionation using a Sephadex LH-20 concentrated and purified the flavonoid antioxidants in SF3. Flavonoids are widely distributed compounds in many plants with health-related properties, which are based on their antioxidant activity (Benavente-García *et al.*, 1997). The comparison between the phenolic acid and flavonoid contents in SF3 showed that the most abundant compounds were the flavonoids, which accounted for 63% of the total compared to phenolic acid at 37% (data not shown). The major flavonoids in SF3 were myricetin and kaempferol, which approximately accounted for 56.4 and 41.6% of the total flavonoid content as determined by RP-HPLC, respectively. A strong positive correlation between TFC and antioxidant activity was also observed in the present study. These findings indicated that the flavonoid compounds, particularly myricetin and kaempferol, could be responsible for the antioxidant activity in *G. procumbens* leaves. The structures of myricetin and kaempferol are similar in that both compounds contain hydroxyl groups at the C3, C5 and C7 positions on the A or C rings of the flavonoids. Previous studies have shown that the number of hydroxyl groups and their positions on the A or C rings of flavonoids are related to their antioxidant activities (Chen *et al.*, 2012). In addition, they suggested that at least two hydroxyl groups were

necessary to contribute to antioxidant potency. Moreover, previous studies have demonstrated that hydroxyl groups at the C3, C5 and C7 positions of flavonoids give better antioxidant activities (Chen *et al.*, 2012; Wang *et al.*, 2013). Thus, the highest antioxidant activity of SF3 was attributed to the flavonoids (myricetin and kaempferol) as well as the chemical structures, substitutions and number of hydroxyl groups of these flavonoids. Myricetin is a flavonoid often found in many plants that has a variety of biological activities, including anti-inflammatory, anticancer, antiviral and antioxidative actions. Moreover, it has potent therapeutic benefits in diabetes and cardiovascular diseases (Kampa *et al.*, 2007; Ong and Khoo, 1997). Meanwhile, kaempferol is also known for its health benefit effects as it reduces cancer, arteriosclerosis, cardiovascular disorders and exerts antioxidant and anti-inflammatory potencies (Chen and Dou, 2008; Park *et al.*, 2006). Based on our findings, we concluded that the fractionation using multiple steps (crude extract, fractions and sub-fractions) purified and concentrated the most responsible phenolic compounds for antioxidant activity from *G. procumbens* leaves. Our findings demonstrated that flavonoids, especially myricetin and kaempferol were the most important contributors to antioxidant activity in *G. procumbens*, rather than phenolic acids.

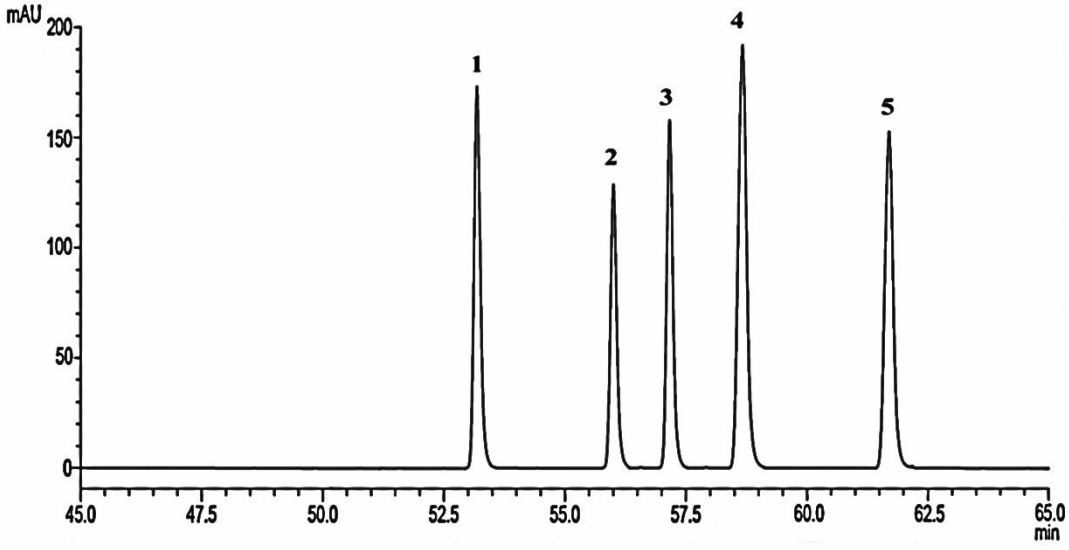



**Fig. 4.8** Chromatogram of standard flavonoids at wavelength 370 nm. Peaks: 1, rutin; 2, myricetin; 3, quercetine; 4, apigenin; 5, kaempferol.

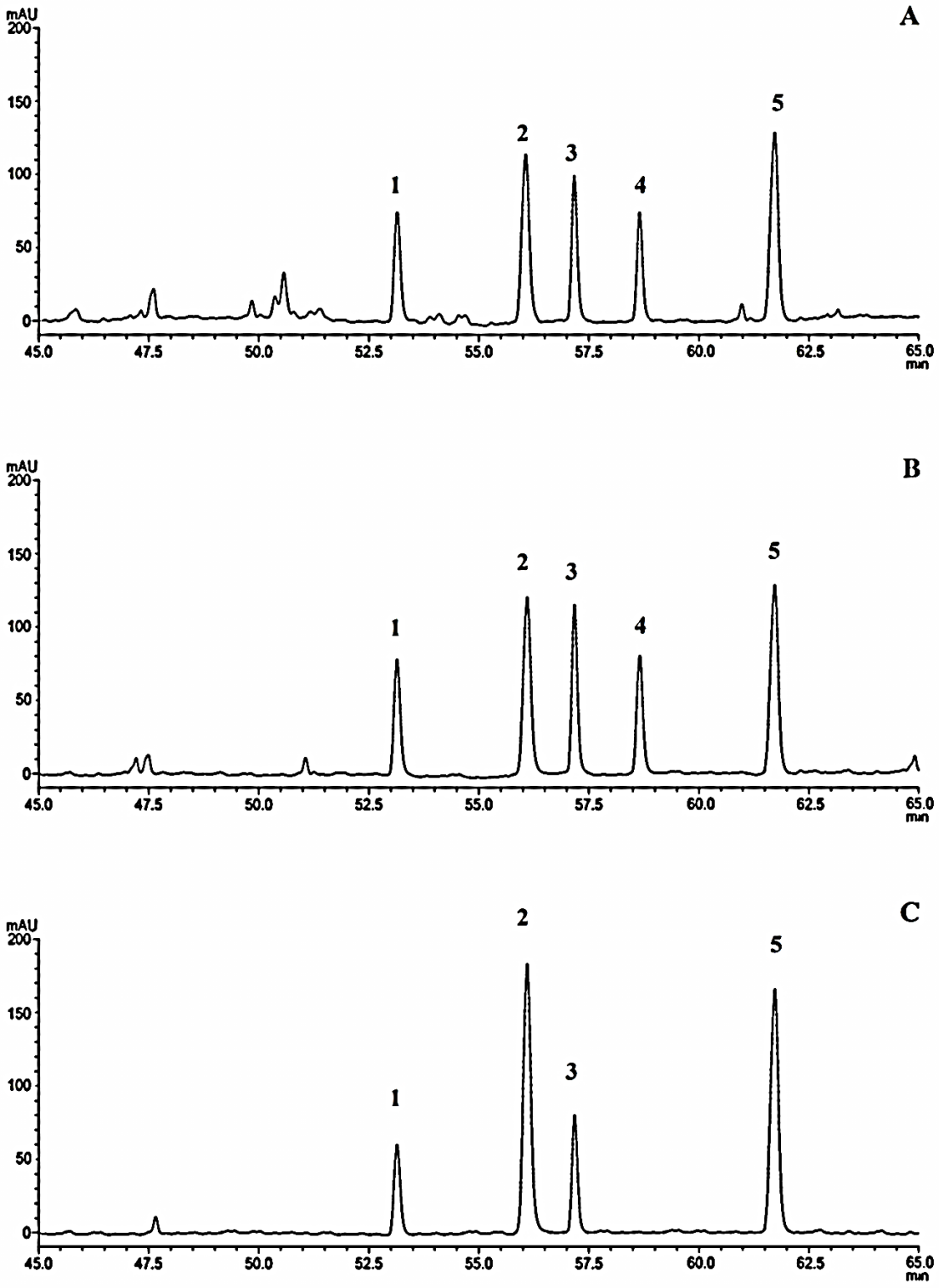



**Fig. 4.9** HPLC chromatogram at wavelength 370 nm of flavonoids in crude ethanolic extract (A), ethyl acetate fraction (B) and sub-fraction 3 (C). Peaks: Peaks: 1, rutin; 2, myricetin; 3, quercetine; 4, apigenin; 5, kaempferol.

**Table 4.12** Flavonoid compositions of the crude extract, the ethyl acetate fraction and sub-fractions derived from *G. procumbens* leaves.

| Fraction | Individual flavonoid content in extract/fraction (µg/g DW) | | | | | |
|---|---|---|---|---|---|---|
| | Rutin | Myricetin | Quercetin | Apigenin | Kaempferol | Total |
| Crude ethanolic extract | 42.56±0.36$^{e}$ | 251.10±3.67$^{d}$ | 135.87±0.40$^{f}$ | 49.92±0.73$^{c}$ | 464.53±1.81$^{d}$ | 943.98±9.91$^{d}$ |
| Ethyl acetate fraction | 84.38±0.24$^{a}$ | 261.18±1.65$^{c}$ | 193.22±1.47$^{e}$ | 85.92±1.45$^{a}$ | 192.60±0.67$^{e}$ | 817.30±6.69$^{e}$ |
| Sub-fractions | | | | | | |
| SF1 | 61.23±0.01$^{b}$ | 175.99±0.02$^{e}$ | 28.25±0.01$^{d}$ | ND | 98.88±0.03$^{f}$ | 364.35±0.02$^{f}$ |
| SF2 | 52.29±0.01$^{c}$ | 27.96±0.01$^{f}$ | 13.85±0.67$^{g}$ | ND | 25.62±0.01$^{g}$ | 119.72±0.05$^{g}$ |
| SF3 | 47.10±0.01$^{d}$ | 3518.04±0.01$^{a}$ | 74.86±0.02$^{a}$ | ND | 2593.11±0.03$^{a}$ | 6233.11±0.03$^{a}$ |
| SF4 | 30.91±0.19$^{f}$ | 710.76±1.06$^{b}$ | 50.55±0.03$^{b}$ | ND | 1119.41±3.01$^{b}$ | 1911.63±4.07$^{b}$ |
| SF5 | 30.86±0.01$^{f}$ | 713.61±3.67$^{b}$ | 48.59±0.03$^{c}$ | 62.07±0.03$^{b}$ | 871.89±0.38$^{c}$ | 1727.02±1.43$^{c}$ |

Results are expressed as mean ± SD (n = 3). Values with different letters in the same column represent significant differences at $p < 0.05$.

ND: not detected.

# CHAPTER V

# CONCLUSIONS

## 5.1 Conclusions

The findings of the present study can be concluded as follows:

5.1.1 The phenolic compounds in the leaves of *G. procumbens* were firstly extracted with absolute ethanol and then sequentially partitioned by increasing the polarity of solvent, including chloroform, ethyl acetate and *n*-butanol, respectively. The antioxidant and anti-glycation activities of various fractions were evaluated using *in vitro* assays. Among all solvent fractions, the ethyl acetate fraction (EAF) possessed the highest contents of total phenolics and total flavonoids when compared to other fractions with the best potent antioxidant activities in scavenging $DPPH^{\bullet}$, $ABTS^{+\bullet}$, $^{\bullet}OH$ and hydrogen peroxide. The EAF also had the strongest anti-glycation activity and showed superior values for lipid peroxidation inhibition to the other fractions, including ascorbic acid. Moreover, EAF showed a significantly stronger protective effect against oxidative protein damage induced by hydroxyl radicals generated in the Fenton reaction than that of ascorbic acid and trolox as synthetic antioxidants. Our findings suggest that the EAF of *G. procumbens* is a potential source of natural antioxidant and anti-glycation agents in health-care products, such as nutraceuticals, supplements and pharmaceuticals.

5.1.2 According to correlation analysis, the contents of phenolic and flavonoid were strongly positively correlated with antioxidant activities against $DPPH^{\bullet}$, $ABTS^{+\bullet}$, $^{\bullet}OH$ and hydrogen peroxide. In addition, high positive correlations were also found between anti-glycation activity, antioxidant activity and phenolic content. Base on HPLC analysis, the individual phenolic compounds in various fractions were remarkably different, especially gallic, *p*-coumaric and ferulic acids for phenolic acids, and myricetin, quercetin and kaempferol for flavonoids. These findings indicate that phenolic compounds could be the key determinants that were responsible for the anti-glycation and antioxidant activities in derived fractions from this plant. In addition, the anti-glycation and antioxidant capacities of the phenolic compounds also depend on their chemical structures, especially the number and position of hydroxyl group.

5.1.3 The ethyl acetate fraction was further sub-fractioned into five sub-fractions (sub-fractions 1–5) using Sephadex LH-20 column chromatography. Of all ethyl acetate sub-fractions, sub-fraction 3 (SF3) exhibited the strongest antioxidant activities as determined using CUPRAC, $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and FRAP as well as its TPC and TFC. Moreover, the sub-fraction 3 showed the higher $DPPH^{\bullet}$ ($IC_{50}$ = 19 µg/ml) and $ABTS^{\bullet+}$ ($IC_{50}$ = 12 µg/ml) scavenging activities than those of synthetic antioxidants, including ascorbic acid and trolox. These findings indicate that the SF3 from *G. procumbens* leaves is a good potential source of the natural antioxidant. Based on our findings, we concluded that the fractionation using multiple steps (crude extract, fractions and sub-fractions) purified and concentrated the best phenolic compounds responsible for antioxidant activity from *G. procumbens* leaves.

5.1.4 Base on HPLC analysis, the predominant phenolic acids identified in SF3 were ferulic acid and sinapic acid, whereas dominant flavonoids were myricetin and kaempferol. There was a strong positive correlation between antioxidant activity and TFC; however, no correlation existed between those and TPC. Thus, the strongest antioxidant activity in SF3 of *G. procumbens* leaves came from two flavonoids, namely myricetin and kaempferol. Our findings suggest that *G. procumbens* could potentially be developed into functional food products, such as tea or functional drinks, and may also be used as natural ingredients in foods for human health promotion.

**5.2 Suggestions**

According to these results, the leaves of *G. procumbens* have exhibited a potential good source of flavonoids, especially myricetin and kaempferol, which were excellent the antioxidant and anti-glycation activities. Therefore, further studies should be conducted on the evaluation of the biological activities (antioxidant and anti-glycation) by *in vivo* experiments, and to investigate their clinical effects in the human body. Furthermore, the effect of consuming a product produced from this plant on stress and immunity in human need also to be further studied to fully realize the application of this plant for use as functional foods.

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# BIOGRAPHY

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**Research output**

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