CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmacognosy

BIOLOGICAL ACTIVITIES AND CHEMICAL CONTENT OF GLYCYRRHIZA SPECIES

PhD THESIS

JOSÉ CARLOS CHEEL HORNA

Supervisor: Doc. PharmDr. Lenka Tůmová, CSc.

HRADEC KRÁLOVÉ

2010

ACKNOWLEDGEMENTS

This study was carried out at the Department of Pharmacognosy, Faculty of Pharmacy, Charles University, Czech Republic. The research work was financially supported by the Czech Republic Government (Project MSM 0021620822) and the grant SVV–2010–261–002. I thank my supervisor, Doc. Lenka Tůmová, for her support throughout the research process. I am grateful to UNESCO for a PhD fellowship as well as to the Wallonia-Brussels International (IN.WBI) Program, Belgium for a research grant (Bourses d'excellence–research grant IN.WBI). I wish to express my deepest gratitude to PharmDr. Doris Vokurková, PhD. (Institute of Clinical Immunology and Allergology, Faculty of Medicine, Charles University) for her fruitful and pleasant collaboration. Finally, I would particularly like to thank Ing. Jarmila Neugebauerová, Ph.D. (Faculty of Horticulture, Mendel University in Brno) for collection of licorice samples. The metereological data provided by the Solar and Ozone Observatory of the Czech Hydrometeorological Institute is also thankfully acknowledged.

"Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány".

In Hradec Králové, 9 November 2010

José Carlos Cheel Horna

Pharmacist

CONTENT

1. INTRODUCTION……………………………………………………………………........1

2. OBJECTIVES……………………………………………………………………....…..4

3. THEORETICAL FRAMEWORK..5

3.1. Historical perspective of the therapeutical use of *Glycyrrhiza* species...5

3.2. Description, occurrence and sources of licorice...6

3.3. Chemical composition...7

3.3.1. Triterpene saponins…………………………………………………..…………………..8

3.3.2. Flavonoids and other phenolic compounds……………………………………….........11

3.4. Biosynthesis of secondary metabolites of *Glycyrrhiza* species (licorice)……………………...………16

3.4.1. Biosynthesis of triterpene saponins………………………………………………….....16

3.4.2. Biosynthesis of flavonoids……………………………………………………………..20

3.5. Pharmacological activities of licorice……………………………..……………….……….......23

3.5.1. Antiinflammatory activities………………………………………….…………..….24

3.5.2. Immunomodulatory activities…………………………………………………...………..24

3.5.3. Antimicrobial and antiviral activities……………………………………….…..……..26

3.5.4. Hepatoprotective activities…………………………………………………….….….…..26

3.5.5. Antiulcer activities………………………………………………………………….….….…...27

3.5.6. Antioxidant activity…………………………………………………….………….….….28

3.5.7. Antidiabetic activity…………………………………………………………..….….28

3.5.8. Antitumor activity…………………………………………………………...….…….30

3.5.9. Side effects and toxicity…………………………………………………….....…...30

3.5.10. Phytopharmaceutical formulations containing licorice…………………….….……….31

3.6. Industrial use of licorice………………………………………………………...……….31

3.7. Methods of immunomodulating and antioxidant evaluation of plants……………………......………..32

3.7.1. Methods of immunomodulating evaluation of plants…………………………………………….....32

3.7.1.1. Activations of cell surface markers on immune cells…………….……..…………….32

3.7.1.2. Effects on T cells………………………………..…………………………….…………33

3.7.1.3. Effects on cytokine production and other immune effector cells…………….…….…...33

3.7.1.4. Effects on antibody production………………………………..…………….……...34

3.7.1.5. Effects on programmed cell death (apoptosis)………………………………………...35

3.7.2. Methods of antioxidant evaluation of plants…………………………………………………….…..35

4. MATERIALS AND METHODS…………………........………………………………..…38

4.1. Equipments, instruments and glass materials…………………………..……………………….……..…38

4.2. Chemicals..39

4.3. Collection and preparation of samples..40

4.3.1. Samples of *Glycyrrhiza* species for comparative study...40

4.3.2. Sample of *Glycyrrhiza glabra* for study of infusion...41

4.3.3. Samples of *Glycyrrhiza glabra* for study at different harvest times..41

4.4. Chemical analytical methods..42

4.4.1. Determination of total content of phenols………………………………………………......….42

4.4.2. Determination of total content of tannins……………………………………………………....43

4.4.3. Determination of total content of flavones and flavonols……………………………..……….43

4.4.4. HPLC-DAD analysis……………………………………………………………………......….44

4.4.5. HPLC-ESI-MS analysis…………………………………………………………..….......…45

4.5. Biological assays...47

4.5.1. Free radical scavenging activity...47

4.5.1.1. DPPH free radical-scavenging activity..47

4.5.1.2. Superoxide anion radical-scavenging activity...48

4.5.2. Antioxidant activity……………………………...............…………..……….……..…...49

4.5.2.1. Inhibition of β-carotene-linoleate bleaching..49

4.5.2.2. Hypochlorous acid-scavenging activity....51

4.5.2.3. Inhibition of myeloperoxidase-chlorinating system....52

4.5.3. Effects on immune cells....53

4.5.3.1. Activation of immune cells....53

4.5.3.2. Cell cycle progression of lymphocytes....54

4.6. Statistical analysis....54

5. RESULTS AND DISCUSSION....55

5.1. Phenolic content profiles and free radical-scavenging activity of roots of *Glycyrrhiza* species....55

5.1.1. Total content of polyphenols....55

5.1.2. HPLC-DAD analysis....58

5.1.3. Free radical-scavenging activity....60

5.1.3.1. DPPH free radical-scavenging activity....60

5.1.3.2. Superoxide anion radical-scavenging activity....61

5.1.4. Correlation, free radical-scavenging activity and content of TP, TF and TT....63

5.2. Free radical-scavenging, antioxidant and immunostimulating effects of licorice infusion (*Glycyrrhiza glabra* L.)....68

5.2.1. Total content of polyphenols....68

5.2.2. HPLC-DAD analysis....71

5.2.3. Free radical-scavenging activity....72

5.2.3.1. DPPH free radical-scavenging activity....72

5.2.3.2. Superoxide anion radical-scavenging activity....74

5.2.4. Antioxidant activity....75

5.2.4.1. Inhibition of β-carotene-linoleate bleaching....75

5.2.4.2. Hypochlorous acid-scavenging activity....79

5.2.4.3. Inhibition of myeloperoxidase-chlorinating system....81

5.2.5. Effects on immune cells....84

5.2.5.1. Activation of immune cells……………………….……….……...............………84

5.2.5.2. Cell cycle progression of lymphocytes……………..……………….……………..........87

5.3. Variations in the chemical profile, free radical scavenging and antioxidant activities of licorice (*Glycyrrhiza glabra* L.), as influenced by harvest times...90

5.3.1. Extraction yield...90

5.3.2. HPLC-DAD and HPLC-ESI-MS analysis..90

5.3.3. Total content of polyphenols…………………..……………..……………..…………......…....96

5.3.4. Free radical-scavenging activity……………………….……………….…………..................98

5.3.4.1. DPPH free radical-scavenging activity..98

5.3.4.2. Superoxide anion radical-scavenging activity...99

5.3.5. Antioxidant activity…………………………………………..……..……...............................100

5.3.5.1. Inhibition of β-carotene-linoleate bleaching……….……......……....……………100

5.3.5.2. Hypochlorous acid-scavenging activity...105

5.3.5.3. Inhibition of myeloperoxidase-chlorinating system………..………….........………106

6. CONCLUSIONS………………………………...………………...………….........112

6.1. Phenolic content profiles and free radical-scavenging activity of roots of *Glycyrrhiza* species..112

6.2. Free radical-scavenging, antioxidant and immunostimulating effects of licorice infusion (*Glycyrrhiza glabra* L.)……………………………………………………………………….........…112

6.3. Variations in the chemical profile, free radical-scavenging and antioxidant activities of licorice (*Glycyrrhiza glabra* L.), as influenced by harvest times..113

7. ABSTRACT………………………………….........…………………….……………114

8. ABSTRAKT…………………………………...……………………..……………......117

9. LIST OF ABBREVIATIONS...120

10. REFERENCES..122

11. PUBLICATIONS AND CONGRESS PRESENTATIONS…..…………….…..........145

12. ATTENDANCE AT SEMINARS AND SCIENTIFIC MEETINGS……….........149

13. GRANTS AND HONOURS…………………………………………….……….........149

1. INTRODUCTION

In Chinese Pharmacopoeia, the species *Glycyrrhiza uralensis*, *G. glabra* and *G. inflata* are listed as licorice, while in Japanese Pharmacopoeia, *G. uralensis* and *G. glabra* are prescribed **(Kondo *et al.*, 2007)**. In the European Pharmacopoeia, licorice is the name applied to *G. glabra* roots **(European Pharmacopoeia, 2002)**. The other *Glycyrrhiza* species such as *G. echinata* and *G. pallidiflora* are rarely used for medical and commercial purposes **(Kajiyama *et al.*, 1993)**. Until now, the phenolic profile and free radical scavenging activity of roots of *G. glabra, G. uralensis, G. echinata,* and *G. pallidiflora* species had not been comparatively analyzed.

The high rate of mortality originated by chronic diseases and the increasing danger of global pandemics caused by viral infections are encouraging the evaluation of herbal drugs in widespread traditional use. Licorice, the dry roots of *Glycyrrhiza glabra* L. (Fabaceae), is considered one of the oldest and most widely used herbal drugs around the world, being present in most pharmacopoeias of Eastern and Western countries. It has been traditionally used for respiratory, gastrointestinal, cardiovascular, genitourinary, eye and skin disorders, and for its antiviral effects **(Fiore *et al.*, 2005)**. The antiulcerogenic action of licorice and its consumption as a food ingredient have also been reported **(Isbrucker *et* Burdock, 2006)**. Glycyrrhizin and flavonoids such as liquiritin, isoliquiritin and their aglycones have been reported as the major constituents of licorice and they are perceived as the active principles responsible for its pharmacological efficacy **(Zhang *et* Ye, 2009)**. Reactive oxygen species are implicated in the development of many chronic disorders, such as cancer, atherosclerosis, nephritis, diabetes mellitus, rheumatism, and cardiovascular diseases, as well as in gastrointestinal tract disorders and inflammatory injury **(Scheibmeir *et al.*, 2005)**. Since

licorice is traditionally used for the treatment of some of these conditions, its free radical scavenging and antioxidant potential deserves to be explored. There is evidence of the antioxidant activity of alcoholic and acetonic extracts of licorice **(Di Mambro *et* Fonseca, 2005; Fuhrman *et al*., 2002; Vaya *et al*., 1997)**. The inhibiting effect of licorice extracts on oxidative enzymes such as xanthine oxidase (XOD) and monoamine oxidase (MAO) has also been explored **(Hatano *et al*., 1991)**. However, the antioxidant potential of the infusion of licorice and the relation of such effect with its major constituents had not been extensively investigated.

Given that traditional sources mention its ability to treat symptoms attributable to viral infections, licorice is gaining attention as a potential immunomodulating agent. Most studies on the immunomodulating effects of licorice have focused mainly on glycyrrhizin **(Fiore *et al*., 2008)**. Only recently was the activation effect of a licorice tincture on T cells reported, as measured by CD69 expression **(Brush *et al*., 2006)**. The CD69 glycoprotein is one of the earliest cell surface markers expressed by T, B, NK cells, and granulocytes, following activation. Once expressed, CD69 acts as a costimulatory molecule for the immune cells activation and proliferation **(Ziegler *et al*., 1994)**. Immunity has two components, innate (non-specific) and adaptive (specific). Macrophages, dendritic cells, NK cells, and granulocytes, such as neutrophils, eosinophils, and basophils, are cellular components of the innate immune system, and have been shown to be important regulatory and effector cells in host defence against viral, parasitic, and bacterial infections **(Melvold *et* Sticca, 2007; Nopp *et al*., 2002)**. Innate immunity recently has received attention, because, despite a certain lack of specificity, it effectively distinguishes self from non-self, and activates adaptive immune mechanisms through the provision of specific signals **(Melvold *et* Sticca, 2007)**. This last event can give rise to the clonal expansion of adaptive immune cells, such as T lymphocytes,

which play important roles in the generation of immune responses **(Simms *et* Ellis, 1996)**. To date, little is known about the effects of licorice infusion on innate and adaptive immune cells. Licorice is popularly consumed in the form of teas and infusions **(Obolentseva *et al*., 1999)**, but the biological properties of these aqueous preparations have been little explored.

Recently, the demand for licorice has been increasing, whilst the availability of wild licorice has declined **(Yamamoto *et* Tani, 2006)**. The promotion of cultivation for licorice as an additional and stable source of the medicinal plant requires to determine the influence of enviromental factors on its chemical content and biological properties. The harvesting of licorice root occurs in the autumn of its third or fourth year of growth **(Isbrucker *et* Burdock , 2006)**, which is mainly dependent on the total yield of the plant rather than on their chemical composition. Botanicals could have variable chemical compositions because of variations in soil types, climates as well as harvest time or growing period **(Incerti *et al*., 2009; Yesil-Celiktas *et al*., 2007; Çyrak *et al*., 2007)**. The effects of enviromental factors on the chemical and biological conditions of licorice have been a little explored, except for a few studies, which revealed seasonal variation in the glycyrrhizin and isoliquiritin contents **(Hayashi *et al*., 1998)** and the influence of UV radiation on glycyrrhizin biosynthesis **(Afreen *et al*., 2005)**.

2. OBJECTIVES

- ✓ To determine the total content of phenols, flavonoids and tannins, and the free radical scavenging activity of extracts of *G. glabra, G. uralensis, G. echinata and G. pallidiflora* roots. Furthermore, to investigate the relationship between the total content of polyphenols and the antiradical activity.

- ✓ To evaluate the free radical-scavenging and antioxidant effects of licorice infusion (*Glycyrrhiza glabra*) and its major constituents. Moreover, to investigate their effects on the NK cells and granulocytes activation as well as on the cell cycle progression of lymphocytes.

- ✓ To investigate the variation in the chemical composition, free radical-scavenging and antioxidant activities of extracts of licorice obtained from *Glycyrrhiza glabra* plant collected at different harvest times during a one-year period. In addition, to investigate the correlation between chemical composition and biological activities.

3. THEORETICAL FRAMEWORK

3.1. Historical perspective of the therapeutical use of *Glycyrrhiza* species

Licorice is the name applied to the roots and stolons of some *Glycyrrhiza* species (Fabaceae) and has been used by human beings for at least 4000 years **(Nomura *et al.*, 2002)**. It has a long and storied history of use in both Eastern and Western cultures pre-dating the Babylonian and Egyptian empires **(Fenwick, 1990)**. The genus name *Glycyrrhiza* is derived from the ancient Greek word for "sweet root" (Gr. *glykos* (sweet) + *rhiza* (root)), which was later latinized to *liquiritia* and eventually to licorice. The two principal forms in commerce are licorice root (*Liquiriti radix*) and the extract (*Glycyrrhizae extractum crudum* or *Succus liquiritiae*) **(Isbrucker *et* Burdock, 2006)**. The ancient Greeks and Romans are known to have cultivated the plants in the third century. Licorice was a prescriptive agent of Hippocrates in the treatment for asthma, dry cough and other "pectoral diseases", and was also thought to be effective in preventing thirst. In Chinese traditional medicine, licorice (*Gan Cao*) remains one of the oldest and most commonly prescribed herbs and has been used in the treatment of numerous ailments ranging from tuberculosis to peptic ulcers **(Davis *et* Morris, 1991)**. The latter indication of liquorice features also prominently in the Indian Ayurveda system. The uses of *Glycyrrhiza glabra* (in Sanskrit: klitaka, madhuka, yasti or yastimadhuka) reported here apart from its use in antidote mixtures for a variety of acute and chronic poisonings, include improvement of the voice, an indication mentioned in the context of viral respiratory tract infections, wound infections, operation wounds of the ear, excessively bleeding punctures from blood letting and acute and chronic liver diseases like hepatitis. European, Indian and Chinese traditions all contain references to antiviral effects in the

context of viral induced voice changes in laryngitis, pharyngitis, most likely viral induced cough, viral hepatitis and viral skin diseases like condyloma and ulcers **(Fiore *et al*., 2005)**.

3.2. Description, occurrence and sources of licorice

There are four main varieties of *Glycyrrhiza glabra. Glycyrrhiza glabra* L. var. *typica* Reg. et Herd., having blue flowers and being found in the Mediterranean and Caucasian regions. This variety is frequently termed Spanish or Italian liquorice. *Glycyrrhiza glabra* L. var. *violacea* Boiss (Persian or Turkish liquorice) and *Glycyrrhiza glabra* L. var. *pallida* Boiss., both found wild in the regions of old Mesopotamia. *Glycyrrhiza glabra* L. var. *glandulifera* (Waldst. et Kit.) Reg. et Herd. (sometimes called *G. glandulifera, G. hirsuta* or *G. brachycarpa),* having violet flowers and occurring in Hungary, Southern Siberia, Turkestan and Afghanistan, from which the common name Russian liquorice is derived **(Fenwick, 1990)**.

Glycyrrhiza uralensis grows wild from Xinjiang to Mongolia, eastern Inner Mongolia and eastern Siberia, and is the main raw material of medicinal Kanzo, including Dongbei-Gancao, which is used mainly in Japan. It was observed wild *G. uralensis* plants possessing falcate fruits with prickly hairs in both the southern and northern peripheries of the Tianshan Mountains. It has already been reported that putative intermediates between *G. uralensis* and *G. glabra* have been observed in Kazakhstan, which is located on the western border of Xinjiang. The distribution area of *G. uralensis* is from Central Asia to China.

Glycyrrhiza inflata, is found in the western part of China. *G. inflata* is the typical *Glycyrrhiza* plant growing naturally in Xinjiang. The chinese name of the *G. inflata* plant is ZhangGuo-

Gancao meaning Gancao (licorice) with swollen fruits, which are clearly distinguishable from the fruits of *Glycyrrhiza uralensis* (**Yamamoto *et* Tani, 2006**).

In addition, reference is sometimes made to *G. pallidiflora* Maxim (Manchurian licorice) and *G. echinata*, but these are regarded as separate species of *Glycyrrhiza* (**Fenwick, 1990**). These species do not produce glycyrrhizin but produce macedonoside C as a major triterpene saponin (**Hayashi et *al*., 2005**). Roots of these plants are rarely used for medical and commercial purposes. *Glycyrrhiza pallidiflora* Maxim. native to the Amur region of Far East Siberia, is called "inu-kanzo" ('Dog-licorice', if translated verbatim) in Japanese (**Kajiyama *et al*., 1993**).

Some local production of *Glycyrrhiza* plants has been reported, such as *G. yunnanensis* P.C. Li (Malay licorice) in southwestern China and *G. squamulosa* Franch in Central Asia region (**Shibata, 2000**).

3.3. Chemical Composition

During the past century, a number of components, including both complexes of biologically active substances and hundreds of individual compounds belonging to various chemical classes and representing groups possessing different pharmacotherapeutic properties, were isolated from licorice. Among these components, the first to be mentioned is a water-soluble complex of biologically active substances which account for 40–50% of the total dry raw material weight. In this complex, a considerable part is made up of triterpene saponins, flavonoids, polysaccharides, pectins, simple sugars, amino acids, mineral salts, microelements, and some other substances. The list of individual compounds isolated by now

from various licorice herbs includes more than 50 triterpenoids, above 200 individual phenolic compounds, dozens of polysaccharides and amino acids **(Obolentseva *et al.*, 1999)**. The genus *Glycyrrhiza* consists of about 30 species, and chemical studies have so far been carried out on 15 of them. Glycyrrhizic acid (Glycyrrhizin) is the major triterpenoid saponin in licorice root and the main sweetener of the herb. The saponin is used frequently as a tool for recognizing the herb and has been obtained from *G. glabra*, *G. uralensis* and *G. inflata*, and thus, these plants are generally accepted as licorice **(Nomura *et al.*, 2002)**.

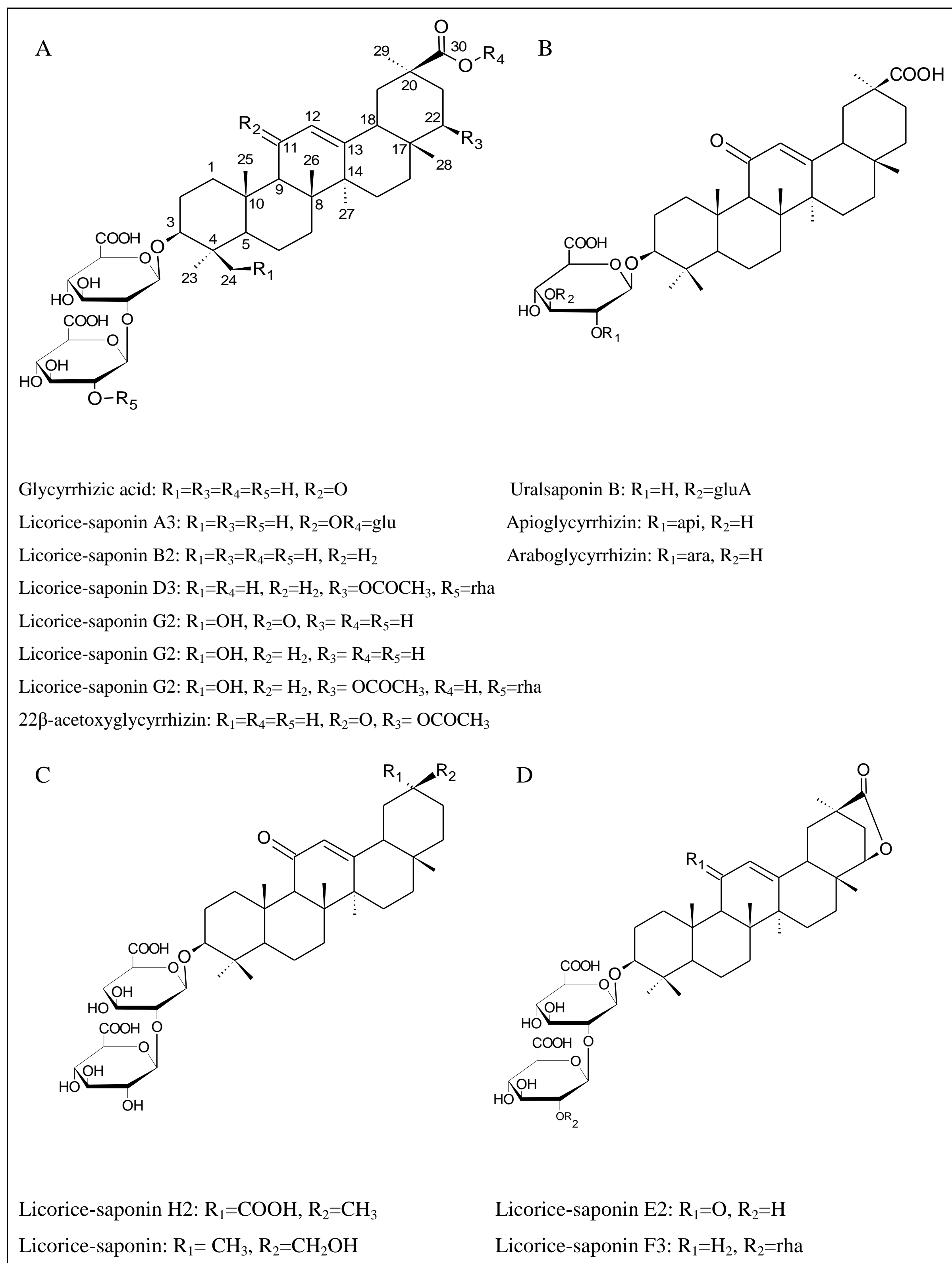
3.3.1. Triterpene saponins

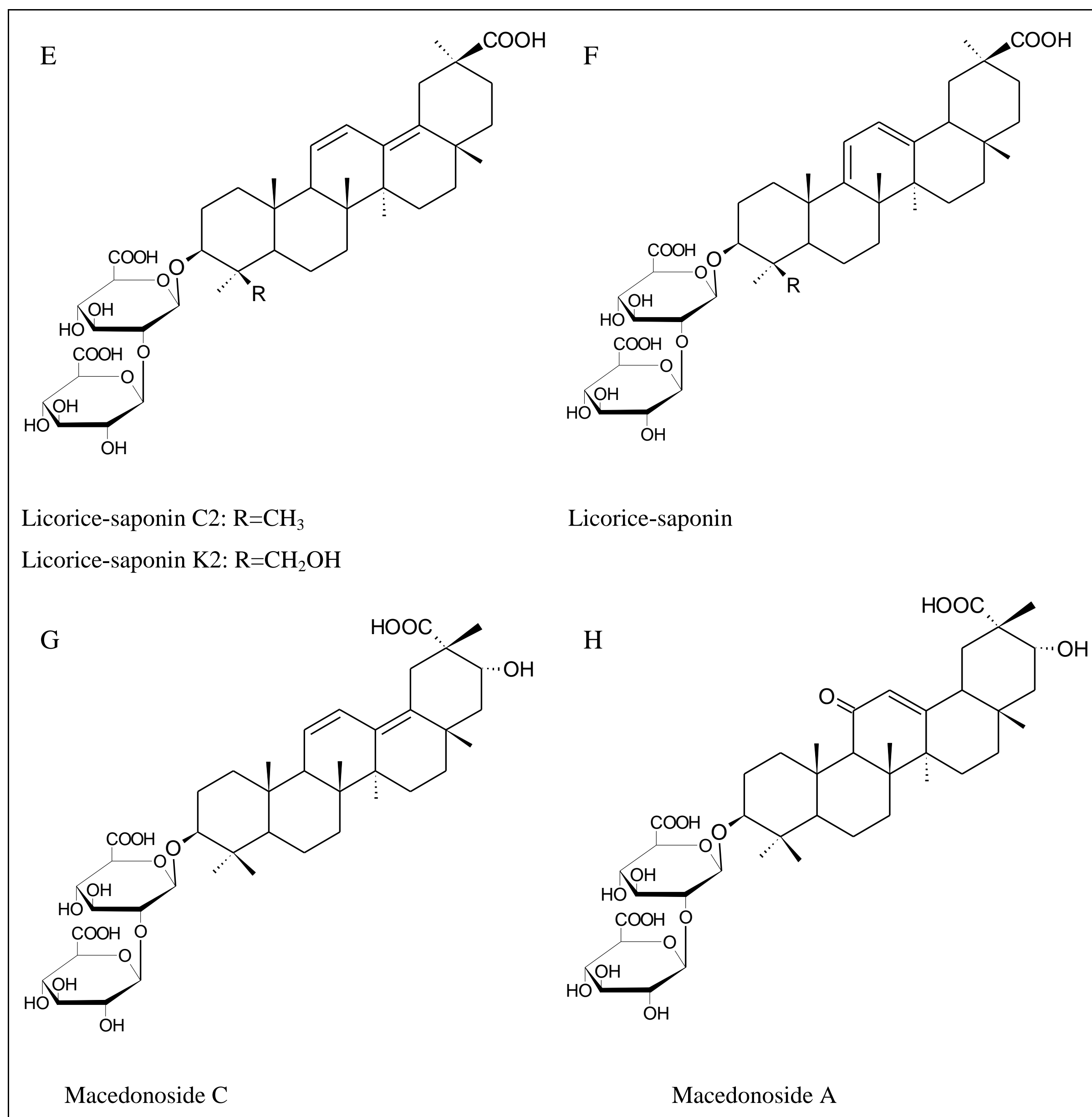
Triterpene saponins are the major characteristic constituents of licorice, and they are responsible for the sweet taste. So far, at least 18 saponins have been obtained from the three official species of licorice **(Zhang *et* Ye, 2009)**. The chemical structures of these saponins are shown in **Figure 1A-F**. Most licorice saponins are present as glucuronides. The aglycones are oleanane type pentacyclic triterpenes with 11-oxo-12-ene, 12-ene, 11, 13(18)-diene or 9(11), 12-diene skeletons, and 3β-OH, 24-OH, 22β-acetoxy, 30-COOH or 29-COOH as functional groups. Licorice saponins E2 and F3 contain unique lactone rings between 30-COOH and 22-OH **(Zhang *et* Ye, 2009)**.

Although the three *Glycyrrhiza* species, *G. glabra*, *G. uralensis*, and *G. inflata*, produce glycyrrhizin as a major saponin, the other three *Glycyrrhiza* species, *G. echinata*, *G. macedonica*, and *G. pallidiflora*, do not produce glycyrrhizin but instead produce macedonoside C **(Figure 1G)** as a major saponin **(Hayashi *et al.*, 2000)**. On the other hand, *G. lepidota*, American licorice, produces licorice-saponin H2 **(Figure 1C)** and macedonoside A **(Figure 1H)** as the two major saponins. Licorice-saponin H2 is a minor saponin isolated

from the glycyrrhizin-producing species, and macedonoside A is a minor saponin isolated from the macedonoside C-producing species. In addition, *G. lepidota* produces trace amounts of glycyrrhizin and macedonoside C, which suggests that *G. lepidota* is a chemotaxonomical intermediate of the glycyrrhizin-producing and macedonoside C-producing species. To confirm the relationship of these *Glycyrrhiza* species, it was determined the nucleotide sequences of a chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*). Based on the *rbcL* sequences, the seven *Glycyrrhiza* species were divided into three groups: the three glycyrrhizin producing species (*G. glabra*, *G. uralensis*, and *G. inflata*), the three macedonoside C-producing species (*G. echinata*, *G. macedonica*, and *G. pallidiflora*), and *G. lepidota* **(Hayashi, 2009)**.

Figure 1. Saponins from licorice. Ara:α-L-arabinopyranosyl; api: β-D-apiofuranosyl; glu: β-D-glucopyronosyl; gluA: β-D-glucuronopyronosyl; rha: α-L-rhamnonopyronosyl (**Zhang *et* Ye, 2009**).





Licorice-saponin C2: R=CH_3
Licorice-saponin K2: R=CH_2OH

Licorice-saponin

Macedonoside C

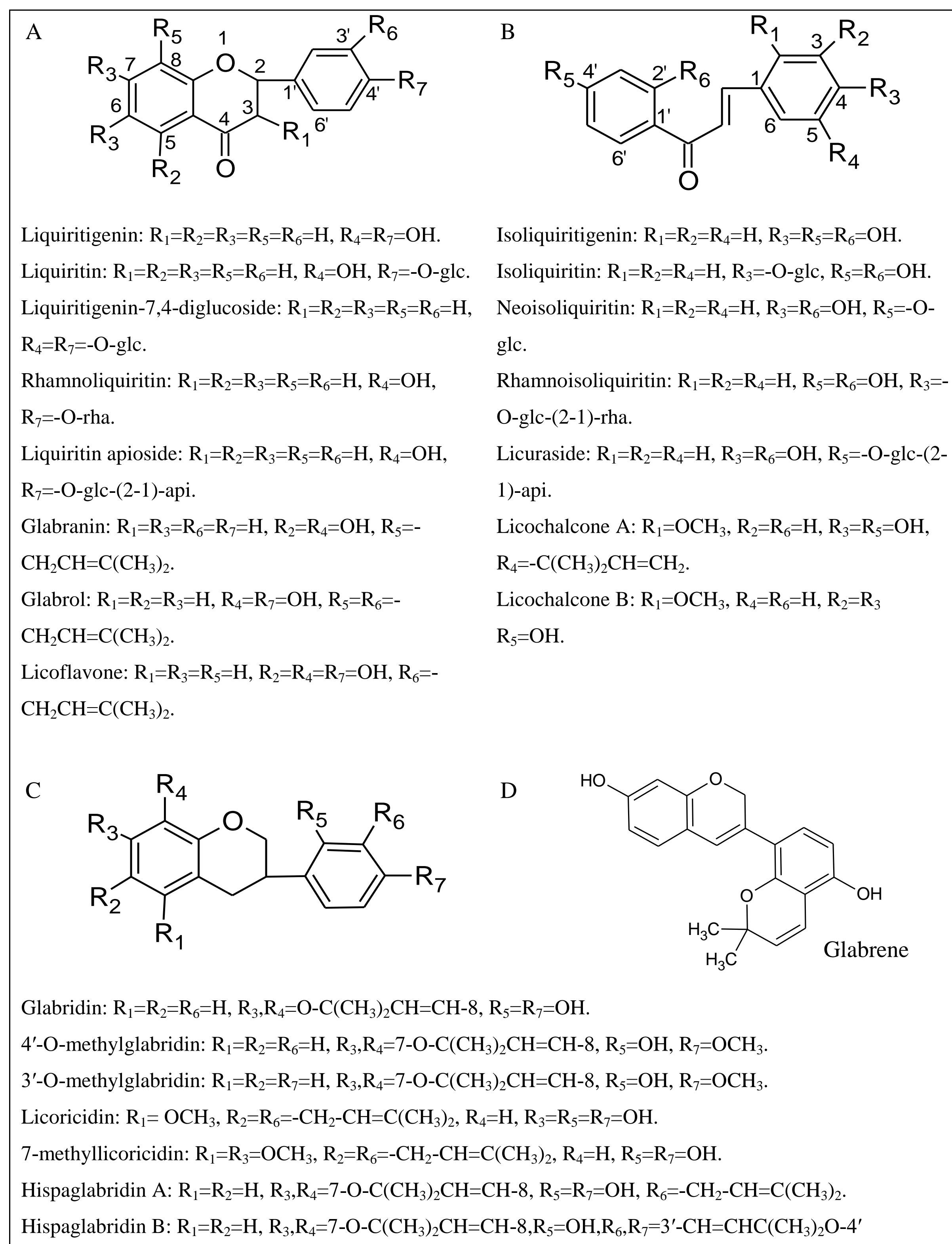
Macedonoside A

3.3.2. Flavonoids and other phenolic compounds

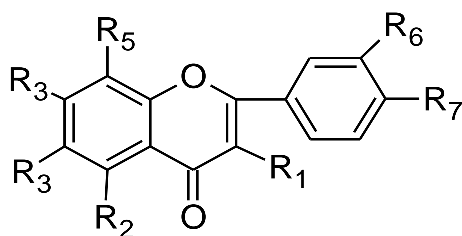
More than 300 flavonoids have been isolated from *Glycyrrhiza* species. These flavonoids belong to various types, including flavanones or flavanonols **(Figure 2A)**, chalcones **(Figure 2B)**, isoflavans **(Figure 2C)**, isoflavenes **(Figure 2D)**, flavones or flavonols **(Figure 2E)**, isoflavones **(Figure 2F)** and isoflavanones **(Figure 2G)**. Among them, flavanones and chalcones are the major types. Aside from flavonoids, less popular phenolic compounds, like

3-aryl-coumarins **(Figure 2H)**, coumestans **(Figure 2I),** benzofurans **(Figure 2J)** and retrochalcones **(Figure 2K)** have also been obtained from *Glycyrrhiza* species **(Zhang *et* Ye, 2009)**. The phenolic compounds in licorice could be chemotaxonomic markers to differentiate *Glycyrrhiza* species, especially the three official species (*G. uralensis*, *G. glabra*, and *G. inflata*). Such main constituents as liquiritigenin, liquiritin, isoliquiritigenin and isoliquiritin are present in all the three official species. However, some compounds may be regarded as the species-specific components, which mean that they may only be present in specific species. For instance, glabridin and glabrene are only present in *G. glabra*, glycycoumarin is only in *G. uralensis*, and licochalcones A and B only in *G. inflata* **(Hayashi *et al.*, 2000)**. While most of isoflavonoids obtained from other *Glycyrrhiza* species are prenylated, all of isoflavonoids found in *G. pallidiflora* were simple. What is most noteworthy among compounds isolated from *G. pallidiflora* is, perhaps, the occurrence of echinatin **(Figure 2K)**. This is an unusual chalcone lacking oxygenfunctionality at C-2' and clearly differs from normal chalcones, in sense of it undergoes no isomerization into flavanone. This characteristic chalcone was first isolated from G. *echinata* callus and was later obtained from *G. inflata*. Echinatin appears to have reversed A- and B-rings, and is thus given the name of "retrochalcone". The occurrence of retrochalcones in nature is still rare, and a search of the literature reveals that it has been found only in *G. echinata*, G. *pallidiflora* and *G. inflata* **(Kajiyama *et al.*, 1993)**.

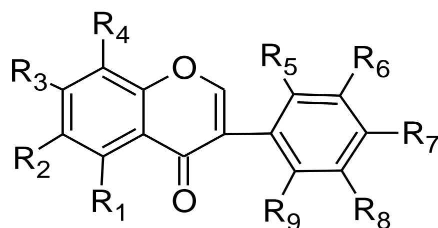
Figure 2. Flavonoids from licorice. Api: β-D-apiofuranosyl; glu: β-D-glucopyronosyl; rha: α-L-rhamnonopyronosyl (**Zhang *et* Ye, 2009**).



E



F



Licoflavone A: R_1=R_2=R_5=H, R_3=R_6=-$CH_2CH=C(CH_3)_2$, R_4=R_7=OH.

Licoflavone B: R_1=R_3=R_6=H, R_2=R_4= R_7 =OH, R_5=-$CH_2CH=C(CH_3)_2$.

Licoflavone C: R_1=R_2=R_5=R_6=H, R_4=R_7= OH, R_3=-$CH_2CH=C(CH_3)_2$.

Licoflavonol: R_1=R_2=R_4=R_5=R_7=OH, R_3=-$CH_2CH=C(CH_3)_2$, R_6=H.

Isolicoflavonol: R_1=R_2=R_4=R_7=OH, R_3= R_5=H, R_6=-$CH_2CH=C(CH_3)_2$.

Glabrone: R_1=R_2=R_4=R_5=R_6=H, R_3=R_9=OH, R_7,R_8=3′-CH=CHC$(CH_3)_2$-O-4′.

Glycyrrhisoflavone: R_1=R_3=R_6=R_7=OH, R_2=R_4=R_5=R_9=H, R_8=-$CH_2CH=C(CH_3)_2$.

Licoisoflavone A: R_1=R_3=R_5=R_7=OH, R_2=R_4=R_8=R_9=H, R_6=-$CH_2CH=C(CH_3)_2$.

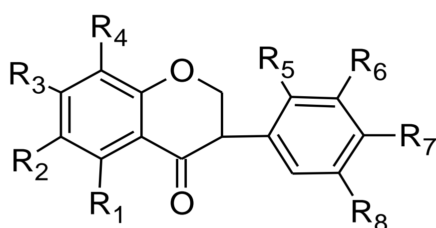
Licoisoflavone B: R_1=R_3=R_5=OH, R_2=R_4=R_8=R_9=H,R_6,R_7=3′-CH=CHC$(CH_3)_2$-O-4′.

Licoricone: R_1=R_2=R_4=R_6=H, R_3=R_5=OH, R_7=R_9=OCH_3, R_8=$CH_2CH=C(CH_3)_2$.

Gancaonin H= R_1=R_3=R_6=OH, R_2=-$CH_2CH=C(CH_3)_2$, R_4=R_5=R_9=H, R_7,R_8=3′-CH=CHC$(CH_3)_2$-O-4′.

Gancaonin G: R_1=R_7=OH, R_2=-$CH_2CH=C(CH_3)_2$, R_3=OCH_3, R_4=R_5=R_6, R_8=R_9=H.

G



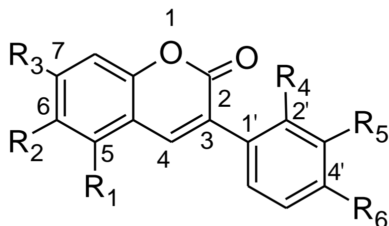
Glyzaglabrin: R_1=R_2=R_4=R_8=H, R_3=R_5=OH, R_6,R_7=-O-CH_2-O-.

Licoisoflavanone: R_1=R_3=R_5=OH, R_2=R_4=R_8=H, R_6,R_7=3′-CH=CHC$(CH_3)_2$-O-4′.

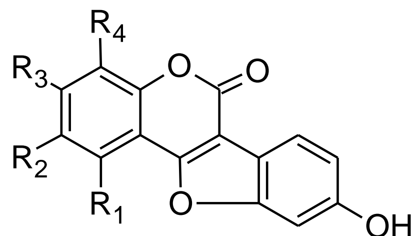
Glabroisoflavanone A: R_1=R_2=R_6=R_8=H, R_3,R_4=7-O-C$(CH_3)_2$CH=CH-8, R_5=R_7=OH.

Glabroisoflavanone B: R_1=R_2=R_6=R_8=H, R_3,R_4=7-O-C$(CH_3)_2$CH=CH-8, R_5=OH, R_7=OCH_3.

H



I



Glycycoumarin: $R_1=OCH_3, R_2=-CH_2CH=C(CH_3)_2, R_3=R_4=R_6=OH, R_5=H$.

Glabrocoumarin: $R_1=R_2=H, R_3=R_6=OH, R_4,R_5=2'-O-C(CH_3)_2CH=CH-3'$.

Glycyrin: $R_1=R_3=OCH_3, R_4=R_6=OH, R_2=-CH_2CH=C(CH_3)_2, R_5=H$.

Inflacoumarin A: $R_1=R_4=R_5=H, R_2=-CH_2CH=C(CH_3)_2, R_3=R_6=OH$.

Licopyranocoumarin: $R_1=OCH_3, R_2,R_3=-6-CH_2CH_2C(CH_3)(CH_2OH)-O-7, R_4=R_6=OH, R_5=H$.

Licofuranocoumarin: $R_1=OCH_3, R_2,R_3=-6-CH_2CH[C(CH_3)_2(OH)]-O-7, R_4=R_6=OH, R_5=H$.

Glycyrol: $R_1=OCH_3, R_2=-CH_2CH=C(CH_3)_2, R_3=OH, R_4=H$.

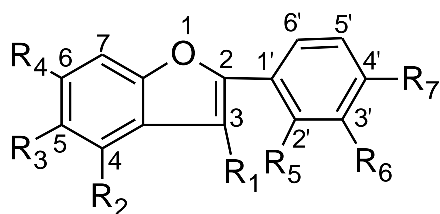
Isoglycyrol: $R_1=OCH_3, R_2,R_3=-6- CH_2CH_2C(CH_3)_2-O-7, R_4=H$.

3-O-methylglycyrol: $R_1=R_3=OCH_3, R_2=-CH_2CH=C(CH_3)_2, R_4=H$.

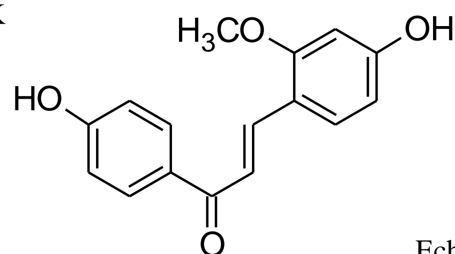
Neoglycyrol: $R_1=OCH_3, R_2=H, R_3=OH, R_4=-CH_2CH=C(CH_3)_2$.

Gancaonin F: $R_1=OCH_3, R_2,R_3=-6-CH=CHC(CH_3)_2-O-7, R_4=H$.

J



K



Echinatin

Licobenzofuran: $R_1=OH, R_2=R_5=H, R_3=R_4=OCH_3, R_6=-CH_2CH=C(CH_3)_2, R_7=OH$.

Licocoumarone: $R_1=H, R_2=OCH_3, R_3=-CH_2CH=C(CH_3)_2, R_4= R_5=R_7=OH, R_6=H$.

Glabrocoumarone A (glainflanin H): $R_1=R_2=R_3=H, R_4=R_5=OH, R_6,R_7=3'-CH=CHC(CH_3)_2-O-4'$.

Glabrocoumarone B: $R_1=H, R_2=R_3=H, R_4=R_7=OH, R_5,R_6=2'-O-C(CH_3)_2CH=CH-3'$.

Gancaonin I: $R_1=H, R_2=R_4=OCH_3, R_3=-CH_2CH=C(CH_3)_2, R_5=R_7=OH, R_6=H$.

Kansonol U: $R_1=H, R_2=R_3=H, R_4=R_7=OH, R_5,R_6=2'-O-C(CH_3)_2CH=CH-3'$.

Kansonol V: $R_1=H, R_2=H, R_3=-CH_2CH=C(CH_3)_2, R_4= R_7=OH, R_5,R_6=2'-O-C(CH_3)_2CH=CH-3'$.

3.4. Biosynthesis of secondary metabolites of *Glycyrrhiza* species (licorice)

3.4.1. Biosynthesis of triterpene saponins

Isoprenoids such as sterols, carotenoids and triterpene saponins are synthesized by consecutive condensations of their five-carbon precursor isopentenyl pyrophosphate (IPP), to its isomer, dimethylallyl diphosphate (DMAPP). These precursors are formed by the mevalonate pathway **(Kuzuyama, 2002)**, as depicted in **Figures 3 and 4**. Triterpenoid saponins are synthesised via the isoprenoid pathway by cyclization of 2,3-oxidosqualene to give primarily oleanane (β-amyrin) or dammarane triterpenoid skeletons. The triterpenoid backbone then undergoes various modifications (oxidation, substitution and glycosylation), mediated by cytochrome P450-dependent monooxygenases, glycosyltransferases and other enzymes **(Haralampidis *et al.*, 2002)**. Glycyrrhizin is an oleanane-type triterpene saponin having β-amyrin as biosynthetic intermediate **(Hayashi, 2009)**, **(Figure 5)**.

Figure 3. Biosynthesis of triterpene saponins – via mevalonate (**Kuzuyama, 2002**).

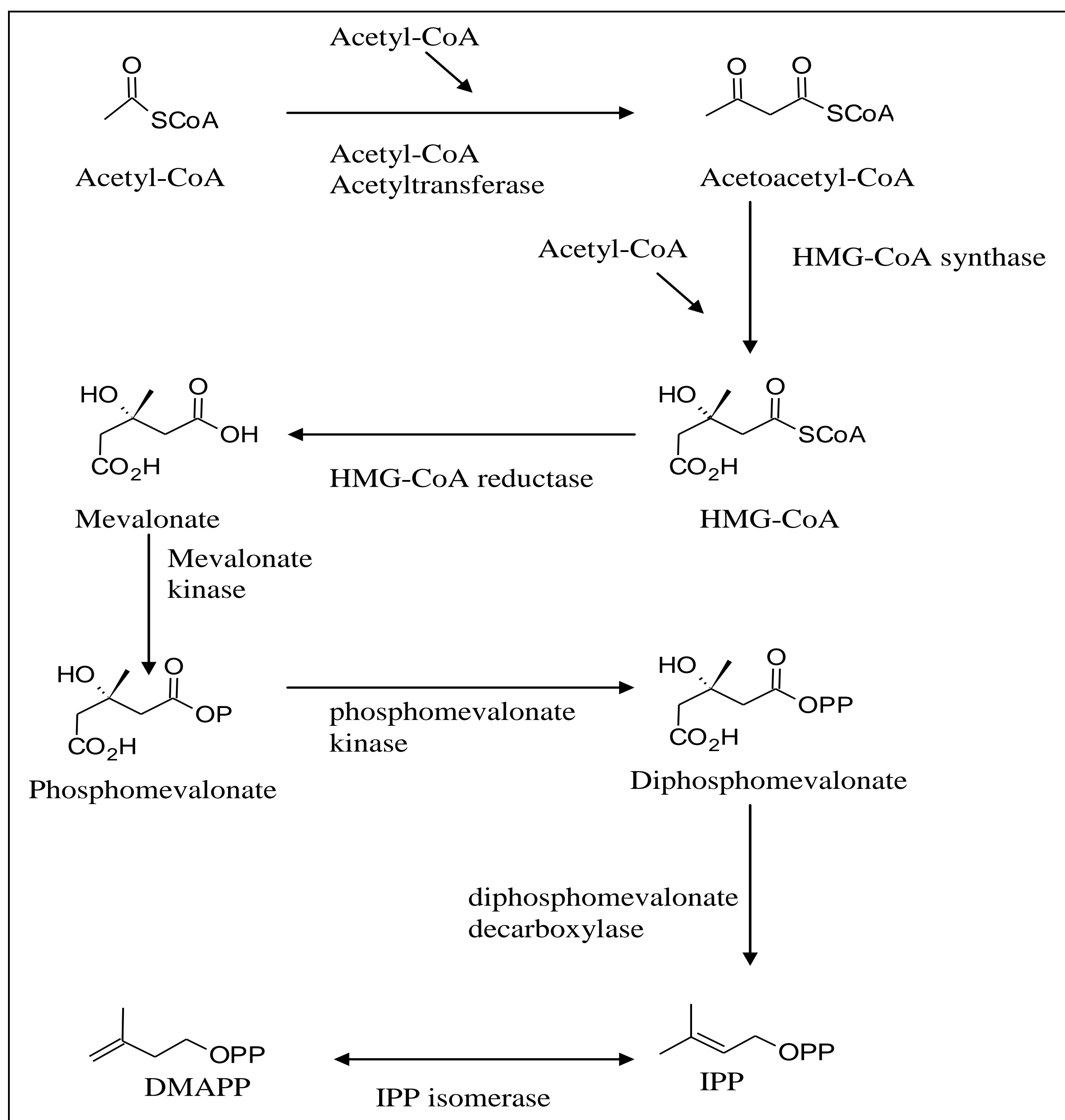


Figure 4. Biosynthesis of triterpenoids and steroids (**Bruneton, 1995**).

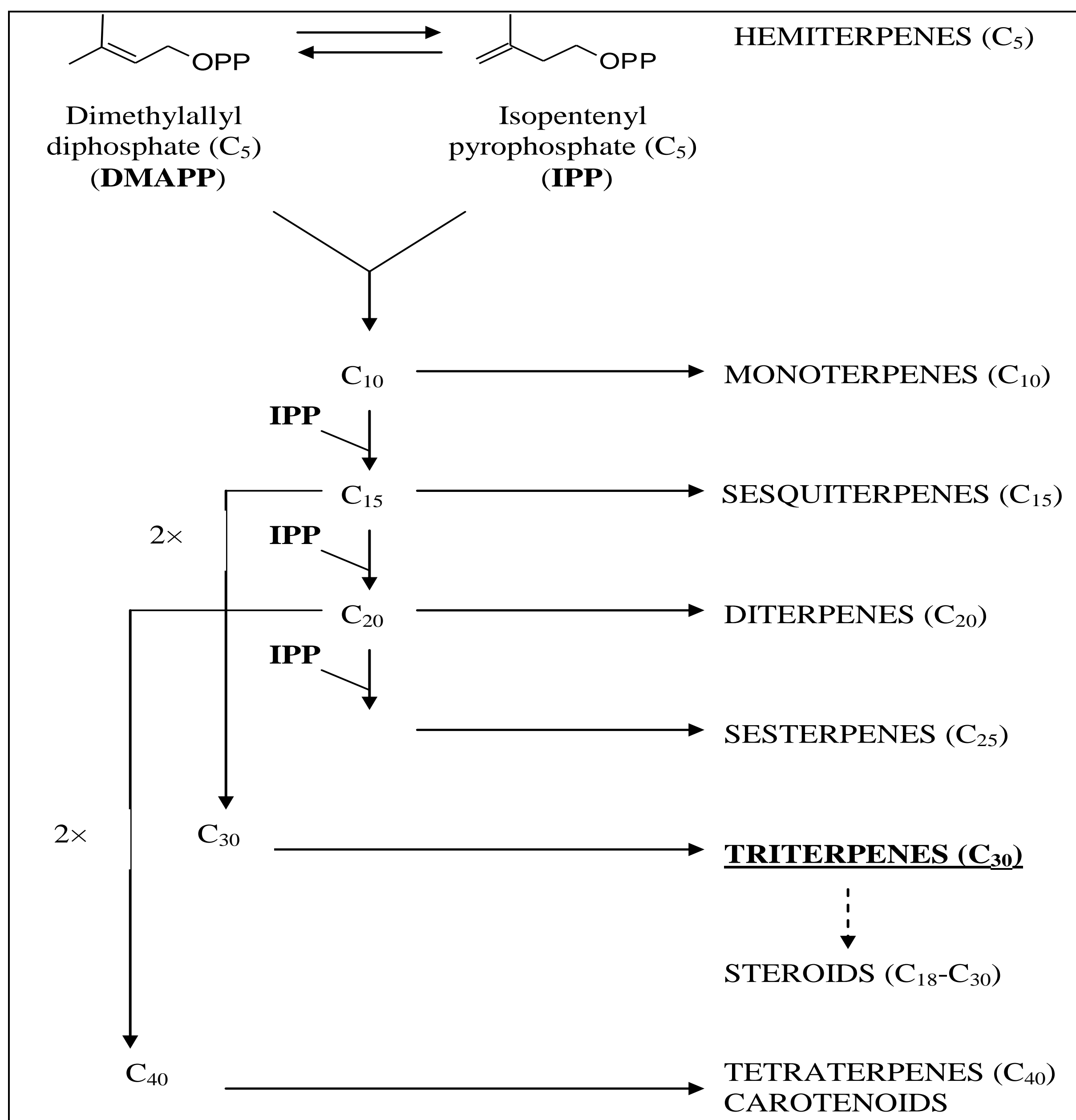
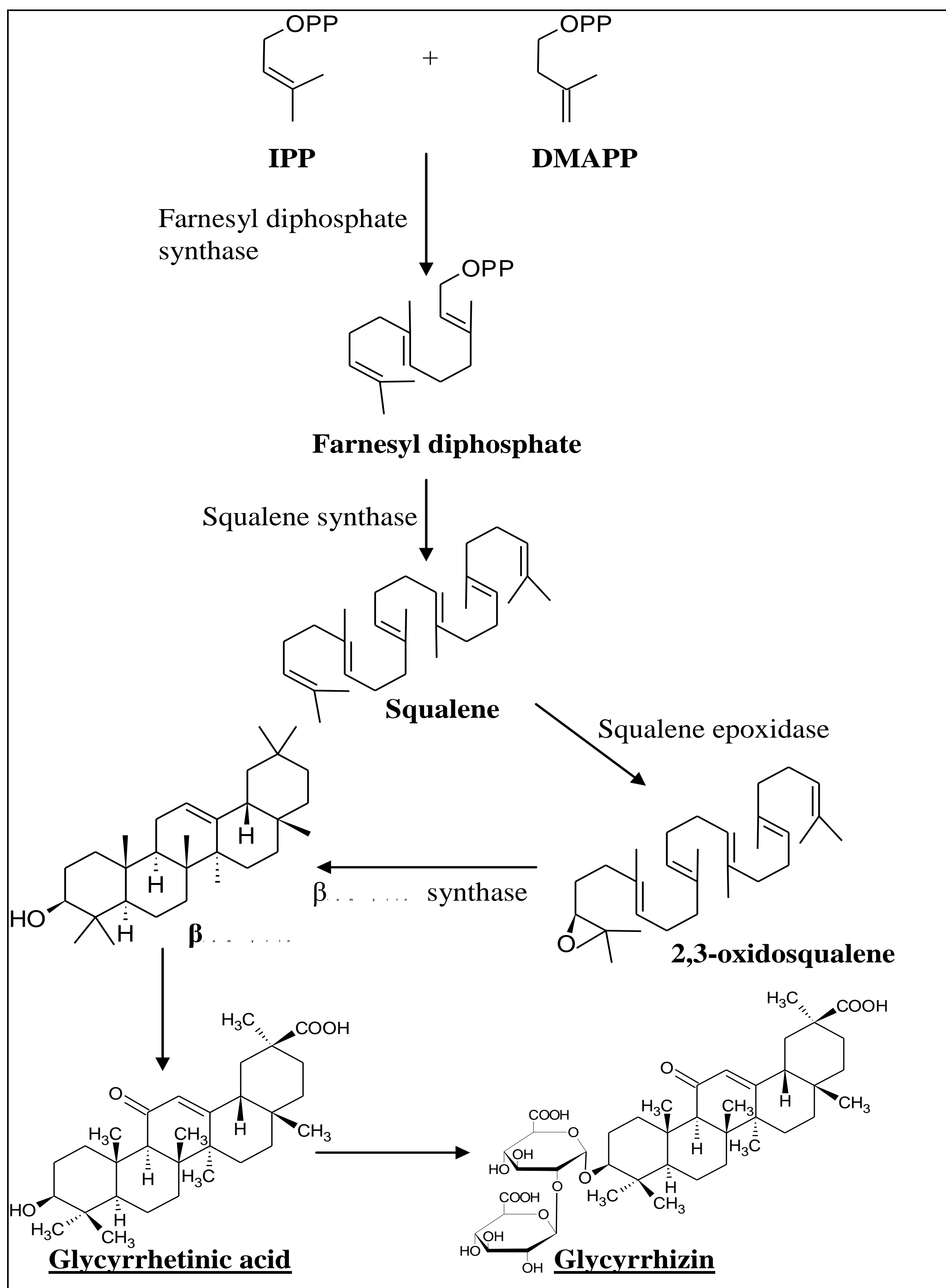


Figure 5. Biosynthesis of triterpenoid saponins (**Phillips *et al*., 2006**).



3.4.2. Biosynthesis of flavonoids

The key step in the formation of flavonoids is the condensation, catalyzed by chalcone synthase (CHS), of three molecules of malonyl-CoA with an ester of coenzyme A and of a hydroxycinnamic acid, as a general rule *p*-coumaryl-CoA (the incorporation of caffeoyl-CoA seems quite exceptional, as the extra hydroxylation of the B ring occurs late in the process). The reaction product is a chalcone most typically 2',4',6',4-tetrahydroxychalcone (THC or naringenin chalcone) **(Ayabe *et al.*, 2010; Bruneton, 1995)**. Malonyl-CoA, one of the two substrates of chalcone synthase (CHS), is synthesized from acetyl-CoA and CO_2. The second substrate of CHS, the CoA ester of cinnamic acid and its derivatives, leads via phenylalanine. This amino acid is exclusively formed by enzymes of the shikimate pathway **(Figure 6)**, which starts from erythrose 4-phosphate and phosphoenolpyruvate, two metabolites of carbohydrate metabolism **(Herrmann, 1995)**. Starting from the amino acid phenylalanine, the enzyme phenylalanine ammonia-lyase (PAL) removes the amine group from the amino acid and produces cinnamic acid. The first of several cytochrome P450 monooxygenases in this pathway, cinnamic acid 4-hydroxylase (C4H), adds a hydroxyl group to form *p*-coumarate. The enzyme 4-coumarate: coenzyme A ligase (4CL) further activates the *p*-coumarate by attaching a CoA at the three-carbon side chain. Next, CHS carries out the condensation of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to form the C15 flavonoid skeleton. In most species, this compound is naringenin-chalcone. The chalcone synthesized by CHS can be converted to the flavanone naringenin (5,7,4′-trihydroxyflavanone) by the enzyme chalcone isomerase (CHI). Naringenin is one of the shared substrates between flavonoid and isoflavonoid pathways **(Figure 7)**. Further modifications of naringenin lead to the production of various flavonoid compounds **(Yu *et* McGonigle, 2005)**.

In licorice, however, an NADPH-dependent chalcone polyketide reductase (PKR) (or chalcone reductase, CHR) coacts with CHS to form 6'-deoxychalcone such as isoliquiritigenin and its derivated 5-deoxyflavonoid liquiritigenin **(Ibrahim *et* Anzellotti, 2003)**. In species that synthesize isoflavones, the enzyme isoflavone synthase (IFS), another cytochrome P450 monooxygenase, acts as the key metabolic entry point for the formation of all isoflavonoids. This enzyme plays two roles: it diverts naringenin formed by CHI into genistein production and, in conjunction with another legume-specific enzyme, chalcone reductase (CHR), forms daidzein. In this case, CHR, CHS, and CHI work in concert to produce isoliquiritigenin and then liquiritigenin, which is the precursor for daidzein **(Yu *et* McGonigle, 2005)**.

Figure 6. Biosynthesis of flavonoids – shikimate pathway (**Bruneton, 1995**).

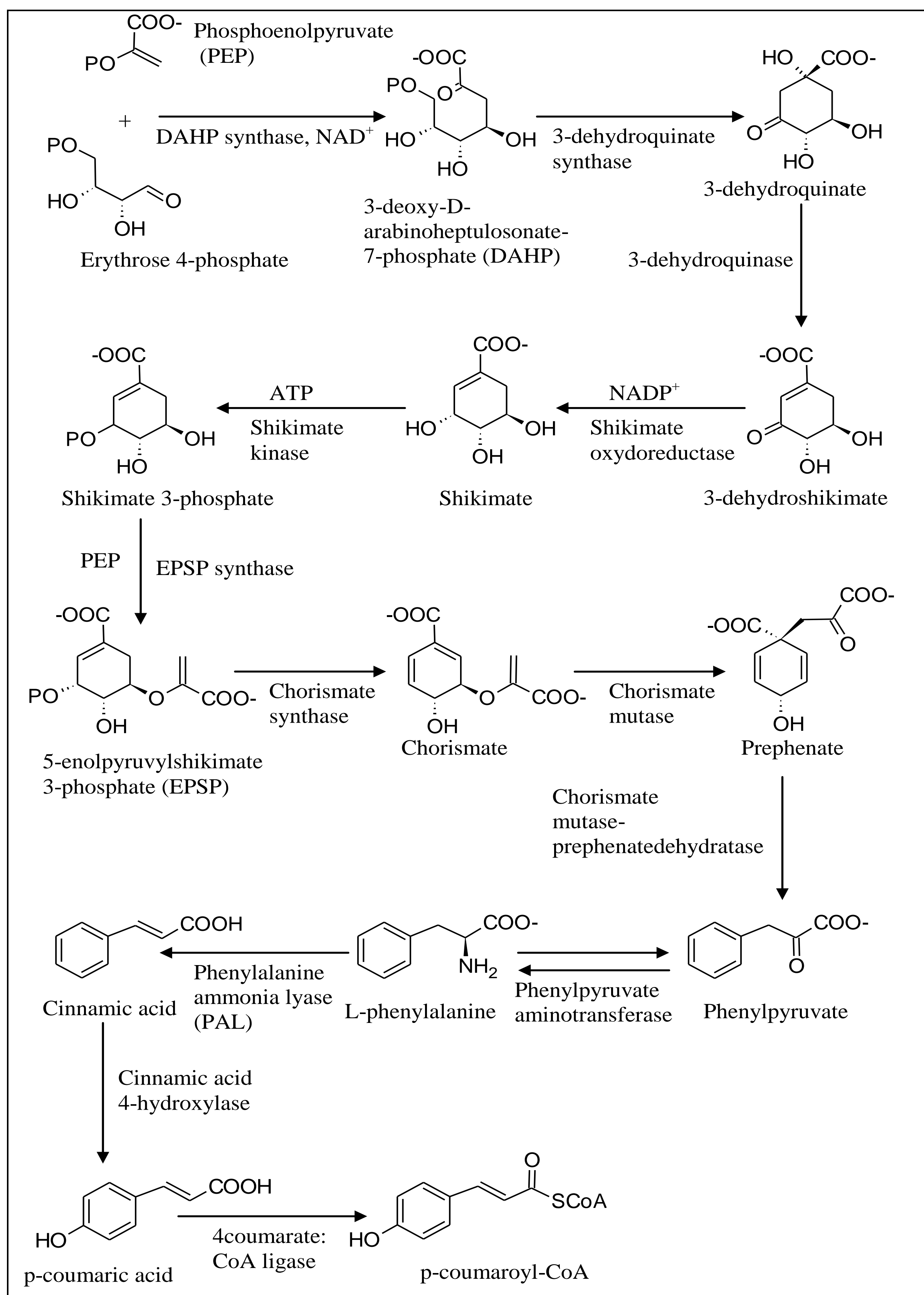
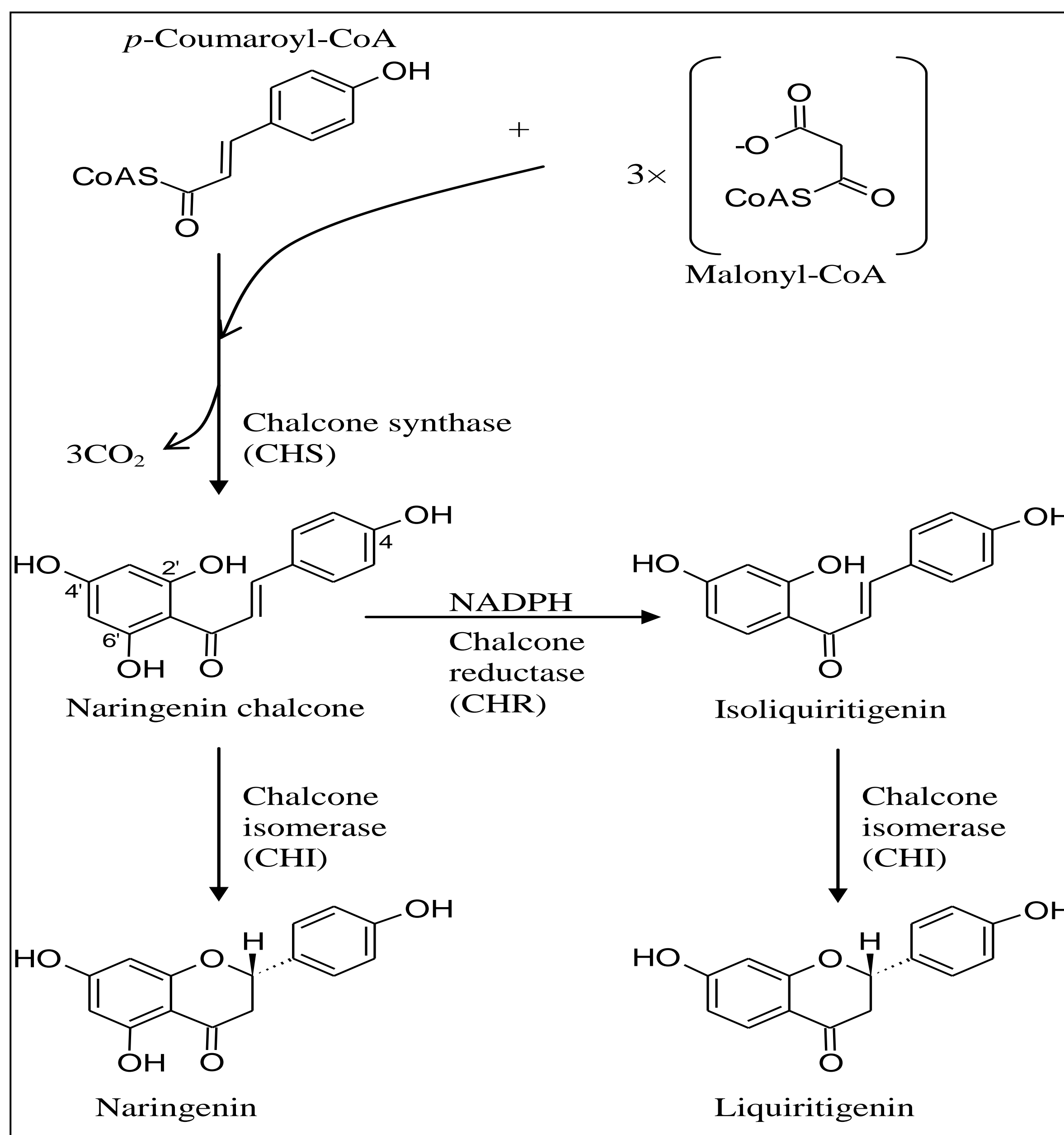


Figure 7. Biosynthesis of flavonoids (**Ayabe *et al*., 2010**).



3.5. Pharmacological activities of licorice

Licorice shows a variety of pharmacological activities, including antiinflammatory, immunomodulatory, antimicrobial, antiulcer, hepatoprotective, antiulcer antioxidant, antidiabetic and antitumor activities.

3.5.1. Antiinflammatory activities

The anti-inflammatory activities of the aqueous licorice extract (*G. glabra*) and glycyrrhizin in comparison with diclofenac sodium (10 mg/kg) was observed, using the carrageenan-induced paw edema model in male albino rats **(Aly *et al.*, 2005).** An herbal preparation (KIOM-79) containing extracts from *Magnolia officinalis*, *Pueraria lobata*, *Glycyrrhiza uralensis*, and *Euphorbia pekinensis*, inhibited the lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) gene in RAW 264.7 cells. Due to the critical role that nitric oxide (NO) release plays in mediating inflammatory responses, the inhibitory effects of KIOM-79 on iNOS suggest that KIOM-79 may represent a useful anti-inflammatory agent **(Jeon *et al.*, 2006)**. Other herbal formulation (TWCCT) of the traditional Chinese medicine containing *Glycyrrhiza uralensis* was also shown to inhibit the LPS-induced NO and prostaglandin E2 (PGE2) production and iNOS expression in RAW 264.7 According to the authors, these findings indicate that TWCCT has anti-inflammatory effects **(Tseng *et al.*, 2006)**. In other investigation, the extracts of roasted licorice obtained by ethanol demonstrated strong anti-inflammatory activity through its ability to reduce nitric oxide and PGE2 production in the LPS-stimulated mouse macrophage cell, RAW264.7. It also inhibited the production of pro-inflammatory cytokines and CD14 expression on the LPS-stimulated RAW264.7 cells. These data suggest that the use of extract of licorice may be a useful therapeutic approach to various inflammatory diseases **(Kim *et al.*, 2006)**.

3.5.2. Immunomodulatory activities

A phytopharmaceutical containing an extract of *Echinacea purpurea* and *Glycyrrhiza glabra* root (Revitonil® tablets) showed immunostimulating potential, using several *in vitro* tests and

the *in vivo* carbon-clearance model in mice. In the *in vitro* phagocytosis test with human granulocytes, Revitonil® showed a 44–53% stimulating effect at a concentration of 100 µg/ml. Whereas in the chemoluminescence test at a concentration of 1.25 µg/ml, Revitonil® tablets exhibited a moderate enhancing effect. A remarkable stimulating activity (30–50%) was observed in the T-lymphocyte CD69 bioassay at a concentration of 100 µg/ml **(Wagner *et* Jurcic, 2002)**. A recent study reported that the consumption of three commonly used herbs: *Echinacea purpurea*, *Astragalus membranaceus* and *Glycyrrhiza glabra*, administered singly and in combination stimulated the CD25 expression on T cells at 24 h with notable increases in activation from *Astragalus* and *Glycyrrhiza* **(Zwickey *et al.*, 2007)**. Other investigations demonstrated that *Echinacea*, *Astragalus* and *Glycyrrhiza* herbal tinctures stimulated immune cells as quantified by CD69 expression on CD4 and CD8 T cells. This activation took place within 24 h of ingestion, and continued for at least 7 days. In addition, these three herbs had an additive effect on CD69 expression when used in combination **(Brush *et al.*, 2006)**. In a prior study, a standardized ethanol extracts of *Allium sativum* (garlic), *Glycyrrhiza glabra* (licorice), *Plantago major* (plantain) and *Hippophae rhamnoides* (sea buckthorn) enhanced the membrane function of avian macrophage. In the same study, small concentrations (20 µg/ml) of licorice proved the comitogenic potential for both T and B avian lymphocytes **(Dorhoi *et al.*, 2006)**. *Glycyrrhiza uralensis* saponins showed a slight haemolytic effect and enhanced significantly a specific antibody and cellular response against ovalbumin (OVA) in mice **(Sun *et* Pan, 2006)**. An herbal preparation containing *Glycyrrhiza uralensis* Fischer, *Glycine max* L. Merr, *Angelica gigas* Nakai and *Cnidium officinale* Makino increased the immune-stimulating activities on T and B cells with cytolytic activity against K562 cells **(Sa *et al.*, 2007)**. In addition, *in vitro* studies showed immunomodulatory effects of polysaccharide fractions obtained from shoots of *G. glabra* and hairy roots of *G. uralensis* **(Nose *et al.*, 1998)**.

3.5.3. Antimicrobial and antiviral activities

The antimycobacterial activity of *Glycyrrhiza glabra* roots was found at 500 μg/ml of concentration. The bioactivity guided phytochemical analysis identified glabridin as potentially active against both *Mycobacterium tuberculosis* H37Ra and H37Rv strains at 29.16 μg/ml concentration **(Gupta *et al.*, 2008)**. The antibacterial and antifungal activities of the extracts from nine samples of *Glycyrrhiza glabra* collected in various sites of Calabria, Italy were reported **(Statti *et al.*, 2004)**. A licorice preparation made from the commercially available oil-based extract of *G. glabra* showed a low MIC (25 μg/ml) against five tested strains of filamentous fungi, but not against *Aspergillus fumigatus* MOO8, in a blended tea **(Sato *et al.*, 2000)**. A recent study was conducted using *Glycyrrhiza uralensis* ethanol extract in a culture of A549 human bronchial epithelial cells infected with influenza virus H1N1. The extract produced an inhibitory effect on the production of RANTES, the potent chemotactic cytokine for monocytes, basophils and T cells, typically detected in nasal secretions of patients with upper respiratory tract infection, and involved in the epithelial cell-mediated inflammatory process **(Ko *et al.*, 2006)**. According to a recent review **(Nassiri-Asl *et* Hosseinzadeh, 2008)**, glycyrrhizin and its preparation under the name of Stronger Neo-Minophagen C® (SNMC) are used intravenously for the treatment of chronic hepatitis (B and C).

3.5.4. Hepatoprotective activities

Fractionated extracts of *Glycyrrhiza glabra* L. and *Schisandra chinensis* Baillon inhibited the acetaminophen or D-galactosamine-induced hepatotoxicity **(Nakagiri *et al.*, 2003).** The protective effect of licorice extract against azathioprine- or cadmium- induced hepatotoxicity

was also informed (**Wu *et al*., 2006; Lee *et al*., 2009**). *Glycyrrhiza* flavonoids provided protection to hepatocytes exposed to carbon tetrachloride and galactosamine. The authors pointed to the antilipid peroxidation effect of *Glycyrrhiza* as the central mechanism contributing to its protective action against carbon tetrachloride-induced hepatotoxicity (**Luper, 1999**).

3.5.5. Antiulcer activities

Revers (1956) was one of the first authors to systematically study anti-ulcer properties of licorice extract. In an un-blinded and un-controlled study, 45 patients with confirmed gastric ulcers were administered 10 g/day of powdered licorice extract. The ulcers were found to disappear in 17 of the cases, were diminished in 22 cases, and were unchanged in six of the cases. Patients with duodenal ulcers did not react as favorably. Approximately 20% of the patients were noted to develop edema, some with complications, including violent headache, dizziness, upper right quadrant pain, compression in the chest, and hypertension. Crude fractionation of the licorice extract revealed that glycyrrhizin was the probable agent responsible for the edematous effect. Later, the anti-ulcer activity of deglycyrrhizinated licorice was demonstrated by using a rat model of aspirin-induced gastric mucosal damage (**Bennett, 1980**). A further study (**Ishii *et* Fujii, 1982**) suggested that the antiulcer effects of licorice extract may be due to reduced gastric secretions caused by an inhibition of gastrin release. The deglycyrrhized licorice was also shown to reduce ulcers induced by oral ibuprofen (**Dehpour *et al*., 1995**). In a more recent study, a water extract of licorice showed a significant anti-gastric ulcer effect in rats and this effect was attributed to liquiritin apioside (**Nakamura *et al*., 2003**). Pepticare, a herbomineral formulation of the Ayurveda medicine consisting of the herbal drugs: *Glycyrrhiza glabra*, *Emblica officinalis* and *Tinospora*

cordifolia, was more potent than *G. glabra* alone in protecting against pylorus-ligation and ethanol-induced ulcers. The authors attributed the antiulcer activity of the formulation to its antioxidant mechanism of action **(Bafna *et* Balaraman, 2005)**.

3.5.6. Antioxidant activity

There is evidence of the antioxidant activity of alcoholic and acetonic extracts of licorice **(Di Mambro *et* Fonseca, 2005; Fuhrman *et al*., 2002; Vaya *et al*., 1997)**. The inhibiting effect of licorice extracts on oxidative enzymes such as xanthine oxidase (XOD) and monoamine oxidase (MAO) has been also explored **(Hatano *et al*., 1991)**. The constituents of *G. inflata*, licochalcone A, B, C, D and echinatin, were effective in preventing microsomal lipid peroxidation induced by Fe (III)-ADP/NADPH and licochalcone B, D showed potent antioxidative and superoxide scavenging activities **(Haraguchi *et al*., 1998)**. Furthermore, the isoflavone derivatives of *G. glabra* such as glabridin inhibited lipid peroxidation in rat liver microsomes and protected mitochondrial functions from oxidative stresses **(Haraguchi *et al*., 2000)**. Moreover, glabridin, an isoflavan of *G. glabra*, was a potent antioxidant toward LDL oxidation in *in vitro* and *in vivo* studies **(Fuhrman *et al*., 1997; Vaya *et al*., 1997; Belinky *et al*., 1998a)**. In addition, other constituents of *G. glabra* such as isoflavones hispaglabridin A, hispaglabridin B and 4′-O-methylglabridin, the two chalcones, isoprenylchalcone derivative and isoliquiritigenin were antioxidants against LDL oxidation **(Vaya *et al*., 1997)**.

3.5.7. Antidiabetic activity

Licorice was reported to markedly inhibit aldose reductase in steptozotocin-induced diabetic rats **(Shen *et al*., 2007)**. An Oriental medical prescription (Wen-Pi-Tang) composed of *Rhei*

rhizoma, Ginseng radix, Aconiti tuber, Zingiberis rhizoma and licorice, which is used clinically as a medicine to treat renal failure, showed inhibitory activity against the protein glycation reaction. Some flavones such as licochalcone A and licochalcone B also showed inhibitory activity **(Nakagawa *et al.*, 2005)**. KIOM-79, a mixture of extracts obtained from *Puerariae lobata*, *Magnolia officinalis*, *Glycyrrhiza uralensis* and *Euphorbia pekinensis*, inhibited vascular endothelial growth factor (VEGF) expression in human retinal pigment epithelial (RPE) cells cultured under high glucose (HG, 25 mM) or S100b (a specific ligand of the receptor for advance glycation end products (RAGE), 5 μg/ml) **(Kim *et al.*, 2007)**. The metabolic syndrome, including type 2 diabetes, insulin resistance, obesity/abdominal obesity, hypertension and dyslipidemia, is a major public health problem. Peroxisome proliferator-activated receptor (PPAR-γ) ligands such as thiazolidinediones are effective against this syndrome **(Mae *et al.*, 2003)**. The EtOAc extract of licorice (*Glycyrrhiza uralensis* roots) exhibited considerable peroxisome proliferator-activated receptors (PPAR-γ) ligand-binding activity. Bioassay-guided fractionation of the extract resulted in the isolation of two isoflavenes, one of which is a new compound named dehydroglyasperin D, an isoflavan, two 3-arylcoumarins, and an isoflavanone as the PPAR-γ ligand-binding active ingredients of licorice. Glycyrin, one of the main PPAR-γ ligands of licorice, significantly decreased the blood glucose levels of genetically diabetic KK-A^y mice in association with PPAR-γ **(Kuroda *et al.*, 2003)**. Non-aqueous fractions of licorice (*Glycyrrhiza uralensis* Fisher) extracted with ethanol, ethyl acetate and acetone, but not an aqueous extract, had PPAR-γ ligand-binding activity. Some prenylflavonoids including glycycoumarin, glycyrin, dehydroglyasperin C and dehydroglyasperin D, a newly found compound, were identified as active compounds with PPAR-γ ligand-binding activity. These findings indicated that licorice ethanolic extract is effective in preventing and ameliorating diabetes, ameliorating abdominal obesity and

preventing hypertension, and suggest that licorice ethanolic extract would be effective in preventing and/or ameliorating the metabolic syndrome **(Mae *et al.*, 2003)**.

3.5.8. Antitumor activity

The aqueous extract of *G. glabra* inhibits the *in vivo* and *in vitro* proliferation of Ehrlich ascites tumor cells and inhibits angiogenesis in *in vivo* assay, peritoneal and chorioallantoic membrane assays **(Sheela *et al.*, 2006)**. Also, the ethanol extract of *G. uralensis* root induced apoptosis and G1 cell cycle arrest in MCF-7 human breast cancer cells **(Jo *et al.*, 2005)**. On the other hand, there are many studies about the anticancer effects of several derivatives of its components both in *in vivo* and *in vitro* studies. Glycyrrhetinic acid could also trigger the proapoptotic pathway by inducing mitochondrial permeability transition and this property may be useful for inducing apoptosis of tumor cells **(Salvi *et al.*, 2003; Fiore *et al.*, 2004)**. Recently, licochalcone E, a new retrochalcone from the roots of *G. inflata*, exhibited the most potent cytotoxic effect compared with the known antitumor agents, licochalcone A and isoliquiritigenin **(Yoon *et al.*, 2005)**.

3.5.9. Side effects and toxicity

Large amounts of licorice may result in severe hypertension, hypokalemia and other signs of mineralocorticoid excess. This hypertension is caused by decreased 11 β-hydroxysteroid dehydrogenase-2 (11β-HSD2) activity. This enzyme is responsible for the renal conversion of cortisol to cortisone. Thus, licorice leads to activation of renal mineralocorticoid receptors by cortisol, resulting in a state of apparent mineralocorticoid excess and suppression of the rennin angiotensin system **(Nassiri-Asl *et* Hosseinzadeh, 2008)**.

3.5.10. Phytopharmaceutical formulations containing licorice

Licorice is reported as a phytopharmaceutical product and as an active ingredient in polyherbal formulations. For instance, one of the flavonoid-rich fractions from the extract of licorice has been developed as an anti-ulcer drug (Aspalon®) in Japan **(Li *et al.*, 2000)**. Another licorice-based phytopharmaceutical product (Caved-S®) was also reported as an anti-ulcer drug **(Nomura *et al.*, 1998)**. A phytopharmaceutical formulation containing an extract of *Echinacea purpurea* and *Glycyrrhiza glabra root* (Revitonil® tablets) is recommended for relief of common cold symptoms **(Wagner *et* Jurcic, 2002)**. The polyherbal formulation STW 5 (Iberogast®) is a fixed combination of nine medicinal plant extracts effective in the treatment of functional dyspepsia and irritable bowel syndrome. Its components are an aqueous-ethanolic fresh plant extract from *Iberis amara* and drug extracts from peppermint leaves (*Menthae piperitae folium*), chamomile flower (*Matricariae flos*), licorice root (*Liquiritiae radix*), angelica root (*Angelicae radix*), caraway fruit (*Carvi fructus*), milk thistle fruit (*Silybi mariani fructus*), lemon balm leaves (*Melissae folium*), and greater celandine herb (*Chelidonii herba*) **(Ammon *et al.*, 2006)**.

3.6. Industrial use of licorice

Commercially, licorice is added to chewing gum, chocolate candy, cigarettes, smoking mixtures, chewing tobacco and snuff as sweetening agent and as a depigmentation agent in cosmetics **(Nomura *et al.*, 2002)**. Also, licorice is frequently employed to mask the taste of bitter drugs such as aloe, quinine and others. The surfactant property of the saponins may also facilitate the absorption of poorly absorbed drugs, such as the anthraquinone glycosides **(Nassiri-Asl *et* Hosseinzadeh, 2008)**. Licorice and licorice derivatives, including

ammoniated glycyrrhizin, are affirmed as Generally Recognized as Safe (GRAS) for use in foods by the U.S. FDA (21 CFR 184.1408). Both the Council of Europe and the UK Food Additive and Contaminants Committee consider licorice as a natural plant product intended for use in small quantities as a food additive with the intention that its consumption be limited by the glycyrrhizin levels and not to exceed those occurring naturally in foods **(Fenwick, 1990)**. A limit of less than 50 ppm glycyrrhizin was established by these organizations. The Dutch Nutrition Information Bureau advised against a daily glycyrrhizin consumption in excess of 200 mg, assumed to correspond to 150 g of licorice confectionery; although it should be cautioned that the glycyrrhizin content of confectionery products can vary by as much as 30-fold **(Isbrucker *et* Burdock, 2006)**.

3.7. Methods of immunomodulating and antioxidant evaluation of plants

3.7.1. Methods of immunomodulating evaluation of plants

3.7.1.1. Activations of cell surface markers on immune cells

Stimulation of lymphocytes leads to upregulation of various cell surface markers at various stages of cellular activation: CD69 (very early), CD71 (early), CD25 (late), and HLA-Dr (very late) **(Reddy *et al.*, 2004)**. Therefore, *in vitro* manipulation of early activation markers is an approach used in many laboratories to explore potential pathways of cellular activation and can be used to measure the immunomodulatory effects of pharmaceutical agents and vaccine antigens **(Borges *et al.*, 2007)**.

3.7.1.2. Effects on T cells

Along the way of investigating the pathogenesis of immune diseases, the activated immune effector cells such as T cells, B cells, monocytes/macrophages, and dendritic cells have been consistently found in the peripheral blood and the involved organs. Among these immune effector cells, T cells have been considered to be the most important because the regulation of T cell activation requires antigen specificity and a great amount of cytokines released in immune responses are from T cells **(Fox, 1997)**. It is clear that full activation of T cells requires the integration of two signals: one is from a T cell receptor signal and the other is from a co-stimulatory signal **(Panayi *et al.*, 2001)**. T cells play an important role in the regulation of immune response and they could be targets for herbal medicine to modulate their activation and inhibition in immune-related diseases.

3.7.1.3. Effects on cytokine production and other immune effector cells

A network of cytokines regulates the growth and function of the cells of the immune system. T cells possess a dominant role in this network since they are the main source of many cytokines. The production of different cytokines is specifically regulated by means of cell interactions and cytokine concentration, which is dependent largely on the state of differentiation of the T cells. Two different types of differentiated T cells can be characterized according to the pattern of cytokine production of T cells: IL-2 and IFN-γ are typically produced by T helper 1 (Th1) cells, whereas predominantly T helper 2 (Th2) cells produce IL-4, IL-5 and IL-10. Th1 and Th2 cytokines exert a mutual cross-regulation on the precursors of Th1- or Th2-type effector cells which are important mediators in directing the immune system towards the appropriate response. The selective activation of either Th1 or Th2 type cells

depends on the antigen and is influenced by cytokines produced partly by antigenpresenting cells and partly by T helper cells. Both Th1 and Th2 cytokines are possibly susceptible to the herb activation or suppression **(Lai *et al*., 1999)**.

3.7.1.4. Effects on antibody production

Antibodies are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses. However, when a person has an autoimmune disease, the body's antibodies are attacking healthy and normal cells. Therefore, not only searching for herb preparations showing stimulating effects on antibody production is needed, but also is necessary for herb preparations showing inhibitory effects. For example, crude preparations of Fun-boi (*Stephania tetrandra*), a traditional anti-rheumatic herb, have been reported to have immunomodulatory effects on both cell-mediated and humoral immunities *in vitro*. Especially, Fun-boi therapy markedly reduced the severity of arthritis and tended to reduce the serum anti-type II collagen antibody level **(Niizawa *et al*., 2003)**. Formosanin-C, a diosgenin saponin, was isolated from a perennial herb, *Paris formosana* Hayata (Liliaceae) which has been used as a folk remedy for snake bite and as an anti-inflammatory or anti-neoplastic agent. It was reported that this agent significantly inhibited the antibody production of S-antigen induced experimental autoimmune uveitis in guinea pigs **(Wu *et al*., 1990)**. Aqueous extract of *Trigonella foenum graecum* (L.), a widely used medicinal and dietary herb, showed a significantly stimulatory humoral immune response in mice **(Bin-Hafeez *et al*., 2003)**.

3.7.1.5. Effects on programmed cell death (apoptosis)

Aside from the activation of immune effector cells, the defective apoptotic mechanism also plays a crucial role in disease progression **(Jacobson *et al.*, 1997)**. Apoptosis is a natural protective mechanism for embryogenesis, for thymic organ to eliminate inappropriate T cells and for immune privilege sites to protect from inflammatory cell invasion. Different from the necrotic process, the apoptotic process does not induce any inflammatory response because dead cells or their degraded products are rapidly phagocytosed before any leakage of cellular contents **(Nagata, 1997)**. In light of the significance of apoptotic process, the apoptosis-based therapy has been suggested as one of the approaches to control the progression of immune diseases **(Ohsako *et* Elkon, 1999)**. Altogether, both the inappropriate activation of immune effector cells and the ineffective deletion (through apoptosis) of these cells may lead to the development and progression of immune disorders. The therapeutic approaches for immune diseases may rely on both the inhibition of cell activation and the maintenance or enhancement of the apoptotic program of immune effector cells.

3.7.2. Methods of antioxidant evaluation of plants

Growing interest in possible healthy effects of antioxidants has led to the development of a large number of assays to determine the antioxidant capacities of plant extracts. Since the antioxidant capacity of foods is determined by a mixture of different antioxidants with different mechanisms of actions, among which there may be synergistic interactions, it is necessary to combine more than one method to determine *in vitro* antioxidant capacity **(Perez-Jimenez *et al.*, 2008)**. On the basis of the chemical reactions involved, major antioxidant capacity assays can be roughly divided into two categories: hydrogen atom

transfer (HAT) reaction based assays and single electron transfer (ET) reaction based assays. Most HAT-based assays monitor competitive reaction kinetics, and the quantitation is derived from the kinetic curves. HAT-based methods generally are composed of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant. The ET-based assays involve one redox reaction with the oxidant (also as the probe for monitoring the reaction) as an indicator of the reaction endpoint. The HAT- and ET-based assays are intended to measure the radical (or oxidant) scavenging capacity, instead of the preventive antioxidant capacity of a sample **(Huang *et al.*, 2005)**.

The trolox equivalent antioxidant capacity (TEAC) assay has been broadly applied in assaying food samples. The 2,2-di(4-*tert*octylphenyl)-1-picrylhydrazyl (DPPH) assay is an easy and accurate method with regard to measuring the antioxidant capacity of fruit and vegetable juices or extracts. The oxygen radical absorbance capacity (ORAC) assay has found even broader application for measuring the antioxidant capacity of botanical samples and biological samples. The total radical-trapping antioxidant parameter (TRAP) assay has also been widely used. These assays differ from each other in terms of substrates, probes, reaction conditions, and quantitation methods. It is extremely difficult to compare the results from different assays. The **Table 1** lists the major antioxidant capacity assays **(Huang *et al.*, 2005)**.

Table 1. *In vitro* Antioxidant Capacity Assays.

Assays involving hydrogen atom transfer reactions	ORAC (oxygen radical absorbance capacity)
$ROO^{\cdot} + AH \rightarrow ROOH + A^{\cdot}$	TRAP (total radical trapping antioxidant parameter)
$ROO^{\cdot} + LH \rightarrow ROOH + L^{\cdot}$	Crocin or β-caronete bleaching assay IOU (Inhibited oxygen uptake) Inhibition of linoleic acid oxidation Inhibition of LDL oxidation
Assays by electron-transfer reaction	
$M(n) + e\ (\text{from } AH) \rightarrow AH^{\cdot +} + M(n-1)$	TEAC (Trolox equivalent antioxidant capacity) FRAP (ferric ion reducing antioxidant parameter) DPPH (diphenyl-1-picrylhydrazyl) Copper (II) reduction capacity

4. MATERIALS AND METHODS

4.1. Equipments, instruments and glass materials

Analytical Balance, Sartorius A2005, Germany

Beakers, Simax, Czech Republic

Boiling flasks, Simax, Czech Republic

Burette, Qualicolor, Czech Republic

Centrifuge, MPW 342, Poland

Cytomics FC500 flow cytometer, Beckman Coulter, USA

Chemistry drier, Binder, USA

Electric grinder (Eta 0010), Czech Republic

Erlenmeyer flasks, Simax, Czech Republic

Funnels, Simax, Czech Republic

Graduated cylinders, Simax, Czech Republic

High performance liquid chromatography coupled with photodiode array detector (**HPLC-DAD**), Jasco, Japan

High Performance Liquid Chromatographer coupled with mass spectrometry (**HPLC-MS)**, ThermoFinnigan, San Jose, CA, USA

Lyophilizator Equipment, Heto PowerDry LL3000 Freeze dryer, Czech Republic

Micro pipettes, Biohit, Czech Republic

Multi-Unit Extraction Heater, GFL, Typ42, Germany

Pipette, Qualicolor, Czech Republic

Reflux extractor, Simax, Czech Republic

Refrigerator, Zanussi, Czech Republic

Rotary evaporator, Laborota 4010, digital

Separating funnel (Decanting funnel), Simax, Czech Republic

Shaker, LT2 Kavalier, Czech Republic

UV/ Vis spectrophotometer, Shimadzu UV-1601, Japan

Ultrasound shaker, Bandelin Sonorex RK 100H, Germany

Volumetric flasks, Fisherbrand, Czech Republic

Water destilator, Milli-Q RG system (Millipore), Molsheim-France

4.2. Chemicals

2,2-diphenyl-1-picryl-hydrazyl (DPPH), Sigma Aldrich (Steinheim, Germany)

β-nicotinamide adenine dinucleotide reduced (NADH), Sigma Aldrich (Steinheim, Germany)

β-carotene, Koch-light Laboratories Ltd. (England)

Acetonitrile, HPLC Grade, Merck (Darmstadt, Germany)

Aluminium chloride hexahydrate, Merck (Darmstadt, Germany)

Chloroform, HPLC Grade, Merck (Darmstadt, Germany)

Folin–Ciocalteu phenol reagent, Merck (Darmstadt, Germany)

Glacial acetic Acid, HPLC Grade, Merck (Darmstadt, Germany)

Hide powder, Sigma Aldrich (Steinheim, Germany)

Linoleic acid, Sigma Aldrich (Steinheim, Germany)

Methanol, HPLC Grade, Merck (Darmstadt, Germany)

Monoclonal antibodies (PE, FITC and APC labelled), Immunotech (France) and Dako (Denmark)

N-Methylphenazonium methyl sulfate (PMS), Sigma Aldrich (Steinheim, Germany)

Nitrotetrazolium blue chloride (NBT), Sigma Aldrich (Steinheim, Germany)

Phytohemagglutinin (PHA), Sigma Aldrich (Steinheim, Germany)

Polyoxyethylene sorbitan monopalmitate (Tween-40), Sigma Aldrich (Steinheim, Germany)

Propidium iodide, Sigma Aldrich (Steinheim, Germany)

Ribonuclease A, Sigma Aldrich (Steinheim, Germany)

Standards (glycyrrhizin and quercetin), Sigma Aldrich (Steinheim, Germany)

Standard liquiritin, Wuhan Sunrise Technology Development Co., Ltd. (Hong Kong, China)

Sodium carbonate, Merck (Darmstadt, Germany)

Tween-20, Sigma Aldrich (Steinheim, Germany)

Ultrapure water, Milli-Q RG system (Millipore, Molsheim-France)

X-vivo medium, Bio-Wittaker (USA)

4.3. Collection and preparation of samples

4.3.1. Samples of *Glycyrrhiza* species for comparative study

Roots of *G. glabra*, *G. uralensis*, *G. echinata* and *G. pallidiflora* roots were collected in February 2007 from the Botanical Garden of the Faculty of Horticulture, Mendel University of Agriculture and Forestry, Czech Republic (situated 164 m above sea level). Genetical resources were identified with the code 0001 (*G. glabra*), 0009 (*G. uralensis*), 0005 (*G. echinata*), and 0007 (*G. pallidiflora*). The plant material was dried at 40 ºC in an oven and was then ground to fine powders (mesh size 20). A precisely weighed amount (1.50 g) of the powder was extracted under reflux with 150 mL of 80% methanol at 60 °C for 6 h. The

obtained extracts were filtered over Whatman N° 1 paper and methanol then was removed under reduced pressure by a rotary evaporator at 35 °C. The resulting aqueous extracts were lyophilised and the extraction yield was calculated, based on the dry weight of the licorice. The licorice lyophilised extracts were assessed for their biological activities and chemical profile.

4.3.2. Sample of *Glycyrrhiza glabra* for study the infusion

Roots of *G. glabra* were collected in February 2008, in the Botanical Garden of the Faculty of Horticulture, Mendel University of Agriculture and Forestry, Czech Republic (situated 164 m above sea level). The genetic resource was identified with the code 0001. The plant material was dried at 40 ºC in an oven and was then ground to fine powders (mesh size 20). The infusion was prepared by adding 150 ml of distilled water (95–100 ºC) to a precisely weighed amount (1.50 g) of licorice powder. The infusion was brewed for 20 min and was then filtered over Whatmann No 1 paper. The resulting aqueous extract was lyophilised and the extraction yield was calculated, based on the dry weight of the licorice. The lyophilised infusion of licorice was assessed for its biological activities and chemical profile.

4.3.3. Samples of *Glycyrrhiza glabra* for study at different harvest times

The thickened roots of 4-year-old cultivated *Glycyrrhiza glabra* L. were collected in the middle of February, May, August and November of 2008 from the Botanical Garden of the Faculty of Horticulture, Mendel University of Agriculture and Forestry, Czech Republic at 164 m above sea level. The mean values for maximum and minimum temperature (°C) for the months of February, May, August and November 2008 were 7.3°C/-0.8°C; 20.5°C/9.5°C;

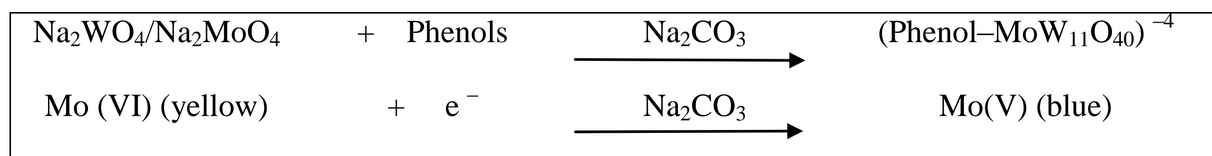
25.7°C/14.1°C and 9°C/4.1°C, respectively. The mean values of global and UV radiations for February, May, August and November of 2008 were 6.73 MJ m^{-2} and 39.57 mJ m^{-2}; 20.21 MJ m^{-2} and 247.09 mJ m^{-2}; 17.77 MJ m^{-2} and 250.97 mJ m^{-2}; and 3.38 MJ m^{-2} and 22.09 mJ m^{-2}, respectively. The plant material was dried at 40 °C in an oven and then was ground to fine powders (mesh size 20). A precisely weighed amount (1.50 g) of the powder was extracted under reflux with 150 mL of 80% methanol at 60 °C for 6 h. Extractions were carried out in triplicate. These extracts were filtered over Whatman N° 1 paper and methanol was then removed under reduced pressure by a rotary evaporator at 35 °C. The resulting aqueous extract was lyophilised and the extraction yield was calculated based on the dry weight of the licorice. The licorice extracts obtained were assessed for their chemical profile and biological activities.

4.4. Chemical analytical methods

4.4.1. Determination of total content of phenols

The total phenolic (TP) content was determined using the Folin-Ciocalteu procedure **(Singleton *et al.*, 1999)**. Briefly, the appropriate extract dilution was oxidized with the Folin-Ciocalteu reagent and the reaction was neutralized with sodium carbonate **(Figure 8)**. The absorbance of the resulting blue color was measured at 760 nm after 30 minutes using a Shimadzu UV-1601 UV/ Vis spectrophotometer. Quantification was plotted on a standard curve of gallic acid. The results were expressed as mg gallic acid equivalents (GAE)/100 mg of extract. Data are reported as means ± standard deviation (SD) to an accuracy of three replicates.

Figure 8. Chemistry of Folin–Ciocalteu method **(Prior *et al.*, 2005)**.



4.4.2. Determination of total content of tannins

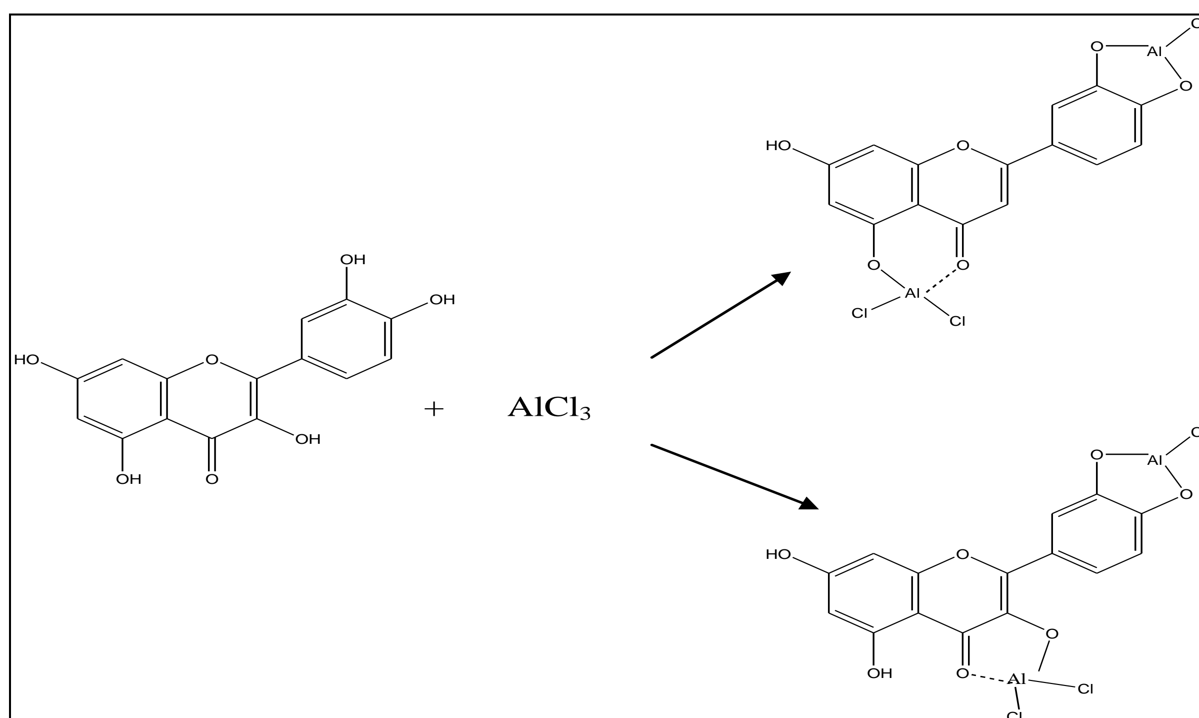
After removal of tannins by adsorption on an insoluble matrix (hide powder), the total tannin (TT) content was determined by Folin-Ciocalteu procedure explained briefly in the previous paragraph (section 4.4.1). Calculated values were subtracted from the total phenolic contents and total tannin contents are expressed as mg gallic acid equivalents (GAE)/100 mg of extract. Data are reported as means ± standard deviation (SD) to an accuracy of three replicates **(Malenčić *et al.*, 2008)**.

4.4.3. Determination of total content of flavones and flavonols

The total flavones and flavonols (TF) content was determined according to the aluminum chloride method **(Chang *et al.*, 2002)**. Quercetin was used as a reference for the calibration curve. A volume a diluted extracts (0.5 ml) were mixed with 1.5 mL of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-1601 UV/Vis spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 0.5 ml of extracts was reacted with aluminum chloride for determination of

flavonoid content as described above. Results were expressed as mg quercetin equivalents (QE)/100 mg of extract. Data are reported as means ± SD after three replicates.

Figure 9. Formation of stable complexes between flavonoids and aluminum chloride **(Chang *et al.*, 2002)**.



4.4.4. HPLC-DAD analysis

The HPLC analysis was performed with a Jasco PU- 2089 pump equipped with a Jasco MD-2015 diode array detector (DAD), and chromatographic separations were performed on a LiChrospher RP-18 column (4.0 × 250 mm i.d., 5 µm). Briefly, two mobile phases were employed for elution: (A) water:methanol:acetonitrile:acetic acid (29:35:35:1, v/v) and (B) acetonitrile. The gradient profile is shown in **Table 2**. Separations were carried out at 25ºC with an injection loop of 20 µl. The DAD detector was operated in the range of 200–650 nm,

and the analysis was performed at 254 nm. Components of extracts were identified by comparing their retention times and UV spectra with those of authentic standards (liquiritin and glycyrrhizin) under identical analysis conditions. Solutions at different concentrations of each standard were injected into the HPLC to check the linearity between concentration and peak areas, and a response factor was calculated. Quantifications of liquiritin and glycyrrhizin were done using these calibration factors.

Table 2. The gradient profile of solvents for HPLC-DAD analysis.

Time (min)	Solution A	Solution B
0.0	100%	0%
5.0	100%	0%
15.0	50%	50%
22.0	50%	50%
22.20	100%	0%
22.20	100%	0%

4.4.5. HPLC-ESI-MS analysis

After HPLC analysis, mass spectra were obtained using a LCQ ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, CA). The HPLC separation was done using a Waters SymmetryShield RP18 column (3.5 µm; 2.1 × 100 mm), at 40ºC under a flow rate of 175 µl/min. Briefly, two mobile phases were employed for elution: (A) water:methanol:acetonitrile:acetic acid (29:35:35:1, v/v) and (B) acetonitrile:acetic acid (99:1, v/v). The gradient profile is shown in **Table 3**. Experiments were performed in both negative

and positive ion modes. Range for scaning was 200–600. The capillary temperature was set to 350 °C both in negative and positive mode. High spray voltage was set at 5300 V for positive mode and 4500 V for negative mode. Nitrogen was used as both sheath and auxiliary gas at a flow rate of 25 arbitrary units (approximately 0.56 L/min) for sheath gas and 5 arbitrary units (approximately 1.5 L/min) for auxiliary gas. MS/MS and MS3 were carried out using helium as the target gas, and the collision energy was set at 50-60 %. Identifications were achieved on the basis of the ion molecular mass, MS*n*, and UV–visible spectra. Data acquisition and processing were carried out using Xcalibur software (version 1.2).

Table 3. The gradient profile of solvents for HPLC-MS analysis.

ime (min)	Solution A	Solution B
0.0	100%	0%
10.0	50%	50%
20.0	50%	50%
20.2	100%	0%
26.0	100%	0%

4.5. Biological assays

4.5.1. Free radical-scavenging activity

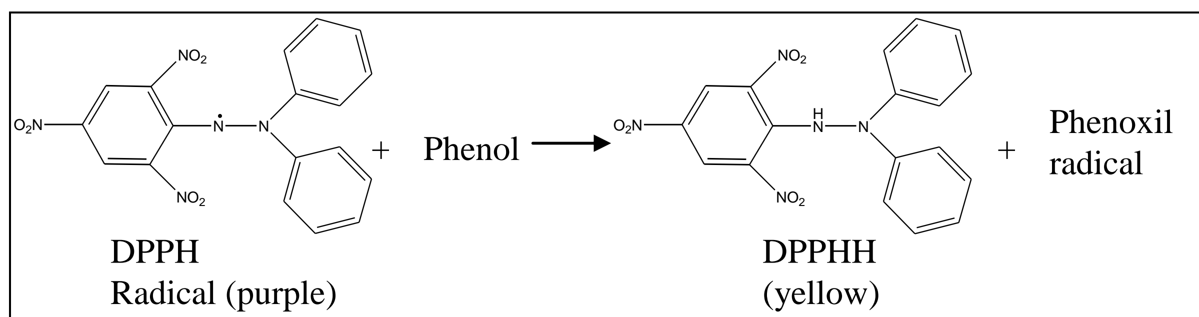
4.5.1.1. DPPH free radical-scavenging activity

The DPPH radical is one of the few stable organic nitrogen radicals, which bears a deep purple colour. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH (**Figure 10**). The ability can be evaluated by electron spin resonance (EPR) or by measuring the decrease of its absorbance (**Prior *et al.*, 2005**). The capacity of extracts and pure compounds of licorice to scavenge DPPH was assessed as previously reported (**Schmeda-Hirschman *et al.*, 2003**). Samples were assayed at concentrations in the range of 10–200 µg/ml. The DPPH-scavenging effect was evaluated spectrophotometrically at 517 nm. The values are reported as means ± SD of three determinations. Quercetin was used as a reference compound. The percentage of DPPH-scavenging effect was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = [\ (E\text{–}S) / (E)\ ] \times 100,$$

where $E = A\text{–}B$ and $S = C\text{–}D$; A, absorbance of the control; B, absorbance of the control blank; C, absorbance of the sample; D, absorbance of the sample blank.

Figure 10. Scavenging of DPPH free radical by a phenolic compound (free radical scavenger). **(Amić *et al.*, 2003)**.



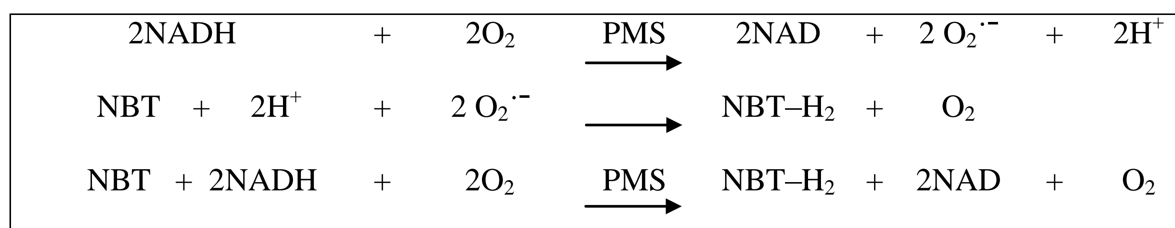
4.5.1.2. Superoxide anion radical-scavenging activity

Superoxide radicals were generated by the NADH/PMS system according to a described procedure **(Valentão *et al.*, 2001)**. The assay involves the production of superoxide from O_2 using NADH as a reductant, and phenazine methosulphate (PMS) as a catalyst in the presence of an indicator, nitro blue tetrazolium (NBT), which turns blue when reduced by superoxide. The color change can be monitored spectrophotometrically in the visible range at 560 nm. The percent inhibition of NBT reduction can be used to quantify superoxide-scavenging. The reaction mixtures in the sample wells consisted of 166 μM NADH, 43 μM NBT, 2.7 μM PMS and test samples (10–200 μg/ml). The assay measures the effect of samples on the reduction of NBT to the blue chromogen formazan by superoxide anion radical at 560 nm. The chemical reaction in the assay is shown in **Figure 11**. Quercetin was used as a reference compound. Values are presented as means ± SD of three determinations. The percentage of superoxide anion-scavenging effect was calculated as follows:

$O_2^{\cdot -}$ scavenging activity (%) = [(E – S) / (E)] × 100,

where $E = A - B$ and $S = C - D$; A, absorbance of the control; B, absorbance of the control blank; C, absorbance of the sample; D, absorbance of the sample blank.

Figure 11. Reduction of nitroblue tetrazolium (NBT) by $O_2^{\cdot -}$ genetared from the NADH/PMS system **(Petyaev *et* Hunt, 1997)**.



4.5.2. Antioxidant activity

4.5.2.1. Inhibition of β-carotene-linoleate bleaching

The antioxidant activity of samples was evaluated using the β-carotene/linoleic acid model system **(Suja *et al.*, 2005)**. A solution of β-carotene was prepared by dissolving 1 mg of crystalline β-carotene in 5 ml of chloroform. One milliliter of this solution was then pipetted into a 50 ml round-bottomed flask, which contained 20 mg of purified linoleic acid and 200 mg of Tween 40 emulsifier. After removal of chloroform at 40 ºC by rotary evaporator, 50 ml of oxygenated distilled water was added to the flask with vigorous stirring. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing 0.2 ml of different concentrations of extracts and compounds, so that their final concentrations in the assay media were in the range of 5–200 µg/ml. Control contained 0.2 ml of corresponding solvent and 4.8 ml of the β-carotene/linoleic acid emulsion. The mixtures were vortexed and

incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a Shimadzu UV-1601 spectrophotometer. Absorbance readings were then recorded at 20 min intervals until the control sample had changed colour (about 120 min). Quercetin was used as an antioxidant reference compound and a blank sample, devoid of β-carotene, was prepared for background subtraction. All determinations were performed in triplicate. The antioxidant activity of samples was based upon three different parameters, namely antioxidant activity (A_{A}), the oxidation rate ratio (R_{OR}) and antioxidant activity coefficient (C_{AA}).

Antioxidant activity (A_{A}) was determined as a percentage of inhibition relative to the control sample.

$A_{\mathrm{A}} = [(R_{\mathrm{control}} - R_{\mathrm{sample}}) / (R_{\mathrm{control}})] \times 100$,

where R_{control} and R_{sample} represent the bleaching rates of β-carotene without and with the addition of antioxidant, respectively. The bleaching rates (R_{control} and R_{sample}) were calculated according to first-order kinetics: $R_{\mathrm{control\ or\ samples}} = \ln (A_t / A_x) \times 1/t$,

where ln is the natural log, A_t is the initial absorbance at 470 nm at $t = 0$ and A_x is the absorbance at 470 nm at $t = 10, 20, 30, \ldots\ldots$ min.

The oxidation rate ratio (R_{OR}) was calculated by

$R_{\mathrm{OR}} = R_{\mathrm{sample}} / R_{\mathrm{control}}$,

where R_{sample} and R_{control} are as described earlier.

The antioxidant activity coefficient (C_{AA}) was calculated using:

$C_{\mathrm{AA}} = [(A_{\mathrm{s(t)}} - A_{\mathrm{c(t)}} / (A_{\mathrm{c(0)}} - A_{\mathrm{c(t)}})] \times 1000$,

where $A_{s(t)}$ is the absorbance of the sample containing antioxidant at t = 10, 20, 30,…… min, $A_{c(t)}$ is the absorbance of the control at t = 10, 20, 30,…… min, and $A_{c(0)}$ is the absorbance of the control at t = 0 min.

4.5.2.2. Hypochlorous acid-scavenging activity

The capacity of samples to scavenge hypochlorous acid (HOCl) was measured by the taurine chlorination assay **(Van Antwerpen *et al.*, 2008a)**. The reaction mixture contained the following reagents, at the concentrations stated between brackets, in a final volume of 1.0 ml: pH 7.4 phosphate buffer (10 mM PO^{3-}_4 /300 mM NaCl), taurine (15 mM) and solutions of test samples of increasing concentrations. Final concentrations of extracts in the assay media were in the range 100–300 µg/ml, whereas the compounds 1 and 2 were at 12 and 25 µg/ml. When necessary, the volume was adjusted to 1.0 ml with water. The mixture was incubated at 37 ºC and the reaction was initiated by the addition of HOCl (60 µM). After 5 min, 100 µl of catalase (4 U/µl) were added. Finally, the quantity of taurine chloramine was measured by the addition of 750 µl of 0.45 mM TNB and 2150 µl of water. The absorbance of the solutions was measured at 412 nm. An HOCl scavenger inhibits the taurine chlorination responsible for the oxidation of TNB into DTNB. The percentage of HOCl scavenged was calculated by taking the absence of inhibitor as the 0% and the absence of HOCl as the 100%. Captopril (4 µg/ml) was used as a reference compound. The results were expressed as means ± SD after three determinations.

4.5.2.3. Inhibition of myeloperoxidase-chlorinating system

The measurement of the inhibition of myeloperoxidase (MPO)-chlorinating activity was performed in a 96-well plate, as previously described **(Van Antwerpen *et al.*, 2008b)**. Briefly, the reaction mixture contained the following reagents, at the concentrations stated between brackets, in a final volume of 200 µl: pH 7.4 phosphate buffer (10 mM PO^{3-}_4 /300 mM NaCl), taurine (15 mM), test samples of increasing concentrations and a fixed amount of recombinant MPO (6 µl of batch solution diluted 10 times, ~40 nM). Final concentrations of extracts in the assay media were in the range 100–300 µg/ml, whereas the compounds 1 and 2 were at 12 and 25 µg/ml. When necessary, the volume was adjusted with water. The mixture was incubated at 37 ºC and the reaction initiated with 7.4 µl of H_2O_2 (99.6 µM). After 5 min, the reaction was stopped by the addition of 10 µl of catalase (8 U/µl). To determine the amount of taurine chloramines produced, 33.3 µl of a 1.0 mM solution of TNB were added and the volume was adjusted to 300 µl. Then, the absorbance of the solutions was measured at 412 nm with a microplate reader and a curve of the absorbance as a fuction of the test sample concentrations was plotted. The percentage of inhibition was calculated by using the absence of the inhibitor as the 0% of inhibition and the absence of H_2O_2 as the 100% of inhibition. Flufenamic acid (0.3 µg/ml) was used as a reference compound. The results were expressed as means ± SD after three determinations.

4.5.3. Effects on immune cells

4.5.3.1. Activation of immune cells

The activation of granulocytes and NK (Natural Killer) cells was analysed by flow cytometry, as measured by CD69 expression **(Brush *et al*., 2006)**. Briefly, an aliquot of peripheral blood was drawn from three healthy volunteers into sodium heparinized tubes. Using sterile 96-well flat-bottomed plates, 100 µl of blood suspension were incubated, along with 100 µl of X-vivo medium containing test samples with increasing concentrations and without test samples (control). The mitogen phytohemagglutinin (PHA) was used as a positive control at 10 µg/ml. Samples were filtered through 0.2 µm filters, before use. Final concentrations of extracts and compounds in the assay media were in the range 100–800 and 12–100 µg/ml, respectively. Plates were then incubated at 37 ºC with 5% CO_2 for 24 h. After incubation, 40 µl of each incubated suspension was labelled with a cocktail of fluorescently-labelled antibodies (CD69 PE, CD69 FITC, CD3 APC, CD56 PE). Flow cytometric analysis was performed on a Cytomics FC500 flow cytometer (Beckman Coulter, USA) and data were analysed by CXP analysis software (Coulter Electronic, USA). Cell activation was measured as increases in mean fluorescence intensities (MFI) due to the expression of CD69 on the cell surface. Fluorescence signals from 10,000 events were obtained and presented as logarithmically amplified signals. Values of activation are presented as activation indices (AI), which were calculated by dividing the MFI of treated cells with test samples by that of untreated cells (control). A positive immune cell response was defined as an AI ≥ 2. Data were presented as the means ± SD after three experiments.

4.5.3.2. Cell cycle progression of lymphocytes

To assess the effect of samples on the lymphocytes cell cycle, the DNA content of individual cells stained with propidium iodide was analysed by flow cytometry (**Xu *et al.*, 2005**). Briefly, 100 µl of diluted blood (1:10) were incubated with test samples for 72 h. Lymphocytes were isolated from red cells by lysis in hypotonic solution, followed by centrifugation. Cells were fixed with 4% paraformaldehyde for at least 5 min and the supernatant was removed by centrifugation at 1100 rpm for 10 min. Five-hundred microliter of 0.5% Tween, 50 µl of ribonuclease A and 50 µl of propidium iodide solution were added and the mixture was incubated for 60 min at 37 ºC with 5% CO_2. Fluorescence (DNA content) was measured on a Cytomics FC500 flow cytometer (Beckman Coulter, USA). A minimum of 10,000 cells analysed in each sample served to determine the percentages of cells in each phase of the cell cycle, using Multicycle AV software (Phoenix Flow Systems Inc., USA).

4.6. Statistical analysis

To determine whether there was any difference between values, one-way analysis of variance (Anova), followed by Tukey's multiple comparison tests were applied. Data values obtained from immunological assays were analysed by the Kruskal–Wallis and the Dunn's multiple comparison tests. To assess the relationship between values, Pearson's correlation coefficients were calculated with 95% confidence. The GraphPad Prism 5 software was used to analyse the data. Differences were considered significant when $p < 0.05$.

5. RESULTS AND DISCUSSIONS

5.1. Phenolic content profiles and free radical-scavenging activity of roots of *Glycyrrhiza* species

5.1.1. Total content of polyphenols

The content of total phenols (TP) was calculated from a calibration curve (r = 0.9992) using gallic acid as a standard, as depicted in **Figure 12**. The TP content, measured by Folin-Ciocalteu method, not varied widely in the plant materials and ranged from 4.15 to 4.71 mg GAE/100 mg (**Table 4**). The highest value was found in *G. uralensis* (4.71 mg GAE/100 mg) and *G. echinata* (4.70 mg GAE/100 mg), whereas the lowest was in *G. glabra* (4.15 mg GAE/100 mg). The TP content of wild-licorice from *G. lepidota* (63.00 mg catequin equivalents/g) and chinese licorice (23.65 mg GAE/g) was previously documented **(Amarowicz *et al.*, 2004; Liu *et al.*, 2008)**. Other investigations **(Wong *et al.*, 2006; Di Mambro *et* Fonseca, 2005)** determined the TP content of *G. uralensis* (14.50 mg GAE/g) and *G. glabra* (7.42 µg/mg).

As far as the content of total flavonoids (TF) were concerned, *G. glabra* roots showed the highest value (1.84 mg QE/100 mg), whereas *G. pallidiflora* had the lowest one (0.53 mg QE/100 mg). The content of total flavonoids (TF) was calculated from a calibration curve (r = 0.9999) using quercetin as a standard, as depicted in **Figure 13**. In an early study, the TF content of *G. pallidiflora* was measured by two different methods. Authors reported values of 2.09 mg QE/100 mg and 4.27 mg QE/100 mg of extract, measured by $AlCl_3$ and $Al(NO_3)_3$ methods, respectively **(Ma *et al.*, 2006)**. The TF content of a chinese licorice (18.51 mg rutin

equivalents/g) and commercial extract from *G. glabra* (0.88 µg/mg) was also published **(Di Mambro *et* Fonseca, 2005; Liu *et al.*, 2008)**. According to an early review, the flavonoids comprise 1.00–1.50% of the water soluble extract of licorice **(Isbrucker *et* Burdock, 2006)**.

Tannins and related plant components have been found to show several biological activities **(Chung *et al.*, 1998)**, however, there is little information of their total content in roots of *Glycyrrhiza* species. In the present study, the TT amount ranged from 0.48 to 0.74 mg GAE/100 mg **(Table 4)**. The extracts of *G. uralensis* and *G. pallidiflora* showed the highest values. In a previous investigation on the licorice components with tannin-like activity of binding with protein, several phenolics of low molecular weight such as licochalcone B and glycyrrhisoflavone were found to bind with hemoglobin **(Hatano *et al.*, 1988)**. According to the authors, although it is inappropriate to call these flavonoids "tannins", they should be responsible to some extent for the activity as "tannins" of the licorice extract.

As can be noted, the values of TP and TF content of licorice, reported by other authors, are not entirely within the range of values exhibited in the present study. The comparison between the values reported by different laboratories can be difficult due to substantial differences in sample preparation, procedure of extraction, selection of end-points and expression of results, even for the same method, as was pointed out above.

Figure 12. Calibration curve of the absorbance values versus concentration of the standard gallic acid.

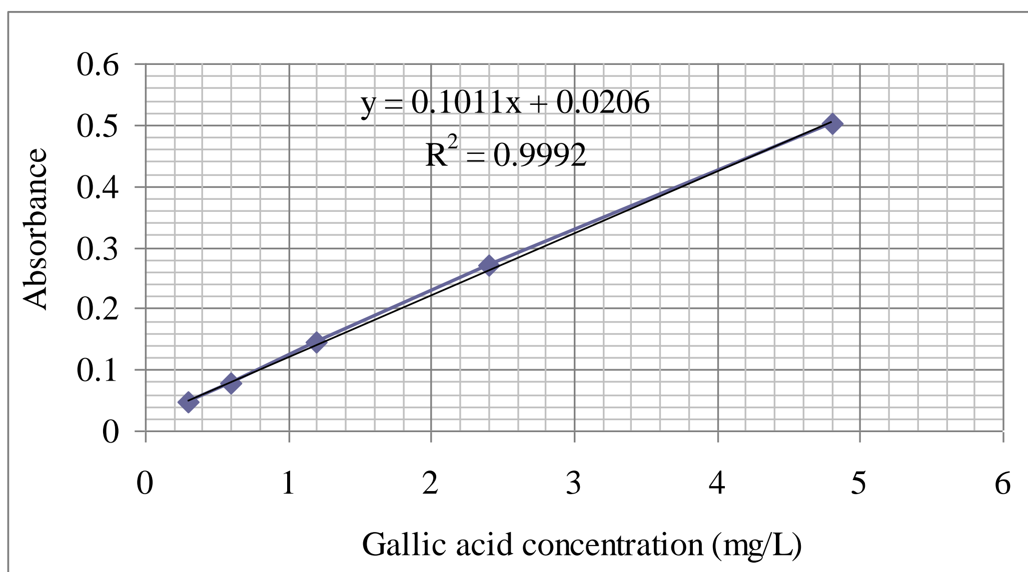


Figure 13. Calibration curve of the absorbance values versus concentration of the standard quercetin.

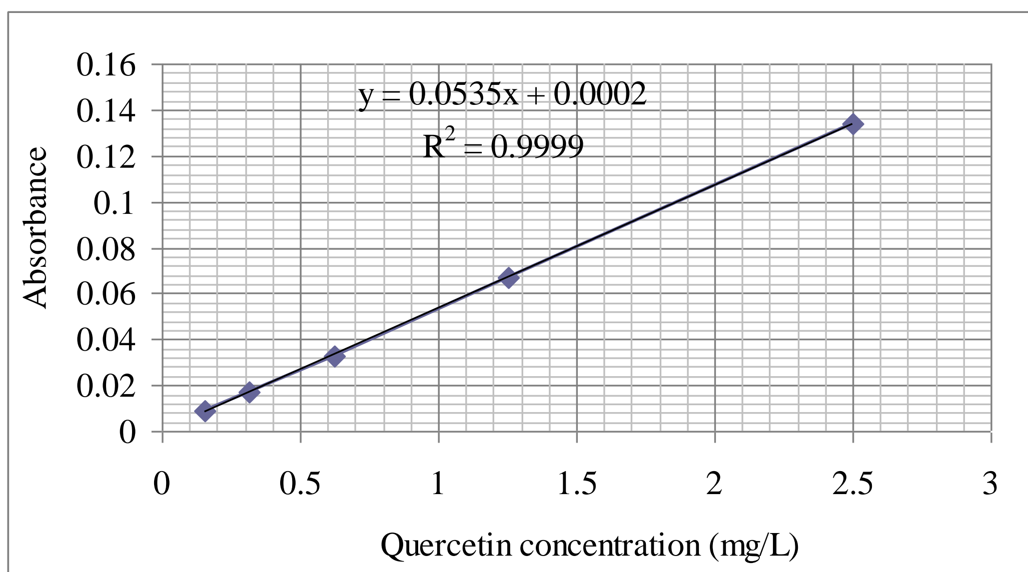


Table 4. Total content of phenols, flavonoids and tannins of extracts from *Glycyrrhiza* species roots.

Samples	Extraction yield (%)	Phenolic content[b]	Flavonoid content[c]	Tannin content[b]
G. glabra	25.65 ± 0.62	4.15 ± 0.04	1.84 ± 0.05	0.48‡ ± 0.06
G. uralensis	25.26 ± 0.72	4.71† ± 0.02	1.09 ± 0.04	0.74* ± 0.05
G. echinata	21.87 ± 0.52	4.70† ± 0.03	0.74 ± 0.01	0.57‡ ± 0.06
G. pallidiflora	25.26 ± 0.82	4.57 ± 0.05	0.53 ± 0.03	0.71* ± 0.06

Each value represents mean ± SD of three determinations. [b]Total contents of phenols and tannins are expressed as mg GAE/ 100 mg dry extract. [c]Total content of flavonoids is expressed as mg QE/ 100 mg dry extract. Values showing the same symbol (†, ‡, *) are not significantly different ($p < 0.05$).

5.1.2. HPLC-DAD analysis

The major components of *G. glabra* and *G. uralensis* were identified as liquiritin and glycyrrhizin. The occurrence of liquiritin was also evident in *G. echinata* and *G. pallidiflora* (**Figure 14**). A second major peak in the HPLC chromatogram of *G. echinata* and *G. pallidiflora* showed UV/Vis spectra compatible with macedonoside C. According to literature (**Hayashi *et al.*, 2005**), *G. glabra* and *G. uralensis* produce glycyrrhizin as a major saponin, whereas *G. echinata* and *G. pallidiflora* do not produce glycyrrhizin but instead produce macedonoside C as a major saponin. In **Figure 15** is shown the relative abundance (area % HPLC) of liquiritin, glycyrrhizin and macedonoside C in extracts of *Glycyrrhiza* species.

Figure 14. HPLC chemical profile of root extracts of *Glycyrrhiza* species.

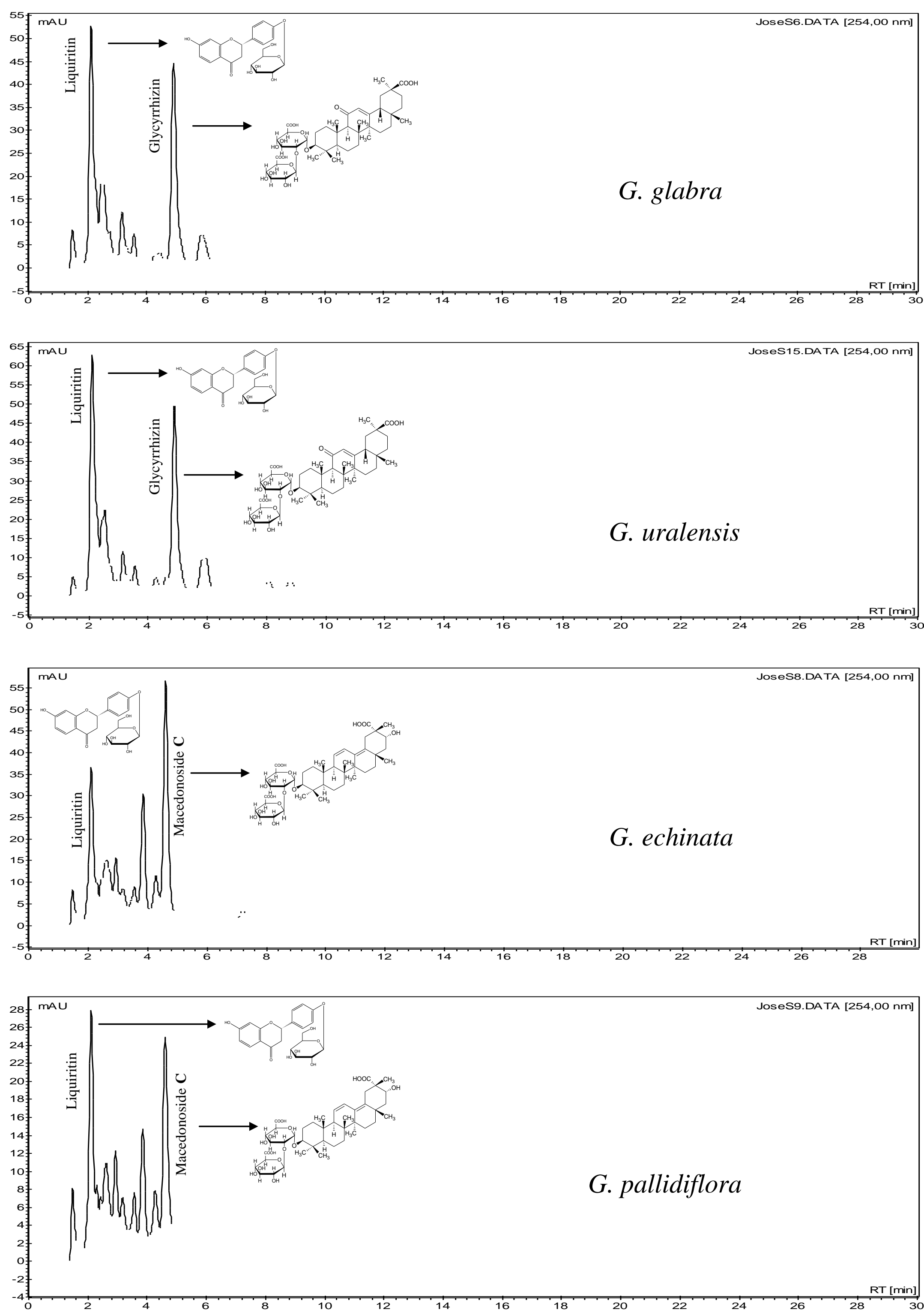
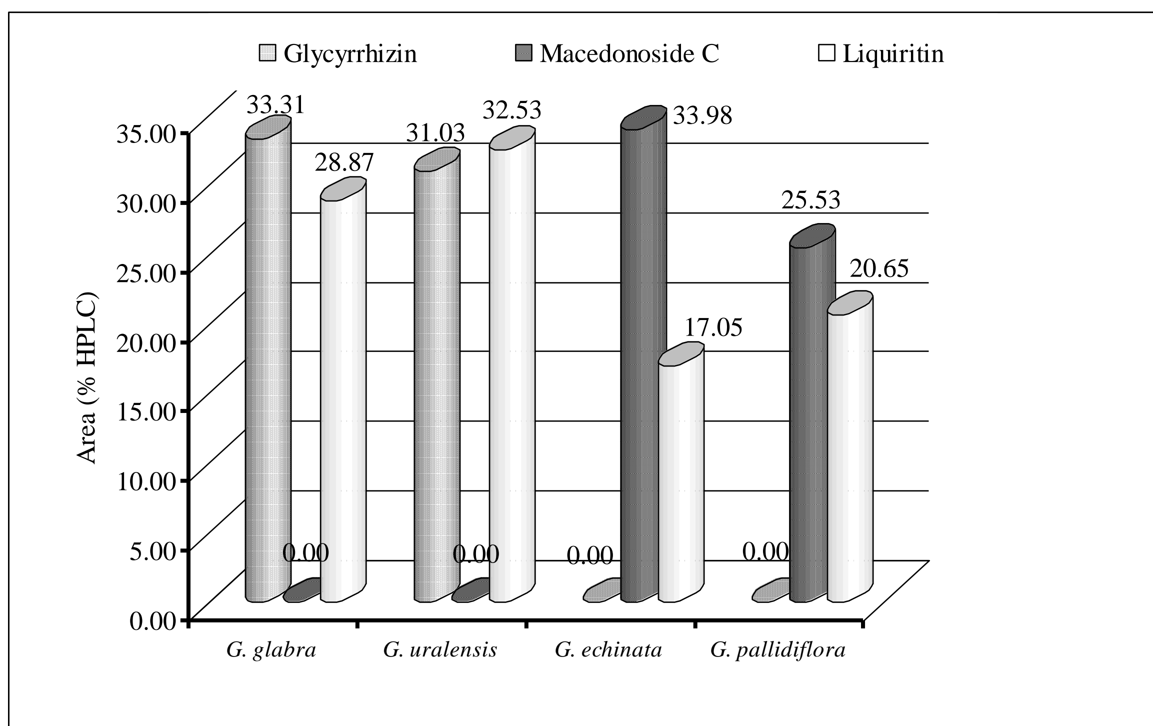


Figure 15. Relative abundance (area % HPLC) of liquiritin, glycyrrhizin and macedonoside C in extracts of *Glycyrrhiza* species.



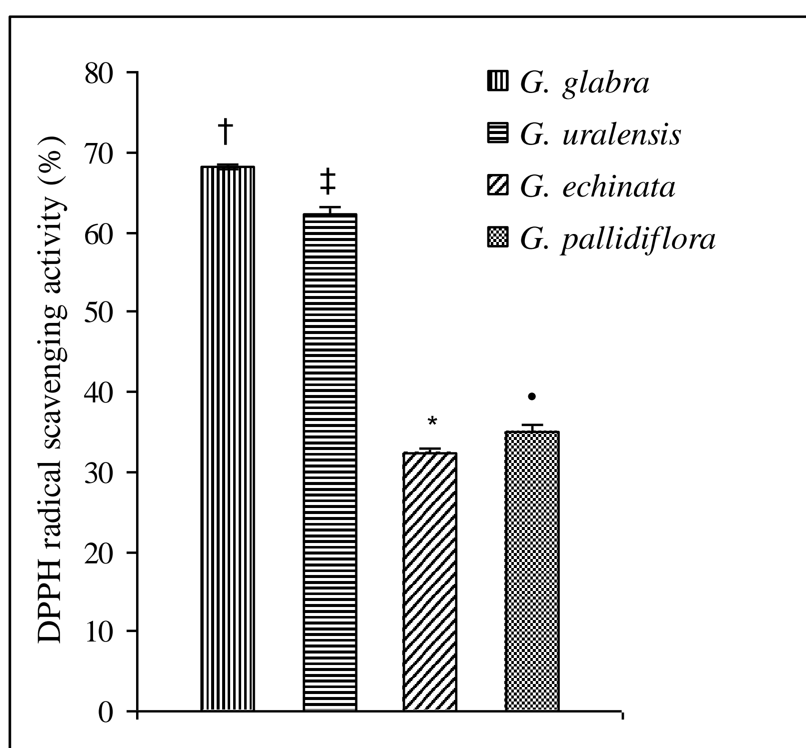
5.1.3. Free radical-scavenging activity

5.1.3.1. DPPH free radical-scavenging activity

All *Glycyrrhiza* extracts showed DPPH scavenging effects **(Figure 16)**. Values varied significantly ($p < 0.05$) ranging from 35.03% to 68.13%. The *G. glabra* extract showed the highest activity (68.13%), while the lowest was exhibited by *G. echinata* (32.31%). In earlier studies the DPPH scavenging effect of *G. uralensis* roots (72.00%) and a licorice extract (27.20%) was reported **(Lee *et al.*, 2003; Liu *et al.*, 2008)**. In other investigation, *G. glabra* extract alone and with the formulations containing this extract showed great free radical scavenging and antioxidant activities using the DPPH free radical assay and the inhibition of lipid peroxidation, respectively **(Di Mambro *et* Fonseca, 2005)**. A herbomineral formulation

of the Ayurveda medicine containing *G. glabra* roots was tested for its anti-ulcer and antioxidant activity in rats **(Bafna *et* Balaraman, 2005)**. The authors attributed the antiulcer activity of the formulation to its antioxidant mechanism of action

Figure 16. DPPH radical-scavenging activity of *Glycyrrhiza* extracts. Each value represents a mean (n = 3) ± SD. Bars showing the same symbol are not significantly different ($p < 0.05$).

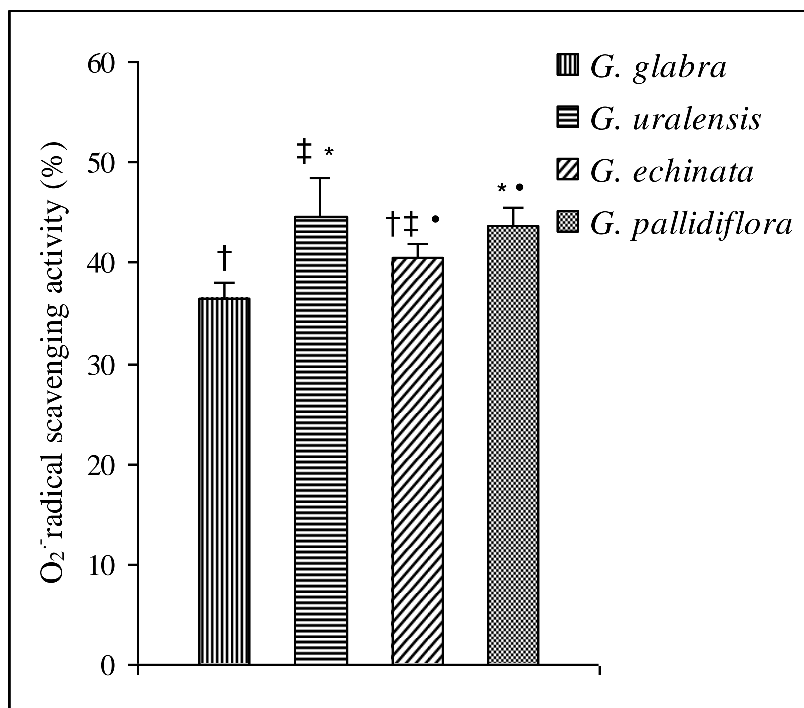


5.1.3.2. Superoxide anion radical-scavenging activity

The superoxide anion radical scavenging activity of *Glycyrrhiza* extracts was also evaluated in this study. The values ranged between 36.43% and 44.52% at 50 µg/ml. The *G. uralensis*, *G. echinata* and *G. pallidiflora* samples showed the highest activity. Values were not statistically different among them ($p < 0.05$). As shown in **Figure 17**, the activity profile of *G.*

glabra and *G. uralensis* was similar to that observed in the Glycyrrhizin-non-producing *Glycyrrhiza* species. Hence, glycyrrhizin does not seem to play a major role in the superoxide anion radical scavenging activity of these plants. In an earlier study **(Tang *et al.*, 2004)**, a bark extract of *G. uralensis* scavenged superoxide anion radical with IC_{50} value above 25 μg/ml. According to literature, it is well known that inhibitory effect on the production of superoxide radical could also be due to inhibitory effect on xanthine oxidase. Licorice extracts have been showed to inhibit xanthine oxidase in about 40–50% at 30 μg/ml **(Hatano *et al.*, 1991)**. Several lines of evidence from both *in vitro* and *in vivo* studies suggest that oxidation of low-density lipoprotein (LDL), critically contributes to human atherosclerosis **(Vaya *et al.*, 1997)**. Superoxide anion radicals have been involved in the oxidative modification of LDL **(Scaccini *et* Jialal, 1994)**. The beneficial effect of licorice consumption in enhancing the resistance of LDL to oxidation as well as in reducing area of atherosclerotic lesions was previously reported **(Fuhrman *et al.*, 1997)**.

Figure 17. Superoxide anion radical-scavenging activity of *Glycyrrhiza* extracts. Each value represents a mean (n = 3) ± SD. Bars showing the same symbol are not significantly different ($p < 0.05$).



5.1.4. Correlation between the free radical-scavenging activity and the content of TP, TF and TT

Several studies have reported strong correlations between the polyphenolic content and their antiradical effects in medicinal plants **(Li *et al.*, 2008; Wojdyło *et al.*, 2007)**. However, others found no such relationship in some plant extracts **(Kähkönen *et al.*, 1999)**. In the present study, positive and significant correlations between TP content and superoxide radical scavenging activity (SRSA) ($r = 0.6881$, $p < 0.05$); and between TT content and SRSA ($r = 0.7754$, $p < 0.05$) were observed. In addition, the TF content significantly correlated with the DPPH free radical scavenging activity (DRSA) with $r = 0.8804$, $p < 0.05$ **(Figure 18)**. The TP

and TT content showed no significant correlation with DRSA. A similar finding was observed between TF content and SRSA.

Furthermore, to determine the relative contribution of phenolic groups to the antiradical activity of samples, the coefficient of determination (R^2) was calculated. It was observed that about 47% of SRSA of samples results from the contribution of total phenols **(Figure 19)**. The association between TT content and SRSA showed a remarkable coefficient of determination ($R^2 = 0.6012$, $p < 0.05$), which suggests that about 60% of SRSA of samples results from the contribution of tannins **(Figure 20)**. Besides, a substantial coefficient of determination between TF and DRSA ($R^2 = 0.7751$, $p < 0.05$) indicated that flavonoids were major contributors (about 77%) to the DPPH activity of samples **(Figure 21)**. According to these findings, it is clear that the free radical scavenging activity of *Glycyrrhiza* species roots is not limited to phenolics. Activity may also come from the presence of other secondary metabolites. Indeed, coumarins and glycyrrhizin present in roots of some *Glycyrrhiza* species, have been also shown to have antiradical activity **(Hatano *et al.*, 1988; Jeong *et al.*, 2002)**. Our results are in agreement with a previous report in that tannins and flavonoids contribute significantly to the free radical scavenging activity in different Chinese medicinal plants **(Hu *et al.*, 2004)**. Given that a possible relation between the antiradical of polyphenolic compounds and some pharmacological effects **(Chung *et al.*, 1998)** of plants, the roots of *Glycyrrhiza* species are likely to play a major role in health promoting and anti-aging activities.

Figure 18. Multiple correlation analysis by Scatter Plot Matrix ($p < 0.05$).

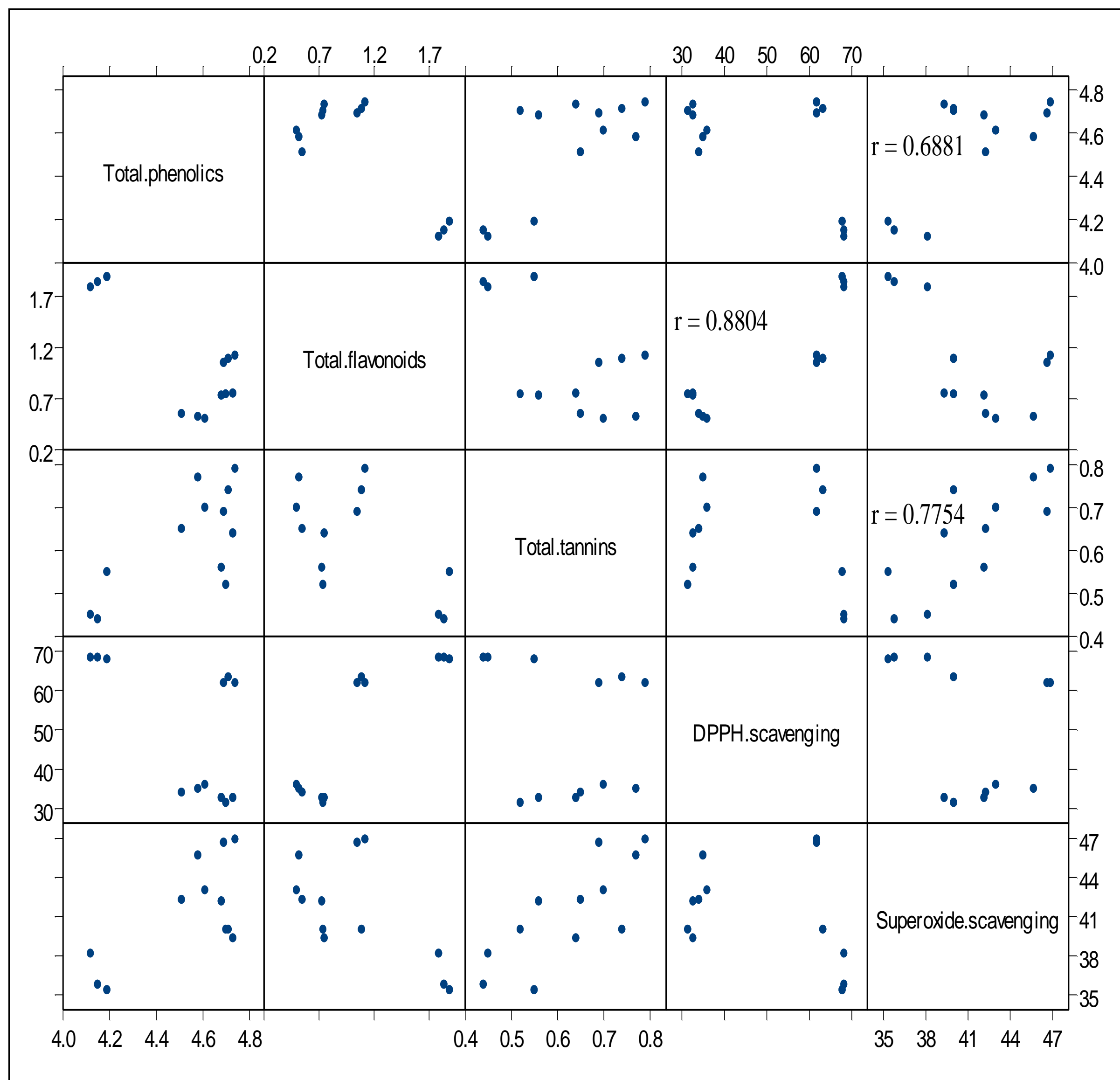


Figure 19. Coefficient of determination ($p < 0.05$) between the superoxide radical scavenging activity and total phenolic content of extracts from *Glycyrrhiza* species roots.

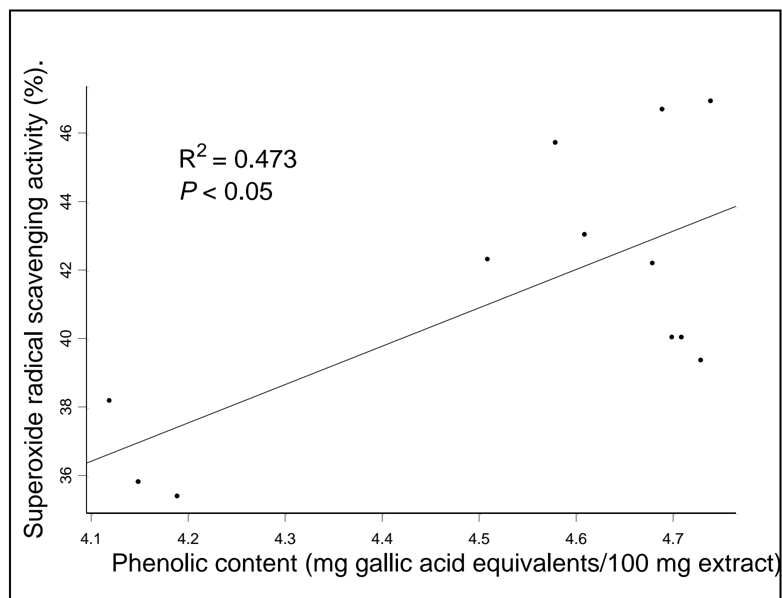


Figure 20. Coefficient of determination ($p < 0.05$) between the superoxide radical scavenging activity and total tannin content of extracts from *Glycyrrhiza* species roots.

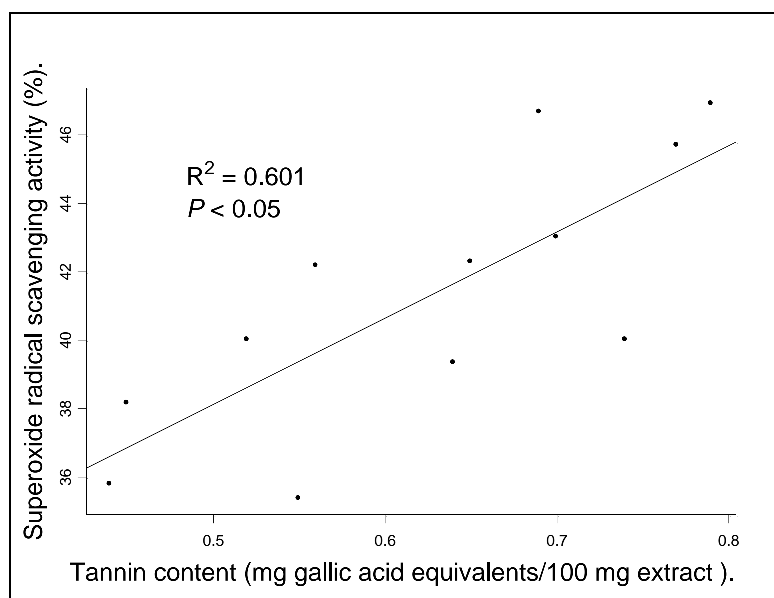
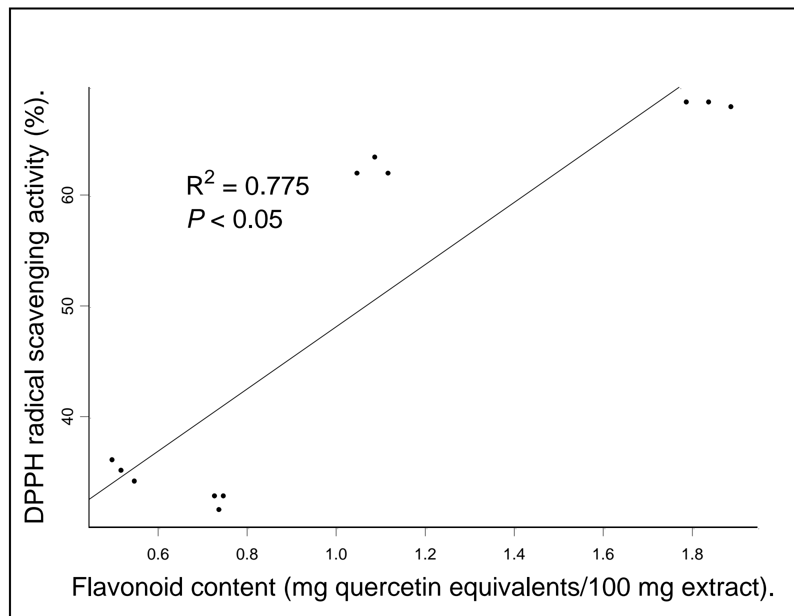


Figure 21. Coefficient of determination ($p < 0.05$) between the DPPH radical scavenging activity and total flavonoid content of extracts from *Glycyrrhiza* species roots.



5.2. Free radical-scavenging, antioxidant and immunostimulating effects of licorice infusion (*Glycyrrhiza glabra* L.)

5.2.1. Total content of polyphenols

Polyphenols are bioactive compounds believed to be involved in the defence process against oxidative damage in biological systems, due at least in part to their antioxidant properties **(Parr *et* Bolwell, 2000)**. About 300 kinds of phenolic compounds have been isolated from various species of *Glycyrrhiza* and many of them are described as exhibiting biological actions which supplement the efficacy of licorice **(Zhang *et* Ye, 2009)**. The **Table 5** shows the results of chemical analyses of licorice infusion (LI). The extraction yield of licorice lyophilised infusion (LI) was about 30%. The TP content in LI was measured by the Folin–Ciocalteu method in which oxidation of phenols by a molybdotungstate reagent yields a coloured product with λmax at 760 nm. The content of TP (7.42 μg/mg) in a licorice extract was previously reported **(Di Mambro *et* Fonseca, 2005)**. Most reports on the flavonoid content in licorice are usually based on flavanones and chalcones, such as liquiritin, isoliquiritin and their corresponding aglycones **(Zhang *et* Ye, 2009)**. Other flavonoid subclasses of licorice, namely flavones and flavonols, have been shown to exhibit strong antioxidant effects **(Hatano *et al.*, 1988)**. In the present study, the total flavone and flavonol (TF) contents in LI (0.70 mg QE/100 mg of LI) were determined by the aluminium chloride ($AlCl_3$) method. The principle of this method is that $AlCl_3$ forms acid-stable complexes with the 4-keto group (ring C) and either the 3-OH group on ring C or 5-OH group on ring A of flavonoids. In addition, the $AlCl_3$ forms acid-labile complexes with the orthodihydroxyl groups in the A- or B-ring **(Chang *et al.*, 2002)**. Liquiritin (1) is a flavanone glucoside bearing unsubstituted and substituted hydroxyl group on rings A and B, respectively.

Therefore, it was not accounted by the $AlCl_3$ method. In a prior study, the content of TF (0.88 µg/mg) in a licorice extract was reported **(Di Mambro *et* Fonseca, 2005)**. In another study the TF content of *Glycyrrhiza pallidiflora* was measured by two different methods. The TF contents were 2.09/100 and 4.27/100 mg of extract, measured by the $AlCl_3$ and $Al(NO_3)_3$ methods, respectively **(Ma *et al.*, 2006)**. In the current investigation, the TT content in LI (0.78 mg GAE/100 mg of LI) was estimated using the protein (hide powder) precipitation method, followed by Folin–Ciocalteu procedure. So far, there is very limited information on the TT content in the roots of *Glycyrrhiza* species. Two flavonoids, named licochalcone B and glycyrrhisoflavone, were isolated from an acetonic-aqueous extract of licorice and they exhibited tannin-like activity and strong DPPH scavenging activity **(Hatano *et al.*, 1988)**.

Figure 22. Calibration curve of the absorbance values versus concentration of liquiritin.

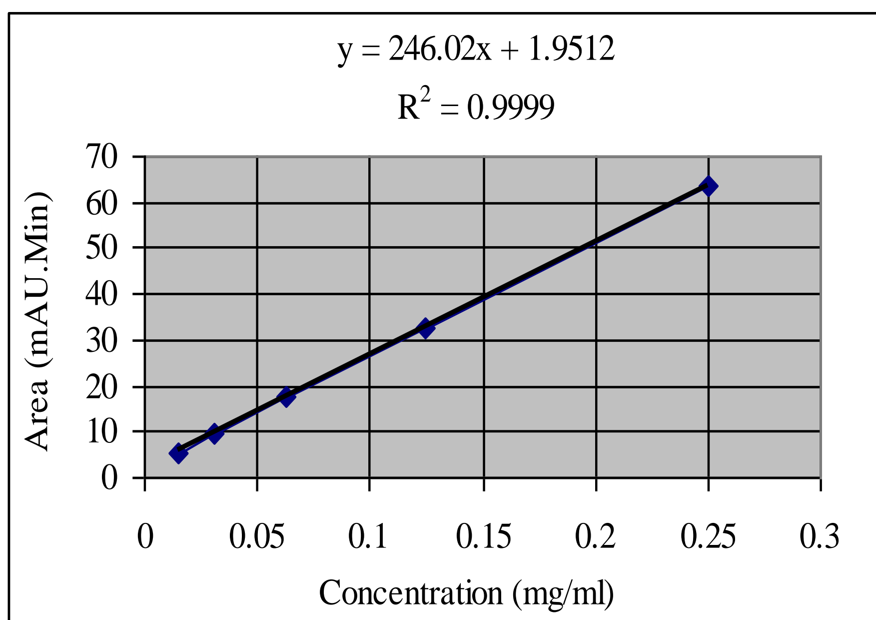


Figure 23. Calibration curve of the absorbance values versus concentration of glycyrrhizin

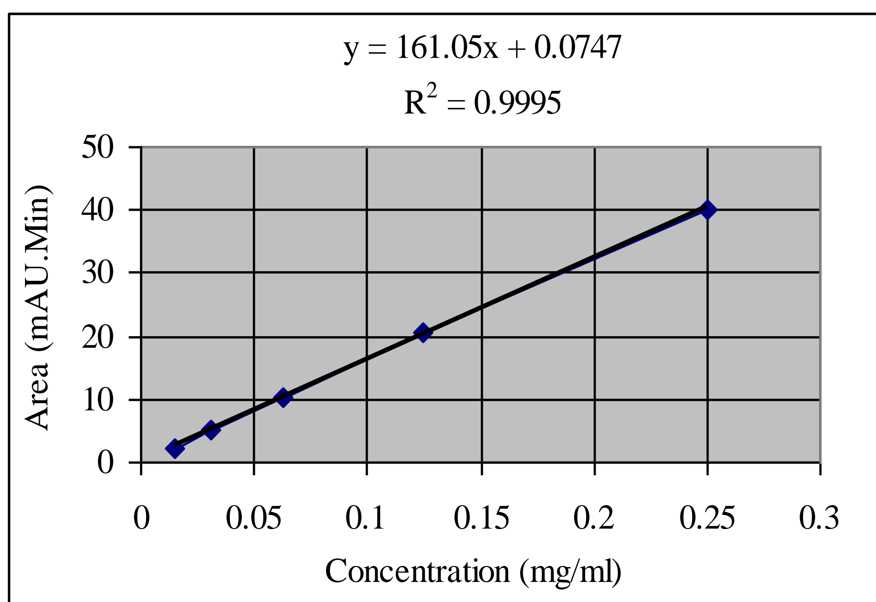


Table 5. Contents of liquiritin, glycyrrhizin, and total polyphenols in licorice infusion (LI).

Compounds	Content in dried roots	Content in lyophilized extract (LI)	Regression equation	Correlation coefficient (r^2)
Liquiritin	1.55 ± 0.05	5.18 ± 0.17	$Y = 246.02X + 1.9512$	0.9999
Glycyrrhizin	2.23 ± 0.04	7.43 ± 0.16	$Y = 161.05X + 0.0747$	0.9995
TP content [(a)]	1.75 ± 0.02	5.83 ± 0.06	$Y = 0.1011X + 0.0206$	0.9992
TF content [(b)]	0.21 ± 0.01	0.70 ± 0.02	$Y = 0.0535X + 0.0002$	0.9999
TT content [(c)]	0.24 ± 0.01	0.78 ± 0.01	$Y = 0.1011X + 0.0206$	0.9992

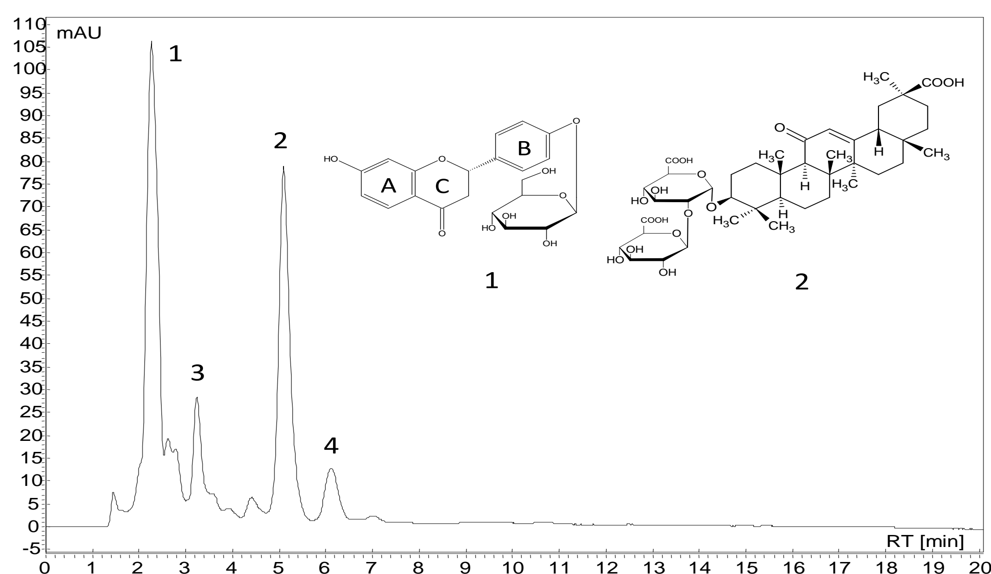
Contents of liquiritin and glycyrrhizin are expressed as mg/100 mg of LI. Each value represents mean (n = 3) ± SD. [a]Total phenols (TP) content is expressed as mg gallic acid equivalents (GAE)/100 mg of LI. [b]Total flavones and flavonols (TF) content is expressed as mg quercetin equivalents (QE)/100 mg of LI. [c]Total tannins (TT) content is expressed as mg GAE/100 mg of LI.

5.2.2. HPLC-DAD analysis

The data from the HPLC-DAD analysis of licorice infusion is presented in **Table 5**, whereas the HPLC chromatogram is presented in **Figure 24.** The two major components of LI were identified as liquiritin (1) and glycyrrhizin (2) by comparisons with the retention times and UV spectra of authentic standards. Compounds 1 and 2 were quantified with calibration curves established with standards **(Figure 22, 23)**. Peaks three and four of the chromatogram **(Figure 24)**, showing UV λmax at 248 nm, were preliminarily identified as derivatives of glycyrrhizin **(Kitagawa *et al.*, 1998)**. Glycyrrhizin has been reported to constitute 10–25% of

licorice extract **(Isbrucker *et* Burdock, 2006)**. The liquiritigenin glycosides constitute 1.6% of licorice aqueous extract **(Kitagawa *et al.*, 1998)**. In the current study, liquiritin was found to comprise about 89% of the TP content in LI.

Figure 24. HPLC chemical profile of licorice infusion (LI). Detection at 254 nm. Peaks: (1) liquiritin; (2) glycyrrhizin; (3) and (4) glycyrrhizin derivatives.



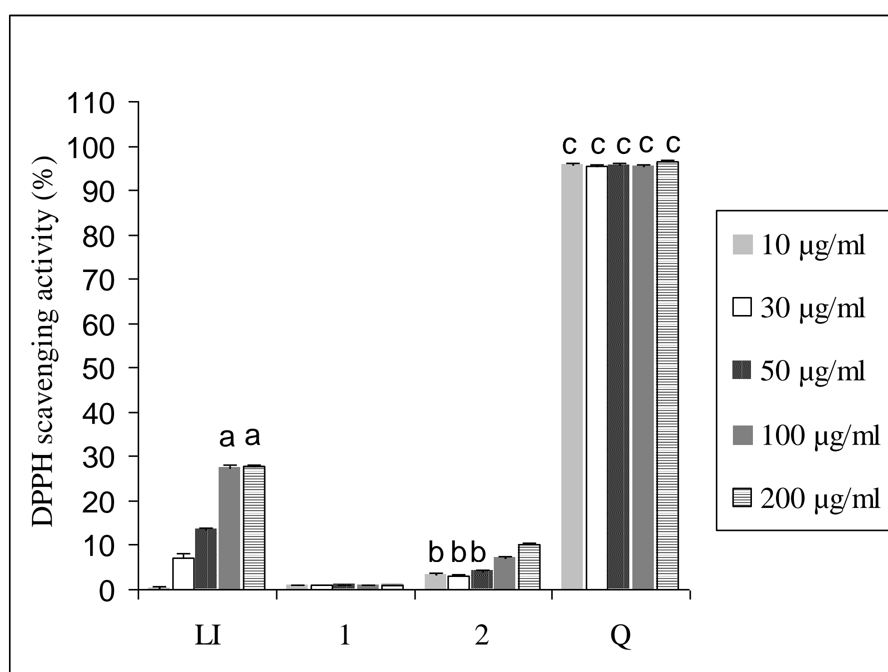
5.2.3. Free radical-scavenging activity

5.2.3.1. DPPH free radical-scavenging activity

This assay is based on the ability of DPPH to react with H-donors. The change in absorbance produced by reduced DPPH is used to evaluate the antiradical ability of the samples. The DPPH-scavenging activities of LI, 1, and 2 are shown in **Figure 25**. The LI exhibited a weak effect in a concentration-dependent manner, in a concentration range of 10–100 µg/ml, which is in accordance with a prior study **(Di Mambro *et* Fonseca, 2005)**. A further increase of LI

concentration caused no increase of activity. Compounds 1 and 2 showed negligible effects, which is consistent with an earlier study **(Kato *et al.*, 2008)**. Quercetin, the reference compound, scavenged DPPH remarkably (about 96%) in a concentration-independent manner (10–200 μg/ml). The DPPH-scavenging activity of flavonoids isolated from an acetonic-aqueous extract of licorice was correlated with their two hydroxyl groups on ring B, in a mutual ortho position **(Hatano *et al.*, 1988)**.

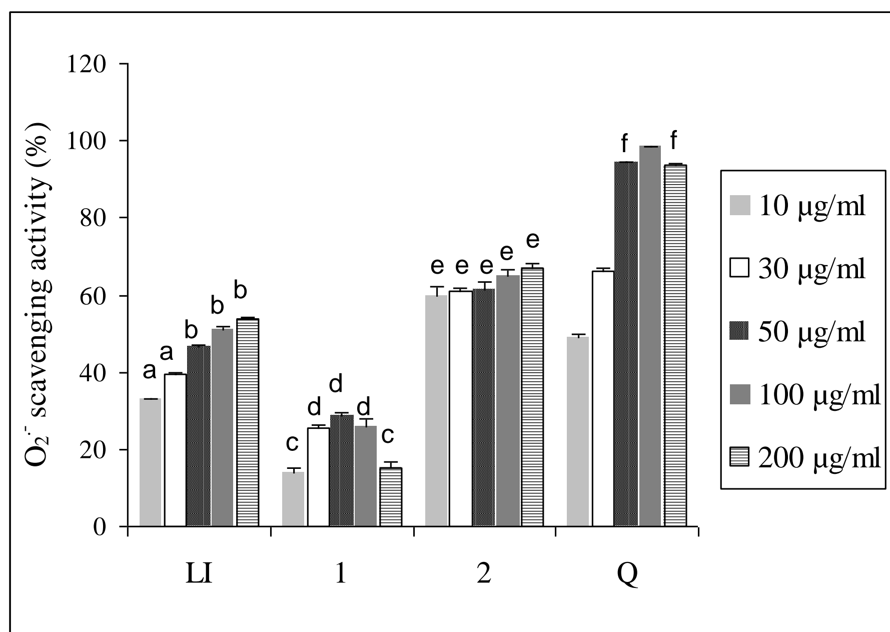
Figure 25. DPPH radical-scavenging activities of licorice infusion (LI), liquiritin (1), and glycyrrhizin (2). Quercetin (Q) was used as a reference compound. Each value represents mean (n = 3) ± SD. Bars showing the same letter (a, b, c) are not significantly different ($p < 0.05$).



5.2.3.2. Superoxide anion radical-scavenging activity

The superoxide anion radical is the most common reactive oxygen species formed *in vivo*. It is known to be very harmful to cellular components as a precursor of more reactive oxygen species, contributing to tissue damage and various diseases **(Nordberg *et* Arnér, 2001)**. The $O_2^{\cdot -}$ scavenging activities of LI, 1, and 2 are shown in **Figure 26**. It was found that LI scavenged superoxide radicals in a concentration range of 10–200 µg/ml. Glycyrrhizin (2) showed an appreciable antiradical effect (about 63%), but no effect dependent on concentration was observed. Compound 1 showed a weak effect (14–26%) in a concentration range of 10–100 µg/ml. A further increase in the concentration of 1 (200 µg/ml) caused a decrease in the scavenging activity. Quercetin, the reference compound, exerted a substantial scavenging effect (49–98%) in a concentration range of 10–100 µg/ml. A further increase in the quercetin concentration (200 µg/ml) caused a slight decrease in the activity (94%). The current study shows that LI has $O_2^{\cdot -}$ scavenging ability, and glycyrrhizin is suggested to be the main compound responsible for this effect. This could be of potential health interest as $O_2^{\cdot -}$ mediated oxidative stress is believed to be involved in the pathogenesis of cardiovascular disorders, diabetes mellitus, acute respiratory distress syndrome and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases **(Ishii *et* Ishii, 2006)**.

Figure 26. Superoxide anion radical-scavenging activities of licorice infusion (LI), liquiritin (1), and glycyrrhizin (2). Quercetin (Q) was used as a reference compound. Each value represents a mean (n = 3) ± SD. Bars showing the same letter (a, b, c, d, e, f) are not significantly different ($p < 0.05$).



5.2.4. Antioxidant activity

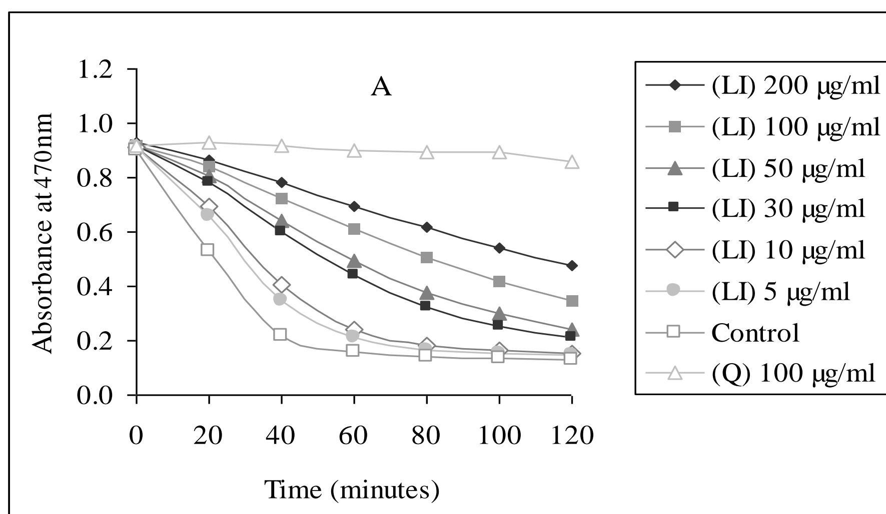
5.2.4.1. Inhibition of β-carotene–linoleate bleaching

The antioxidant activities of LI, and compounds 1 and 2 were evaluated using the β-carotene bleaching method, in which oxidation of β-carotene in the presence of linoleic acid takes place. In this model, during heat-induced oxidation, an atom of hydrogen is abstracted from the active bis-allylic methylene group of linoleic acid located on carbon-11 between two double bonds. The pentadienyl free radical so formed then attacks highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. As the β-carotene molecules lose their conjugation, the carotenoids lose their characteristic orange colour, which can be

monitored spectrophotometrically (**Amarowicz *et al*., 2004**). The effect of LI on the β-carotene consumption at different incubation times is shown in **Figure 27A**. Bleaching inhibition in the presence of LI increased as long as the concentration was high. The system containing LI (200 µg/ml) retained about 83% of the initial β-carotene after 1 h, whereas quercetin at 100 µg/ml retained about 98% of β-carotene. After 2 h, LI at 200 µg/ml retained about 64% of the initial β-carotene. The concentration-effect relationship for LI is shown in **Table 6.** Compounds 1 and 2 showed no antioxidant effect in this system (**Figure 27B**). The greatest antioxidative efficacy found was from the reference compound quercetin, which practically inhibited β-carotene consumption throughout the incubation period. The data for oxidation rate ratio (R_{OR}) and activity coefficient (C_{AA}) support the antioxidant activity index of LI. The oxidation rate ratio bears an inverse relationship to the antioxidant activity index. The activity coefficient increases directly with the increase in value of the antioxidant activity index.

In a prior investigation, a licorice ethanolic extract and glabridin, its major constituent, inhibited β-carotene consumption by 87% and 93%, respectively, after 90 min of incubation (**Vaya *et al*., 1997**). In the same study, the antioxidant effect of the ethanolic extract against the LDL oxidation was mainly atributted to the isoflavonoid glabridin. From our study, the antioxidant activity of LI, measured in the β-carotene/linoleic acid model, could not be attributed to either liquiritin or glycyrrhizin. Various lines of research provide strong evidence that oxidised LDL is involved in the formation of early atherosclerotic lesions. It has been suggested that the oxidation of LDL starts after the depletion of its endogenous lipophilic antioxidants, such as β-carotene, vitamin E, and lycopene (**Belinky *et al*., 1998a**). Therefore, LI may prove to be of potential health benefit by protecting LDL-associated carotenoids and by preventing the oxidation of lipid components within cell membranes.

Figure 27. Effects of licorice infusion (LI), liquiritin (1), and glycyrrhizin (2) on the β-carotene consumption at different incubation times. The β-carotene-linoleic acid emulsion was incubated at 50 ºC in the absence (control), or presence of LI (A) and compounds 1 and 2 (B) at different concentrations. The graphics are representative of three separate experiments. Quercetin (Q) was used as a reference compound at 100 µg/ml.



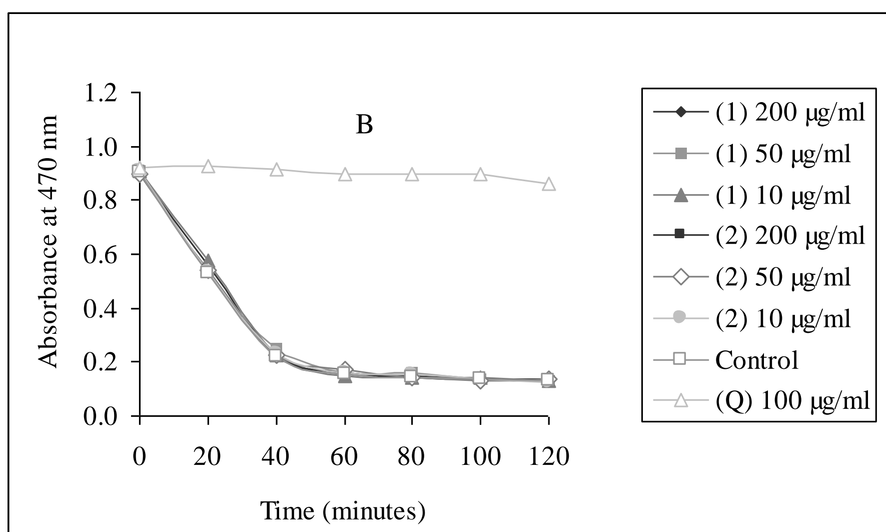


Table 6. Concentration (μg/ml) response of antioxidant activity for the licorice infusion (LI), liquiritin (1), and glycyrrizin (2) by β-carotene-linoleate bleaching method.

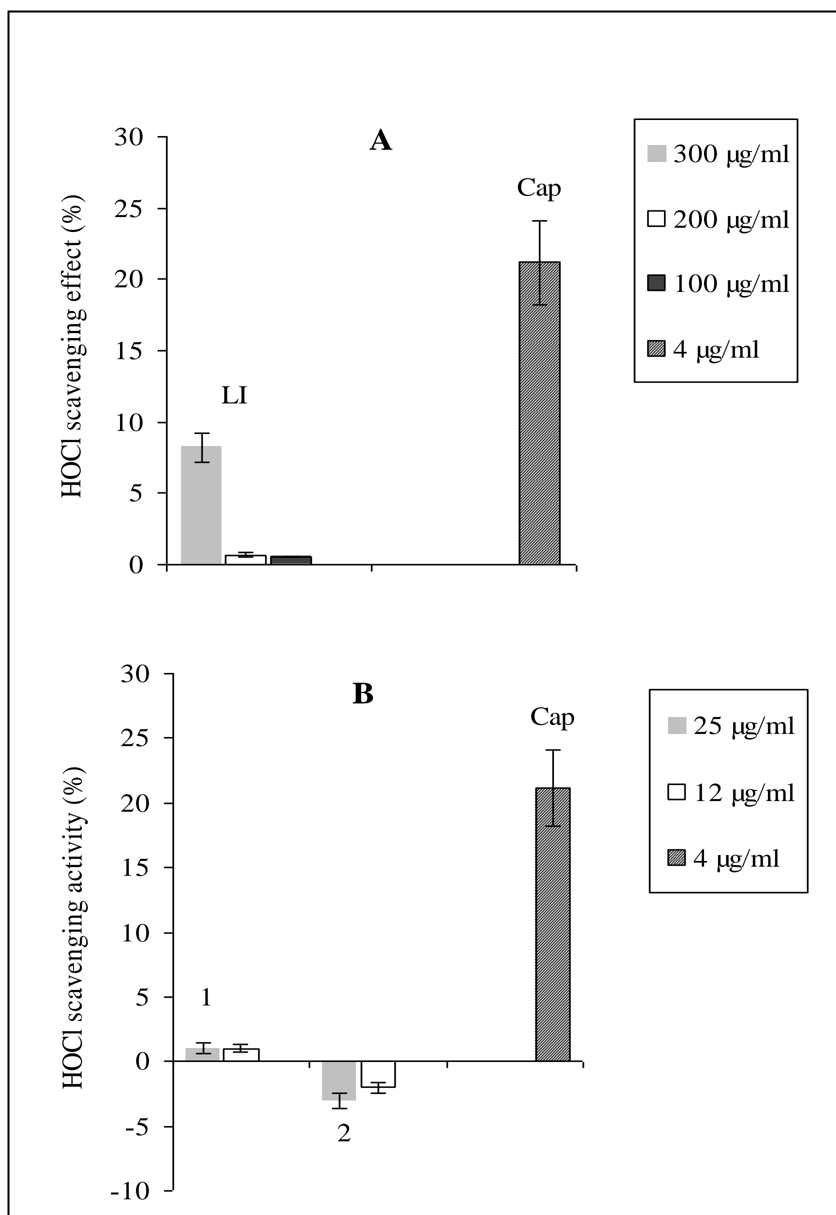
Samples	A_A [(a)]		R_{OR} [(b)]		C_{AA} [(c)]	
	t = 60 min	t = 120 min	t = 60 min	t = 120 min	t = 60 min	t = 120 min
(LI) 200 μg/ml	82.94 ± 0.72	64.13 ± 1.22	0.17 ± 0.01‡	0.36 ± 0.01	721.51 ± 3.32	437.81 ± 8.36
(LI) 100 μg/ml	76.32 ± 0.44	48.77 ± 1.31	0.24 ± 0.00‡	0.52 ± 0.02	613.61 ± 1.53	278.41 ± 6.94
(LI) 50 μg/ml	65.00 ± 0.27	31.88 ± 1.12	0.35 ± 0.00	0.69 ± 0.00	458.35 ± 4.65	144.96 ± 2.28
(LI) 30 μg/ml	56.95 ± 1.10	22.24 ± 1.68	0.43 ± 0.01	0.78 ± 0.02	374.31 ± 5.51	101.36 ± 2.40
(LI) 10 μg/ml	25.55 ± 1.04	7.15 ± 0.58†	0.79 ± 0.04	0.93 ± 0.01	112.84 ± 3.94	27.95 ± 1.88*
(LI) 5 μg/ml	15.12 ± 0.99	4.68 ± 0.45†	0.90 ± 0.06	0.96 ± 0.01	75.07 ± 3.93	18.16 ± 1.29*
(1) 5-200 μg/ml	N.d.	N.d.	N.d.	N.d.	N.d.	N.d.
(2) 5-200 μg/ml	N.d.	N.d.	N.d.	N.d.	N.d.	N.d.
[(d)]Quercetin 100 μg/ml	98.15 ± 0.59	96.33 ± 0.76	0.02 ± 0.00	0.03 ± 0.00	990.15 ± 5.59	944.74 ±12.18

[(a)]Antioxidant activity index (%). [(b)]Oxidation rate ratio. [(c)]Antioxidant activity coefficient. [(d)]Reference compound. N.d., not detected. Each value represents mean (n=3) ± SD. Values within a column with the same symbol (†, ‡, *) are not significantly different ($p < 0.05$).

5.2.4.2. Hypochlorous acid-scavenging activity

Hypochlorous acid (HOCl) is produced in the organism at sites of inflammation by the oxidation of Cl^- ions, catalysed by neutrophil-derived myeloperoxidase, in the presence of H_2O_2. Hypochlorous acid is a potent oxidant implicated in the bactericidal activity of the neutrophils and also in cell-mediated cytotoxicity. They can oxidise a number of cellular targets and promote proteolytic damage at the inflammatory site via the indirect activation of elastase and collagenase, two major proteolytic enzymes released from the lysosomal granules of these cells **(Weiss, 1989)**. The LI **(Figure 28A)** and compounds 1 and 2 **(Figure 28B)** exhibited no meaningful activity against HOCl. Captopril, the reference compound, scavenged HOCl at a physiologically relevant concentration (4 μg/ml). The traditional use of licorice infusion against inflammatory processes could not be associated with the scavenging of HOCl.

Figure 28. HOCl scavenging activity of (A) licorice infusion (LI), (B) liquiritin (1) and glycyrrhizin (2), as a function of concentration. Captopril (Cap) was used as a reference compound. Each value represents mean (n=3) ± SD.

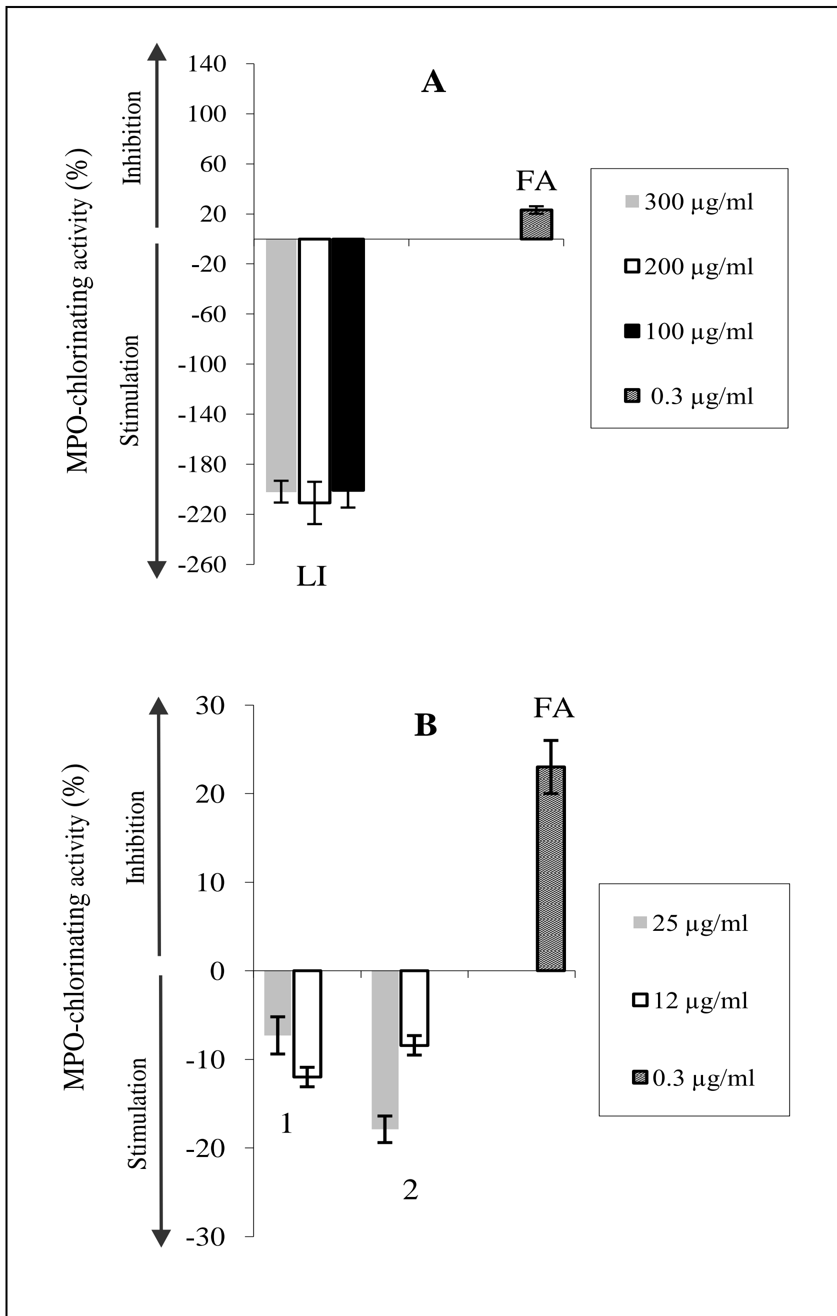


5.2.4.3. Inhibition of myeloperoxidase-chlorinating system

Myeloperoxidase (MPO) is a key enzyme belonging to the complex defence system against exogenous aggressions. It is released from the cytoplasmatic granules of stimulated neutrophils and monocytes. Its role is to ensure the production of hypochlorous acid (HOCl) in the presence of hydrogen peroxide (H_2O_2) and chlorine anions (Cl^-). During phagocytosis, the MPO/H_2O_2/Cl^- system promotes the elimination of microorganisms by HOCl-mediated oxidations. However, MPO and H_2O_2 can also be released to the outside of the cell where a reaction with chloride can induce damage to adjacent tissue and thereby contribute to the pathogenesis of diseases **(Klebanoff, 2005)**. The effects of LI and compounds 1 and 2 on the MPO-chlorinating activity, determined by the measurement of the taurine chlorination, were investigated. Surprisingly, a marked pro-oxidant effect was exhibited by LI when used in the concentration range of 100–300 µg/ml **(Figure 29A)**. Compounds 1 and 2 also exhibited pro-oxidant effects at 12 and 25 µg/ml **(Figure 29B)**. In a preceding investigation, three herbal constituents of STW5 (Iberogast®), *Carum carvi*, *Mentha piperita*, and *Chelidonium majus*, showed a prooxidant effect in the MPO-catalysed chlorination system using the ACC-ethene model. Conversely, the ethanolic extract of *G. glabra*, another STW5 constituent, showed an antioxidant effect **(Schempp *et al.*, 2006)**. Recently, the pro-oxidant effect of a phenolic derivative of flufenamic acid in the MPO system was attributed to quinonimine, its corresponding oxidation product **(Van Antwerpen *et al.*, 2008a)**. In the MPO model, antioxidant activity can be exerted in two different ways: HOCl can be scavenged, or the generation of HOCl can be decreased or blocked by inhibition of the catalytic activity of MPO. In order to relate the effect of LI and compounds 1 and 2 on the MPO system to their scavenging activity of HOCl, we

compared the percentage of activity in the MPO system to the percentage of HOCl-scavenging. As can be observed in **Figure 29A**, LI had a strong pro-oxidant effect in the MPO system, and it weakly reacted with HOCl **(Figure 28A)**. The pro-oxidant effect of compound 1 in the MPO system **(Figure 29B)** could not be related to its behaviour in the HOCl-scavenging assay **(Figure 28B)**. The pro-oxidant effect of 2 in the MPO system was only partially attributable to the same effect observed in the HOCl-scavenging assay **(Figure 28B)**. These observations suggest that MPO could be involved in the pro-oxidant effect of LI. Supporting this assumption, myeloperoxidase has been shown to catalyse the oxidation of phenolics to form phenoxyl radicals **(Kagan *et al*., 2003)**. Therefore, polyphenolic compounds of LI could be substrates of MPO, yielding reactive phenoxyl radicals, responsible for the pro-oxidant effect observed with taurine.

Figure 29. Effect of licorice infusion (LI), liquiritin (1) and glycyrrhizin (2) on the myeloperoxidase-chlorinating system, as a function of concentration. Flufenamic acid (FA) was used as a reference compound. Each value represents mean (n=3) ± SD.



5.2.5. Effects on immune cells

5.2.5.1. Activation of immune cells

CD69 glycoprotein is a very early cell activation molecule expressed on the surface of activated immune cells. It can appear within 1–2 h of activation and exhibits maximal expression levels between 18 and 24 h after stimulation. Although a physiological ligand for CD69 has not yet been identified, its wide distribution, and the observation that crosslinking of the molecule generates intracellular signals, suggest a significant role for CD69 in immune response (**Ziegler *et al.*, 1994**). The effect of LI and compounds 1 and 2 on activation of granulocytes and NK cells, as measured by CD69 expression, was analysed by flow cytometry. As indicated by a shift to the right in the histograms (**Figure 30B**), the CD69 expression on cells was increased after 24 h. As the cells were activated, the amount of the fluorescently-labelled antibodies bound to them was increased. The LI substantially stimulated the expression of CD69 on granulocytes in a concentration-independent manner in the range 100–800 μg/ml (**Figure 30D**). Values of the activation index (AI) of granulocytes treated with LI in the range 100–800 μg/ml did not differ significantly, and they were not significantly different from the AI values exhibited by cells treated with PHA ($p < 0.05$). The LI showed a similar effect on NK cells, but on a lower scale (**Figure 31D**). Compounds 1 and 2, in the range 12–100 μg/ml, did not stimulate the CD69 expression on granulocytes and NK cells (**Figures 30C and 31C**).

Figure 30. Effects of licorice infusion (LI), liquiritin (1), and glycyrrhizin (2) on granulocytes' activation, as measured by CD69 expression. The filled histograms represent the group control (untreated) and the open histograms the stimulated (pretreated) group (B). As compounds 1 and 2 (in range 12–100 µg/ml) had no effect on immune cells, histograms (C) are shown as representatives. Granulocytes were identified on the basis of forward scatter (FSC) and side scatter (SSC). The activated cells were identified in the A circular gate (A). The mitogen, phytohemagglutinin (PHA), was used as a positive control. The cell activation is shown as activation index (AI) in the bar graphic (D). A positive cell response was defined as an AI ≥ 2. Values are presented as means ± SD of three determinations. *$p < 0.05$.

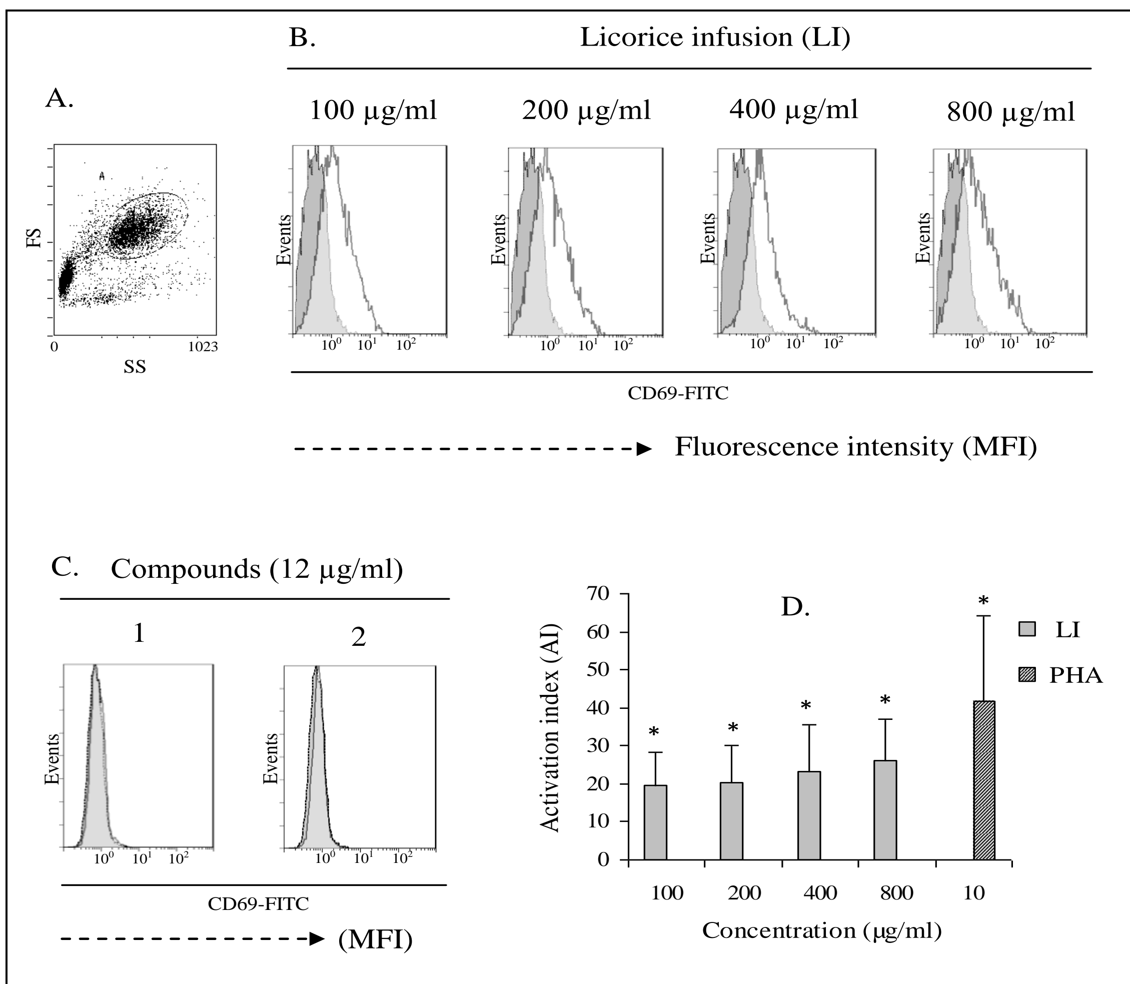
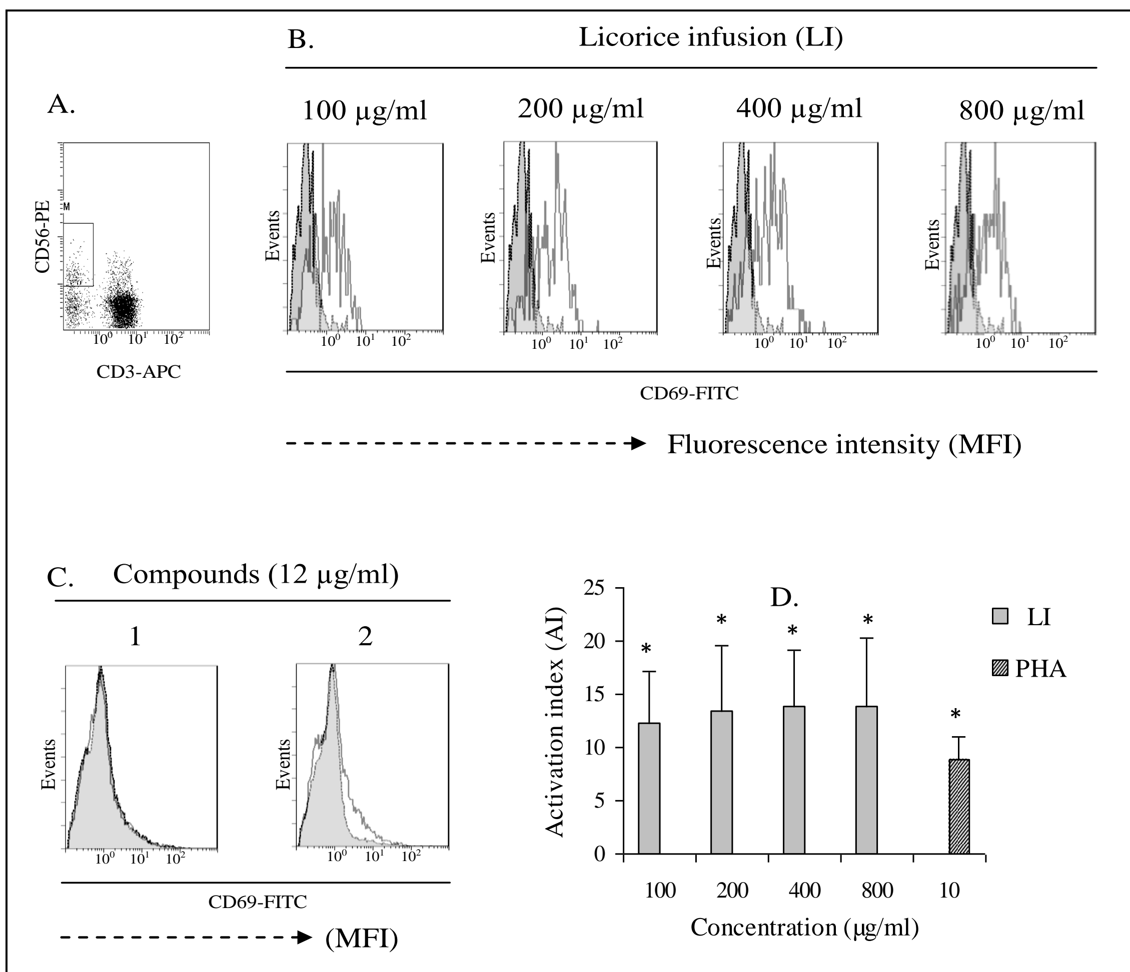


Figure 31. Effects of licorice infusion (LI), liquiritin (1), and glycyrrhizin (2) on NK cells' activation, as measured by CD69 expression. The filled histograms represent the group control (untreated) and the open histograms the stimulated (pretreated) group (B). As compounds 1 and 2 (in range 12–100 μg/ml) had no effect on immune cells, histograms (C) are shown as representatives. NK cells were identified by double surface immunostaining using the combination of CD3/CD56. The activated cells were identified in the M rectangular gate (A). The mitogen, phytohemagglutinin (PHA), was used as a positive control. The cell activation is shown as activation index (AI) in the bar graphic (D). A positive cell response was defined as an AI ≥ 2. Values are presented as means ± SD of three determinations. *$p < 0.05$.

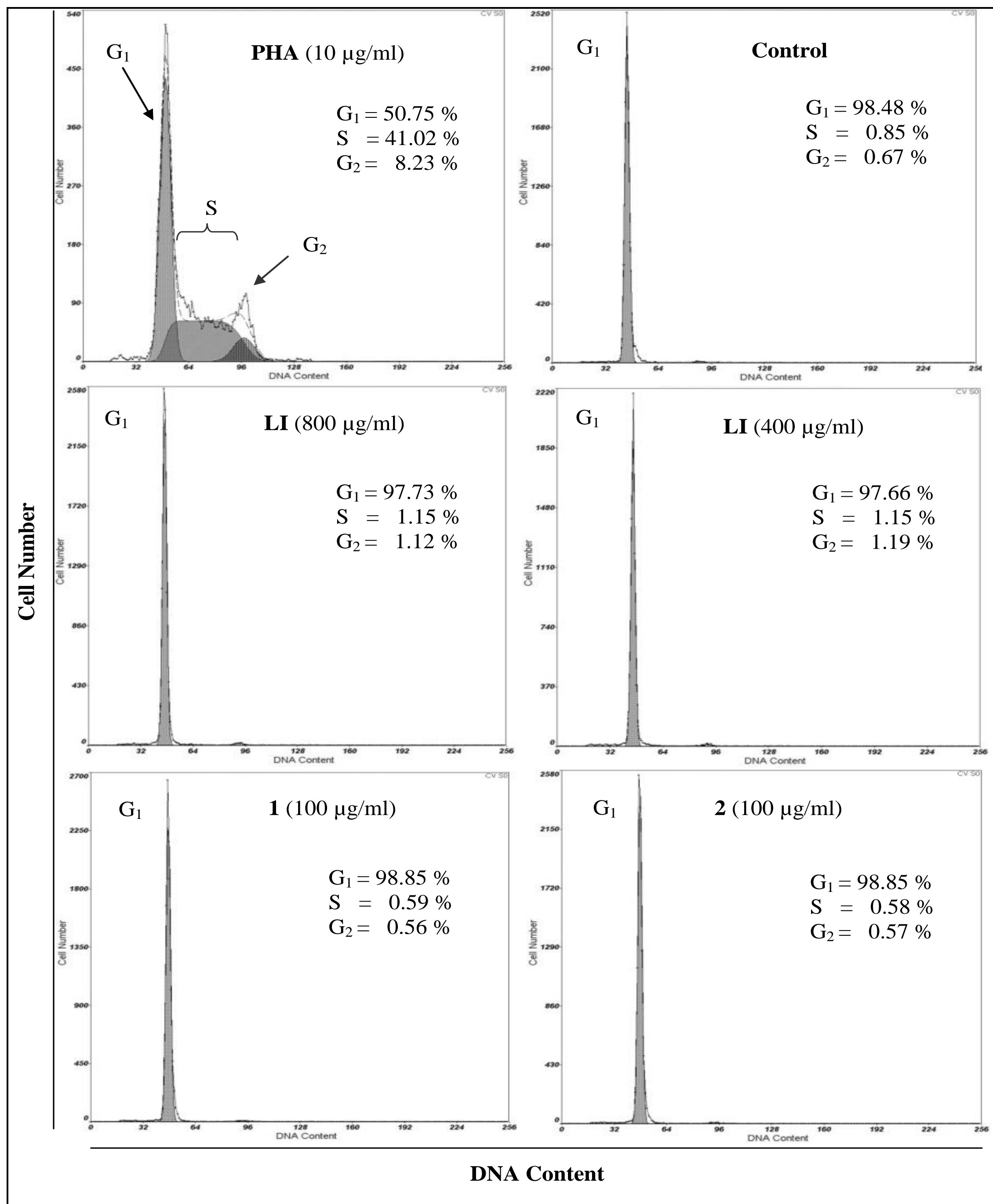


5.2.5.2. Cell cycle progression of lymphocytes

It is known that the interaction of lymphocytes with antigens or PHA initiates a cascade of biochemical events and gene expression, which induces the resting immune cells to enter the cell cycle, and then begin proliferating and differentiating. This event is critical to the development of immunity **(Melvold *et* Sticca, 2007)**. By measuring the incorporation of propidium iodide (PI) in lymphocyte DNA using flow cytometry, the effects of LI, 1, and 2 on cell cycle progression of lymphocytes were investigated. Fluorescent dye (PI) binds to DNA in a manner that accurately reflects the amount of DNA present. The LI, 1, and 2 showed no meaningful effects on cell cycle progression of lymphocytes. Most of the lymphocytes treated with LI, 1, and 2 were in the G_1 phase, and there were only very small numbers of cells in the S or G_2 phases. The PHA stimulated lymphocytes to enter into the S and G_2 phases after 72 h of culture **(Figure 32)**. Among granulocytes, the eosinophil granulocytes have been shown to be important effector cells in the defence against parasitic infections, whereas neutrophil granulocytes have primarily been regarded as part of the defence against bacterial infection **(Nopp *et al.*, 2002)**. The NK cells are important elements in innate defences against virally-infected and cancerous host cells **(Melvold *et* Sticca, 2007)**. By stimulating innate cellular immunity, most pathogens can be eliminated before they fully exhibit their pathological features. In the current investigation, the stimulating effect of LI on the granulocytes and NK cell activation was statistically equal to that shown by PHA, which is considered a strong mitogen. It is questionable whether the LI immunostimulating effect is longterm, because the CD69 expression on the surface of immune cells occurs very rapidly, but it also declines rapidly (after 2 days it disappears). So, this study measured the CD25 expression, a later-expressed activation

marker, but the expression was very low. It seems that the repeated administration of LI would be important for its long-term immunostimulating effect. Recently, tinctures of *Echinacea purpurea*, *Astragalus membranaceus*, and *Glycyrrhiza glabra* were shown to stimulate CD4 and CD8 T cells, as determined by CD69 expression. These three herbs had an additive effect on CD69 expression when used in combination **(Brush *et al.*, 2006)**. In the present study, the stimulating effect of LI on the activation of innate immune cell, as determined by CD69 expression, is reported for the first time. Since licorice has historically been used to treat symptoms attributable to viral infections, the present study suggests that one mechanism for the antiviral properties of licorice could be the activation of NK cells. Furthemore, the traditional use of licorice as an antiulcerogenic agent was reported to be due, in part, to its bactericidal effect on *Helicobacter pylori* **(Krausse *et al.*, 2004)**. Therefore, the granulocyte activation, which plays a major role in host defence against bacterial infection, may be a possible mechanism for the antibacterial action of licorice. These data may support, at least in part, the traditional use of this herb as an antiviral and antiulcerogenic agent.

Figure 32. Effects of licorice infusion (LI), liquiritin (1), and glycyrrhizin (2) on the cell cycle progression of human peripheral lymphocytes. One representative experiment is shown and the percentages of cells in each phase of the cell cycle were observed (G_1, S and G_2 phases).



5.3. Variations in the chemical profile, free radical scavenging, and antioxidant activities of licorice (*Glycyrrhiza glabra* L.), as influenced by harvest times

5.3.1. Extraction yield

In this study, the extraction yield of the licorice extract (LE) varied slightly within the different harvest times of *Glycyrrhiza glabra*. The yield values for the February, May, August and November samples were 25.65%, 21.40%, 26.00% and 26.00%. The lowest value was from the LE obtained from plant at the pre-flowering stage. The highest extraction yields were from the LE of the plant at the fruit-set and vegetative stages. The extract yield did not exhibit regular changes related to plant growth and course time. Similar finding were reported for *Juniperus* species **(Adams, 1987)** and for Turkish oregano **(Ozkan *et al*., 2010)**.

5.3.2. HPLC-DAD and HPLC-ESI-MS analysis

There was no qualitative variation in the chemical composition of LE obtained from the same plant collected at different harvest times, as analysed by HPLC-DAD **(Figure 33)**. Liquiritin (1) and glycyrrhizin (2), the two major peaks in the HPLC chromatogram, varied significantly across harvest times in the ranges of 2.87–6.28 mg/100 mg of extract and 4.18–11.43 mg/100 mg of extract, respectively **(Table 7)**. The LE from February and May showed the highest content of 1, with significantly equal values. The highest 2 content was found in February. The November LE displayed the lowest contents of 1 and 2. The peak 3 with UV λmax at 248 nm was preliminarily identified as a glycoside of glycyrrhetin **(Kitagawa *et al*., 1998)**. The UV spectra of peaks 4 and 5

(Figures 34C and 35C) were compatible to those reported in literature for glabridin and glabrene **(Simons *et al*., 2009)**. The peak 6 (UV λmax: 224, 278, 314 nm) was tentatively identified as a liquiritigenin glycoside by comparison of its UV–visible spectra with that of the corresponding available standard liquiritin as well as with literature data **(Zeng *et al*., 1990)**. The relative contents (area % HPLC) of 3, 4, 5 and 6 significantly varied across harvest times in the range of 0.88–11.38%, 1.86–10.03%, 1.80–18.40% and 5.53–16.31%, respectively **(Table 8)**. The lowest level of 3 was in the November LE and the maximum in the August sample. Compounds 4 and 5 had similar variation patterns over the harvest times, with the highest levels in November (10.03 % and 18.40%, respectively) and the lowest in February (1.86% and 1.80%, respectively). The highest relative content of 6 was detected in May, while the lowest was estimated in November.

The tentative identities of peaks 4 and 5, in the HPLC chromatogram **(Figure 33)**, were confirmed by ESI-MS after fractionation of LE. The extract of licorice (LE) from *Glycyrrhiza glabra* collected in August was used for the MS analysis. The 80% methanol extract of licorice was concentrated in vacuum to remove methanol. The resulting aqueous extract was partitioned with ethyl acetate and the resulting non-aqueous phase concentrated to dryness under reduced pressure. This residue was dissolved in water leading to the formation of precipitate, which was redissolved in methanol. This methanolic fraction was concentrated to dryness and extracted with chloroform. The chloroform fraction enriched in peaks 4 and 5 **(Figure 33E)**, was used for the ESI-MS analysis.

The ESI-MS of peak 4 indicated a molecular ion at *m/z* 325 in positive mode and at 323 *m/z* in negative mode. Its MS^2 fragmentation profile **(Figure 34A, B)** was found to be in accordance with the fragmentation of glabridin, which has a molecular mass of 324 Da **(Simons *et al*., 2009)**. The ESI-MS of peak 5 indicated a molecular ion at *m/z* 323 in positive mode and at 321 *m/z* in negative mode. The ESI-MS^2 spectrum of that ion **(Figure 35A, B)** showed similarity to the spectral profile of glabrene, which has a molecular mass of 322 Da **(Simons *et al*., 2009)**. This finding is also supported by the knowledge that glabridin and glebrene are regarded as species-specific components of *Glycyrrhiza glabra* roots **(Zhang *et* Ye, 2009)**. Therefore, the combination of accurate mass, MS/MS fragments and UV/vis absorbance spectrum indicates that the most likely identities of peaks 4 and 5 are glabridin and glabrene, respectively.

Glycyrrhizin has been reported to constitute 1–7% of hydroalcoholic extracts of licorice from different origins **(Kondo *et al*., 2007)**, whereas the liquiritigenin glycosides were shown to constitute 1.6% of licorice extract **(Kitagawa *et al*., 1998)**. The contents of glabridin and glabrene in licorice extracts were estimated at 11.6 % and 2.8%, respectively **(Vaya *et al*., 1997; Okada *et al*., 1989)**. In the present study, liquiritin and glycyrrhizin showed a similar variation pattern, which suggests that their biosynthesis are similarly regulated. The biosynthesis of 1 and 2 seems to be favored in the cooler harvest time (February), which corresponds to the vegetative stage of *Glycyrrhiza glabra,* prior to the pre-flowering period **(Chrtková, 1995)**. The variation pattern of the 2 content over harvest times was the opposite of that previously reported for the same compound in licorice cultivated in Turkey **(Hayashi *et al*., 1998)**, but in agreement with that of other saponins such as gingsenosides from roots of *Panax notoginseng* **(Dong *et al*., 2003)**.

Figure 33. HPLC chemical profile of licorice extract (LE) from *Glycyrrhiza glabra* harvested during the months of February to November (A-D). HPLC chemical profile of $CHCl_3$ fraction enriched in peaks 4 and 5 (E). Detection at 254 nm. Peaks: (1) liquiritin; (2) glycyrrhizin; (3) glycyrrhizin derivative; (4) glabridin; (5) glabrene and (6) liquiritigenin glycoside.

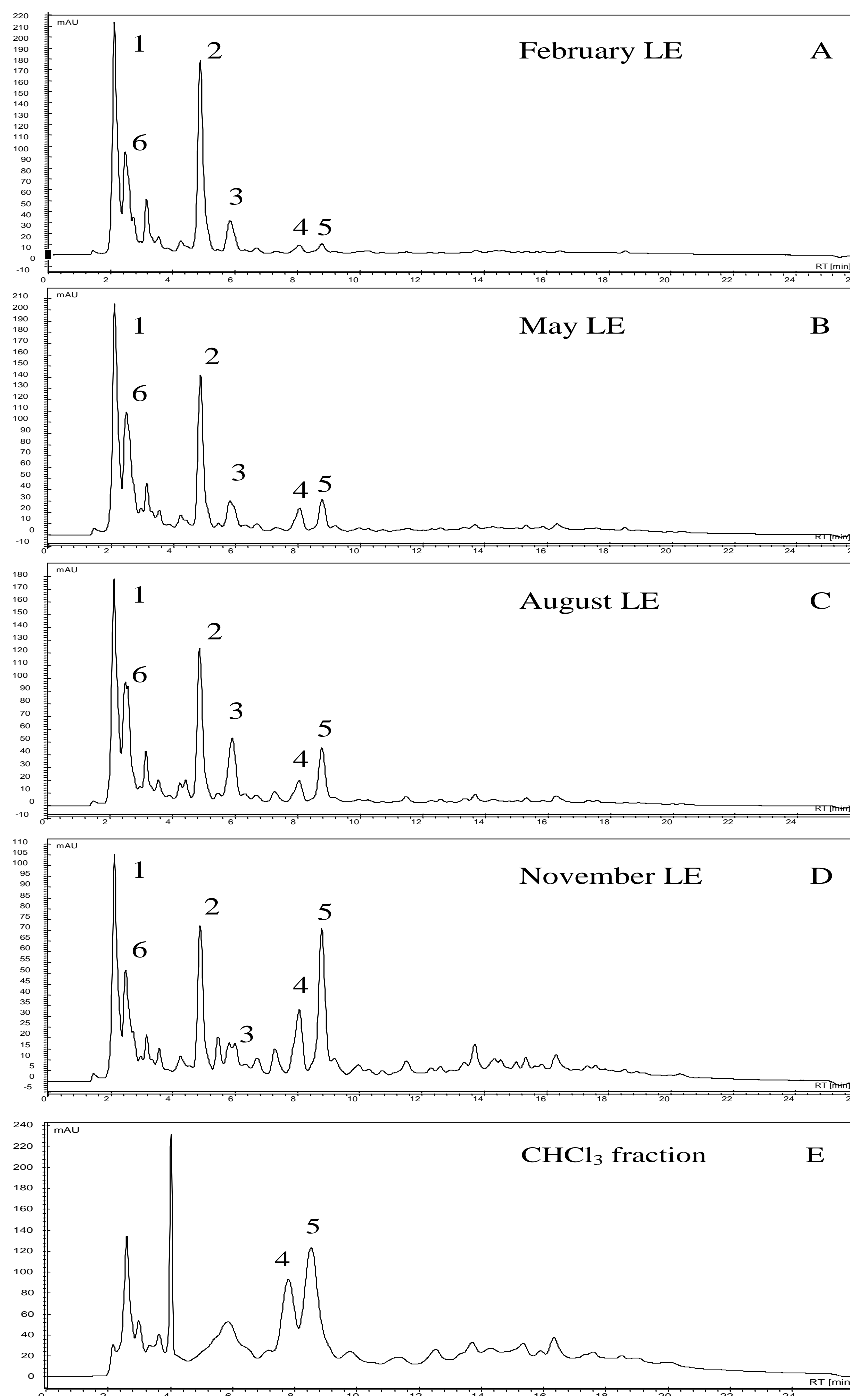


Figure 34. Positive-ion mode ESI-MS^2 spectrum (A), negative-ion mode ESI-MS^2 spectrum (B) and UV spectrum (C) of chromatographic peak 4. Tentative structure: Glabridin (D).

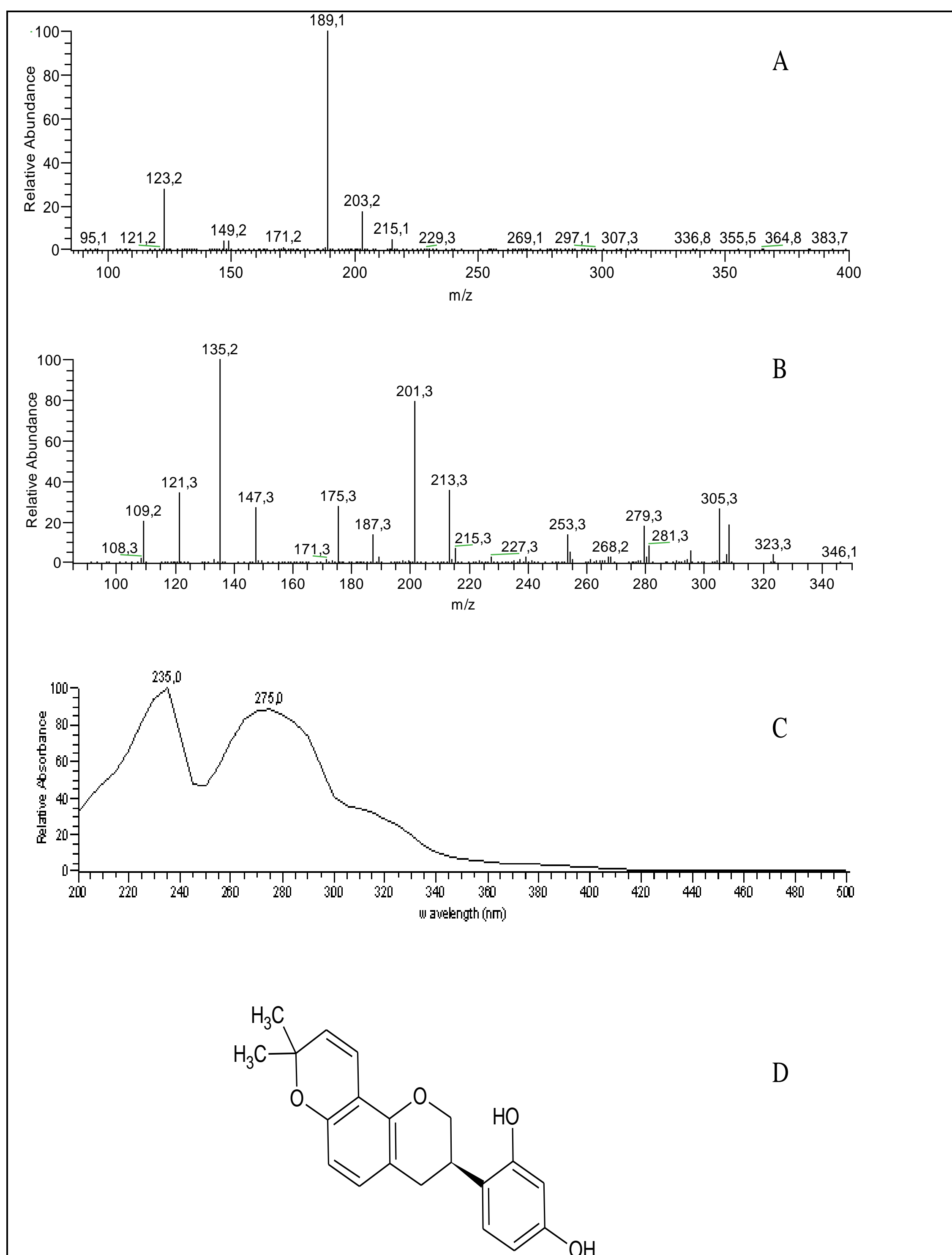
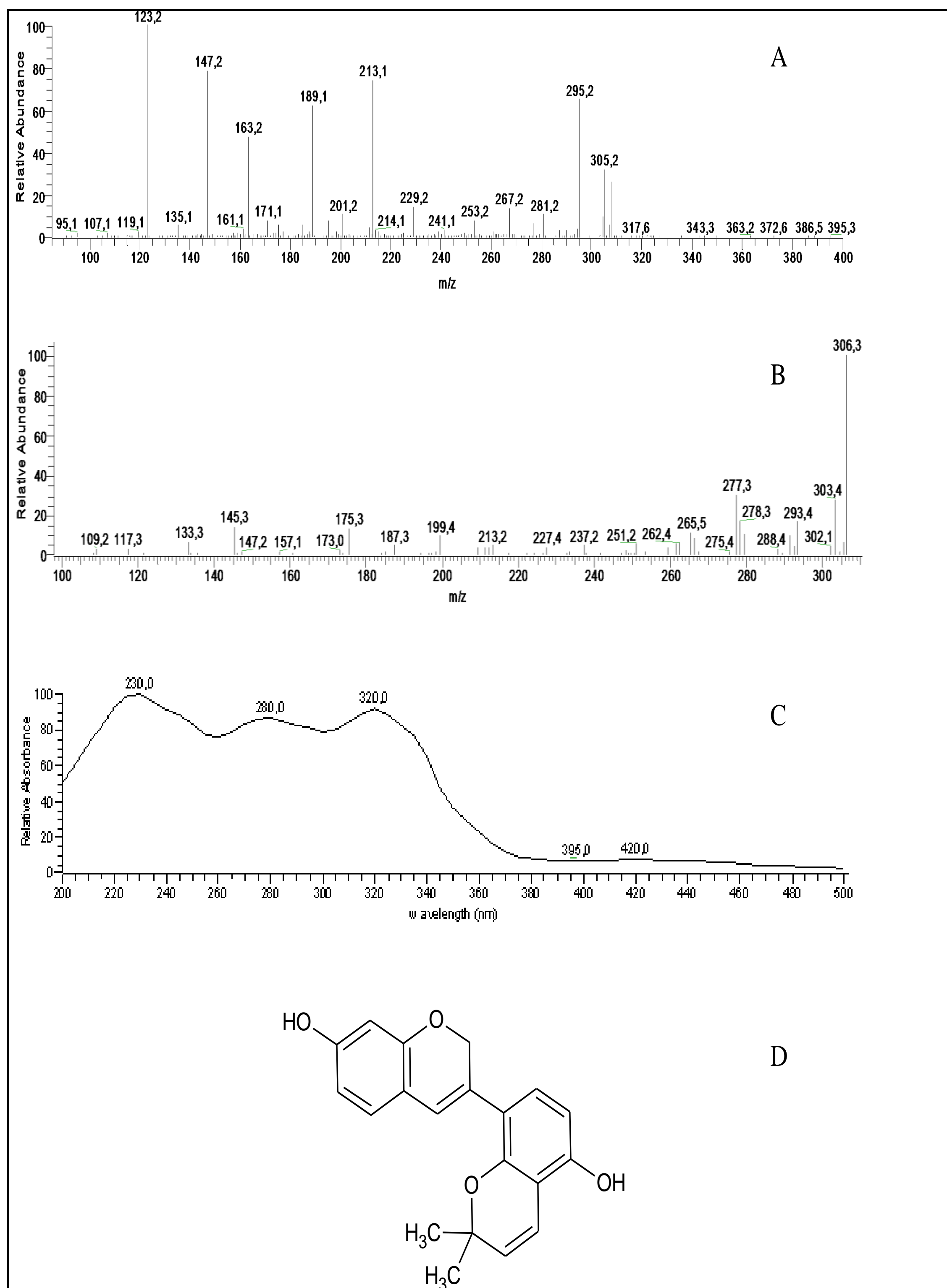


Figure 35. Positive-ion mode ESI-MS^2 spectrum (A), negative-ion mode ESI-MS^2 spectrum (B) and UV spectrum (C) of chromatographic peak 5. Tentative structure: Glabrene (D).



5.3.3. Total content of polyphenols

Polyphenols occur in all plant foods and contribute to the beneficial health effects of vegetables and fruit, due at least in part to their antioxidant properties **(Parr *et* Bolwell, 2000)**. As shown in the **Table 7,** the total contents of phenols (TP), flavones and flavonols (TF), and tannins (TT) in LE significantly varied within different harvest times, fluctuating in the range 7.20–10.79 mg GAE/100 mg of LE, 1.84–4.42 mg QE/100 mg of LE and 0.48–1.28 mg GAE/100 mg of LE, respectively. The highest content of TP, TF and TT were in November, August and May, respectively. All three parameters displayed their lowest values in February.

The variation pattern in the TP content of LE was in agreement with an earlier study of with *Solanum lycopersicum* fruits **(Toor *et al*., 2006)**. Our results could not confirm that phenolic compounds increase with increasing light intensity **(Slimestad *et al*., 2009)** because variations in the content of total polyphenolic of LE across harvest times did not match up with the fluctuations in the global and UV radiation recorded. The low contents of TP, TF and TT in February could be due to decreased active biosynthesis during cooler weather, as was previously remarked **(Yao *et al*., 2005)**. It was further observed that the TP variation pattern was the reverse of that exhibited by liquiritin, but similar to that of glabrene (5). It seems that the 4 and 5 contents quantitatively domine the dynamics in the TP content across the harvest times. The variation pattern in the TF and TT contents of LE were consistent with earlier investigations on some leafy vegetables **(Hertog *et al*., 1992)** and leaves of *Betula pubescens* **(Salminen *et al*., 2001)** respectively. Of particular interest, was the fact that the content of TF (flavones and flavonols) varied inversely respect to that of the flavanone liquiritin. Chalcone reductase

(CHR) is an enzyme that co-acts with chalcone synthase (CHS) to produce precursors of 5-deoxyflavonoids such as liquiritin, whereas CHS is involved in the biosynthesis of 5- hydroxyflavonoids such as flavones and flavonols **(Joung *et al.*, 2003)**. From the results, it is conceivable that enviromental factors associated with harvest times affects the biosynthesis of LE flavonoids by regulating selectively these enzymes.

Table 7. Contents of liquiritin, glycyrrhizin, and total polyphenols in licorice extract (LE) from *Glycyrrhiza glabra* at different harvest times.

Components	Months			
	February	May	August	November
TP	7.20 ± 0.05	8.84 ± 0.05	9.99 ± 0.07	10.79 ± 0.07
TF	1.84 ± 0.05	2.55 ± 0.01	4.42 ± 0.06	3.50 ± 0.06
TT	0.48 ± 0.02	1.28 ± 0.07	0.79 ± 0.01	0.98 ± 0.03
1	6.17 ± 0.22a	6.28 ± 0.21a	5.43 ± 0.15	2.87 ± 0.01
2	11.43 ± 0.25	8.89 ± 0.19	7.68 ± 0.09	4.18 ± 0.02

Contents of liquiritin and glycyrrhizin are expressed as mg/100 mg of LE. Total phenols (TP) content is expressed as mg gallic acid equivalents (GAE)/100 mg of LE. Total flavones and flavonols (TF) content is expressed as mg quercetin equivalents (QE)/100 mg of LE. Total tannins (TT) content is expressed as mg GAE/100 mg of LE. Values followed by the same letter in the same row are not significantly different (Tukey's test, $p < 0.05$, n=3 ± SD).

Table 8. Relative content (area % HPLC) of compounds 3, 4, 5 and 6 in licorice extract (LE) from *Glycyrrhiza glabra* at different harvest times.

Components	Months			
	February	May	August	November
3	7.12 ± 0.08	5.84 ± 0.01	11.38 ± 0.70	0.88 ± 0.02
4	1.86 ± 0.02	4.78 ± 0.01	4.26 ± 0.27	10.03 ± 0.06
5	1.80 ± 0.06	5.32 ± 0.01	9.02 ± 0.61	18.40 ± 0.05
6	10.14 ± 0.23	16.31 ± 0.35	12.82 ± 0.82	5.53 ± 0.10

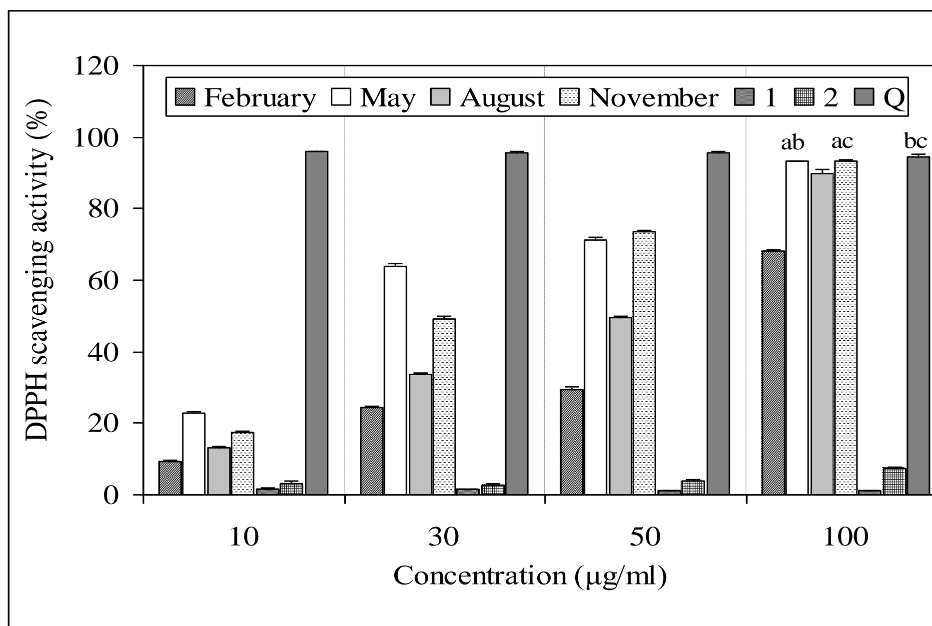
Values followed by the same letter in the same row are not significantly different (Tukey's test, $p < 0.05$, n=3 ± SD). Glycyrrhetic acid derivative (3), glabridin (4), glabrene (5) and liquiritigenin glycoside (6).

5.3.4. Free radical-scavenging activity

5.3.4.1. DPPH free radical-scavenging activity

The DPPH scavenging activity of LE varied significantly within harvest times (**Figure 36**) with the most favorable effect in May and November. LE scavenged DPPH in a concentration-dependent manner in the range of 10–100 µg/ml, which is in accordance with a prior study **(Di Mambro *et* Fonseca, 2005)**. Compounds 1 and 2 showed a negligible effect, which is consistent with an earlier study **(Kato *et al*., 2008)**. The variation pattern in the DPPH effect correlated significantly with changes in the contents of TP, TT, 4 and 5 **(Figure 41A, C, E, F)**. In a previous study, glabridin and glabrene scavenged 31% and 86% of the DPPH radical within 30 min of incubation, respectively **(Belinky *et al*., 1998b)**.

Figure 36. DPPH radical-scavenging activities of licorice extract (LE) from *G. glabra* at different harvest times, liquiritin (1), and glycyrrhizin (2). Quercetin (Q) was used as a reference compound. Bars showing the same letter in the same subfigure are not significantly different (Tukey's test, $p < 0.05$, n=3 ± SD).

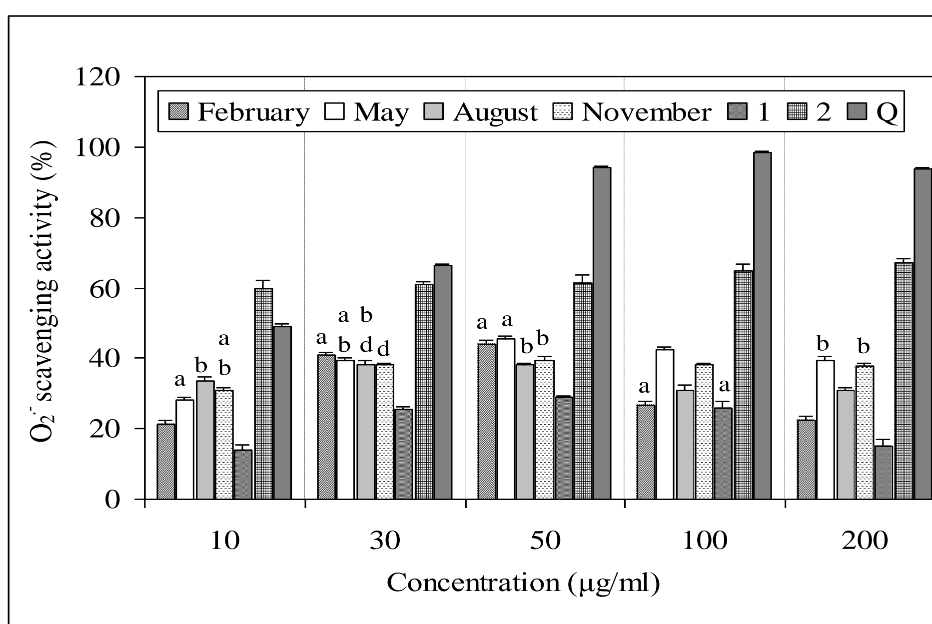


5.3.4.2. Superoxide anion radical-scavenging activity

The superoxide anion radical ($O_2^{\cdot-}$) may contribute to the pathogenesis of many diseases **(Ishii *et* Ishii, 2006)**. The effect of LE toward $O_2^{\cdot-}$ varied significantly across harvest times at a concentration range of 10–200 µg/ml **(Figure 37)**. However, no effect dependent on concentration was observed. It was not possible to determine the LE with the most favourable effect within harvest times, because different variation patterns were observed when different concentrations were used in the assay. As the LE (50 µg/ml) across harvest times showed the highest effect, this extract concentration was used in the correlation analysis. The effect of LE at 50 µg/ml partially correlated with changes in the content of 1 and 2 across harvest times **(Figure 41D)**. The appreciable

antiradical effect (about 63%) of glycyrrhizin (2) followed by a weak effect (14–26%) of liquiritin in the range of 10–100 μg/ml supported these observations.

Figure 37. Superoxide radical scavenging activities of licorice extract (LE) from *G. glabra* at different harvest times, liquiritin (1), and glycyrrhizin (2). Quercetin (Q) was used as a reference compound. Bars showing the same letter in the same subfigure are not significantly different (Tukey's test, $p < 0.05$, n=3 ± SD).



5.3.5. Antioxidant activity

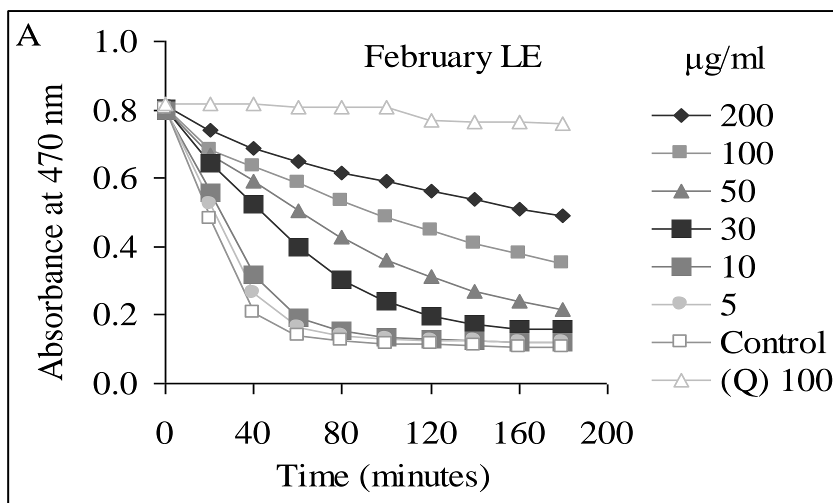
5.3.5.1. Inhibition of β-carotene-linoleate bleaching

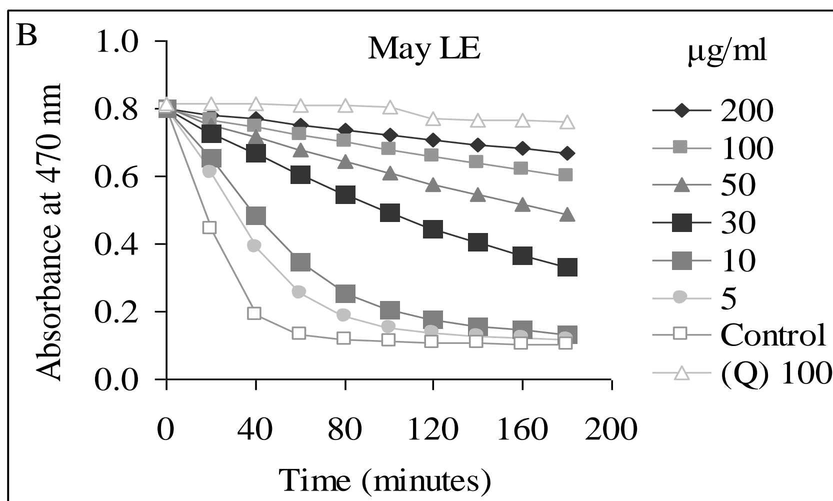
The antioxidant effects LE on the β-carotene consumption varied across harvest times **(Figure 38)**. The concentration-effect relationship for LE in the range of 10–200 μg/ml at different harvest times is shown in **Table 9**. In general, the highest antioxidant effect of LE in the concentration range of 10–200 μg/ml was in November (31–95%) and the

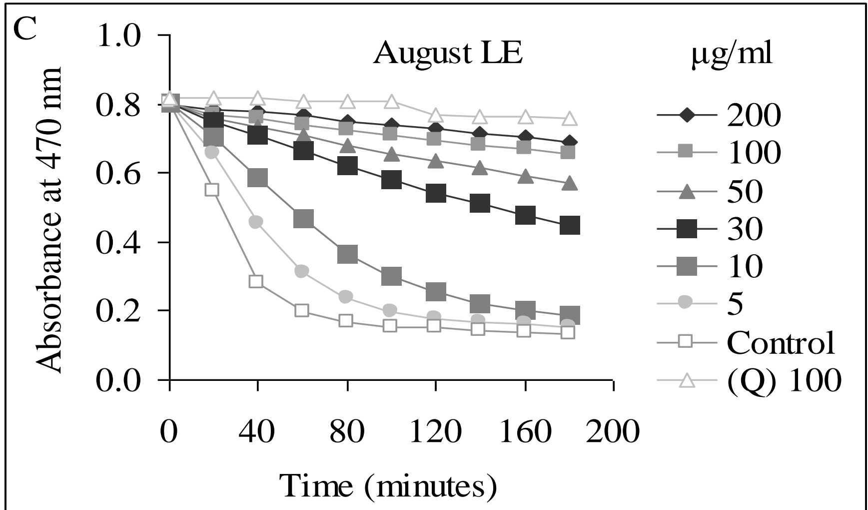
lowest was in February (7–83 %). Compounds 1 and 2 showed no antioxidant effect in this system **(Figure 38E)**. Both the data for oxidation rate ratio (R_{OR}) and activity coefficient (C_{AA}) support the antioxidant activity index of LE. The oxidation rate ratio bears an inverse relationship with the antioxidant activity index. The activity coefficient increases directly with the increase in value of the antioxidant activity index. Variations in the β-carotene bleaching inhibition of LE across harvest times significantly correlated with changes in the contents of 4 and 5 **(Figure 41E, F)**.

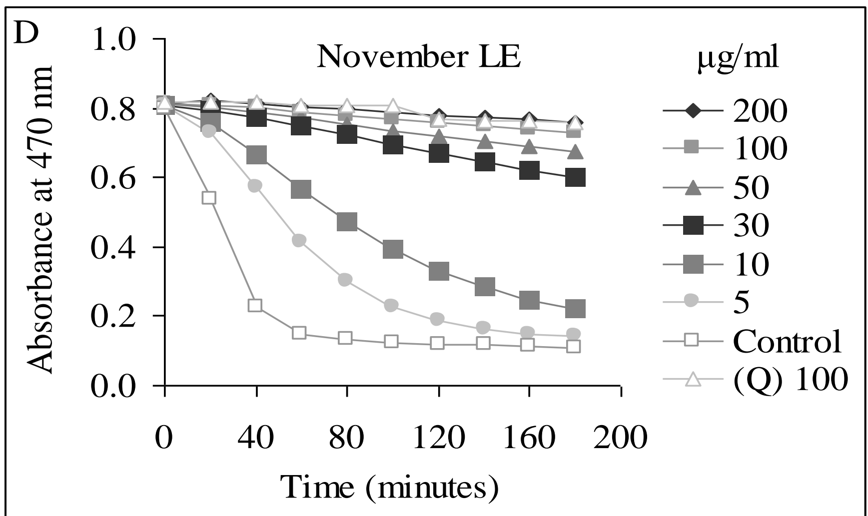
In another investigation, a licorice ethanolic extract and glabridin, its major constituent, inhibited β-carotene consumption by 87% and 93%, respectively **(Vaya *et al.*, 1997)**. In the same investigation glabridin was shown to bind to the LDL and subsequently protect it from oxidation **(Vaya *et al.*, 1997)**. Glabrene was reported to be more potent than glabridin as an inhibitor of LDL oxidation **(Belinky *et al.*, 1998b)**. It has been reported that the oxidation of LDL, that is thought to contribute to atherogenesis **(Witztum *et* Steinberg, 1991)**, starts after the depletion of its endogenous lipophilic antioxidants, such as β-carotene and vitamin E **(Belinky *et al.*, 1998a)**. The November LE may prove to be of potential health benefit by protecting LDL-associated carotenoids.

Figure 38. Effects of licorice extract (LE) from *G. glabra* at different harvest times (A-D), liquiritin (1) and glycyrrhizin (2) (E) on the β-carotene consumption at different incubation times. The β-carotene-linoleic acid emulsion was incubated at 50 ºC in the absence (control), or presence of LE at different final concentrations. The graphs are representative of three separate experiments. Quercetin (Q) was used as a reference compound at 100 μg/ml.






C
August LE
µg/ml
Absorbance at 470 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
40
80
120
160
200
Time (minutes)
200
100
50
30
10
5
Control
(Q) 100


D
November LE
µg/ml
Absorbance at 470 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
40
80
120
160
200
Time (minutes)
200
100
50
30
10
5
Control
(Q) 100

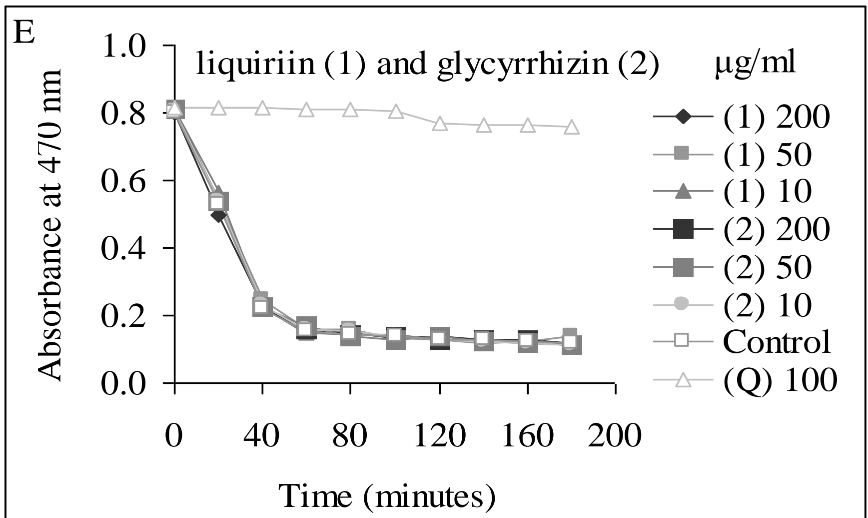

E
liquiriin (1) and glycyrrhizin (2)
µg/ml
Absorbance at 470 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
40
80
120
160
200
Time (minutes)
(1) 200
(1) 50
(1) 10
(2) 200
(2) 50
(2) 10
Control
(Q) 100

Table 9. Concentration (μg/ml) response of antioxidant activity for licorice extract (LE) from *Glycyrrhiza glabra* at different harvest times, by the β-carotene-linoleate bleaching method (at 180 min.).

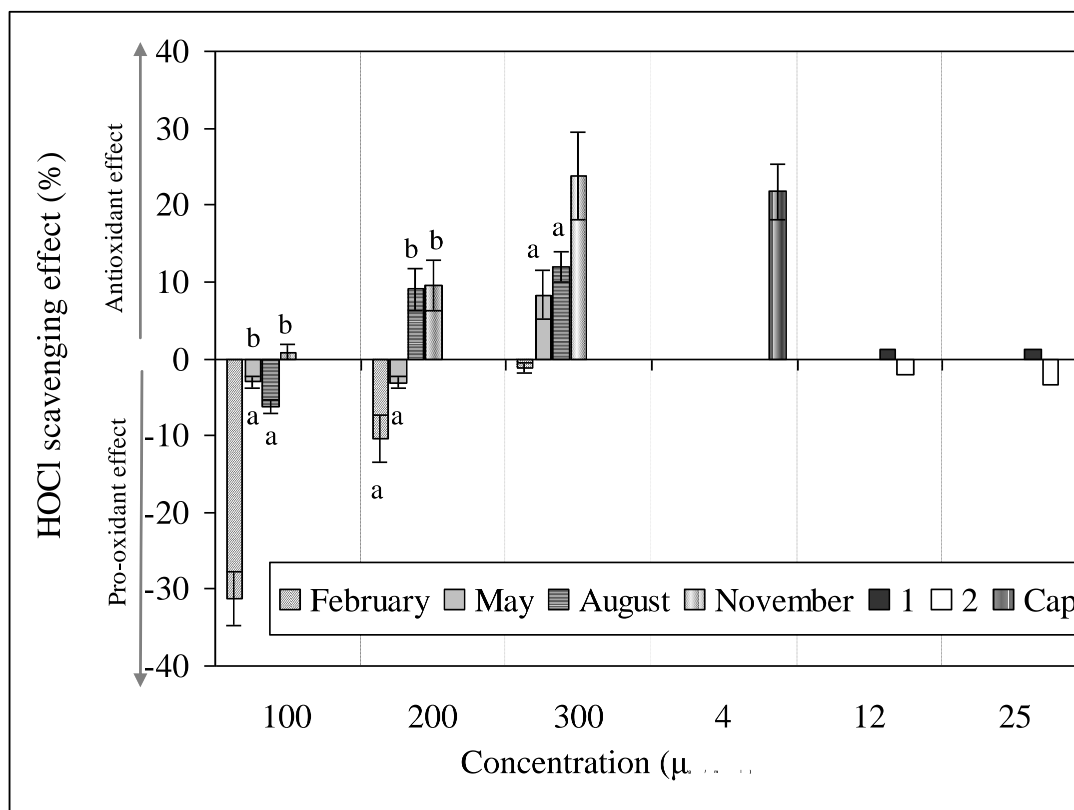
Parameters of antioxidant activity	Samples	Concentration (μg/ml)		
		200	50	10
A_A	February	82.87 ± 2.63	40.99 ± 4.19	7.31 ± 0.47
	May	92.23 ± 0.34ab	78.72 ± 1.77a	20.63 ± 1.76a
	August	91.43 ± 0.30ac	78.05 ± 3.78a	17.75 ± 0.77a
	November	94.52 ± 1.20bc	88.80 ± 2.17b	31.23 ± 3.72
	Q	99.46 ± 0.39	93.60 ± 0.71b	75.92 ± 0.70
R_{OR}	February	0.18 ± 0.03	0.57 ± 0.06	0.93 ± 0.01
	May	0.08 ± 0.01ab	0.22 ± 0.02a	0.80 ± 0.02a
	August	0.09 ± 0.01ac	0.22 ± 0.04a	0.82 ± 0.01a
	November	0.06 ± 0.02bc	0.11 ± 0.02b	0.69 ± 0.03
	Q	0.01 ± 0.00	0.06 ± 0.01b	0.22 ± 0.01
C_{AA}	February	695.53 ± 38.53	229.37 ± 43.36	27.90 ± 0.60
	May	901.32 ± 23.43a	628.63 ± 33.58a	84.00 ± 12.19a
	August	865.56 ± 9.04a	625.81 ± 59.14a	75.54 ± 7.54a
	November	963.19 ± 16.81b	814.58 ± 10.06b	156.28 ± 6.04
	Q	1005.62 ± 10.61b	876.72 ± 11.72b	571.55 ± 7.55

Values followed by the same letter in the same column for the same antioxidant activity parameter are not significantly different (Tukey's test, $p < 0.05$, n=3 ± SD). A_A, antioxidant activity index; R_{OR}, oxidation rate ratio; C_{AA}, antioxidant activity coefficient; Q, Quercetin.

5.3.5.2. Hypochlorous acid-scavenging assay

The myeloperoxidase (MPO)-derived oxidant hypochlorous acid (HOCl) plays a role in tissue injury under inflammatory conditions **(Zhang *et al*., 2002)**. In the present investigation, the HOCl scavenging effect of LE in the range 100–300 µg/ml significantly varied across harvest times with the highest effect being in November and the lowest in February **(Figure 39)**. Surprisingly, the May, August and November samples exhibited a bimodal effect on HOCl. The samples were antioxidant at higher concentrations, whereas they were oxidant at lower concentrations. Compounds 1 and 2 exhibited no meaningful activity against HOCl. Variations in the HOCl scavenging effects of LE across harvest times correlated significantly with changes in the contents of TP, TF, 4 and 5 **(Figure 41A, B, E, F)**. Our results suggest that the traditional use of licorice against inflammatory processes could be favored when harvesting is in November.

Figure 39. Hypochlorous acid (HOCl) scavenging activity licorice extract (LE) from *G. glabra* at different harvest times, liquiritin (1), and glycyrrhizin (2). Captopril (Cap) was used as a reference compound. Bars showing the same letter in the same subfigure are not significantly different (Tukey's test, $p < 0.05$, n=3 ± SD).



5.3.5.3. Inhibition of myeloperoxidase-chlorinating system

Myeloperoxidase (MPO) is an enzyme linked to both inflammation and oxidative stress by its location in leukocytes and its role in catalyzing the formation of the highly chlorinating and oxidizing agent HOCl (**Schindhelm *et al.*, 2009**). The effect of LE on the MPO-chlorinating activity varied significantly at different harvest times (**Figure 40**). In general, the May, August and November samples displayed a bimodal effect: they showed an inhibiting effect at higher concentrations and a stimulating effect (pro-

oxidant effect) at lower concentrations. The February sample at 100–300 µg/ml showed a stimulating effect. Compounds 1 and 2 also exhibited a pro-oxidant effect at 12 and 25 µg/ml, **(Figure 40)**. It was further observed that variations in the effect of LE on the MPO system across harvest times significantly correlated with changes in the contents of TP, TF and TT, 4 and 5 **(Figure 41A, B, C, E, F)**. In a prior investigation, three herbal constituents of STW5 (Iberogast®) such as *Carum carvi*, *Mentha piperita,* and *Chelidonium majus,* showed a pro-oxidant effect in the MPO-catalyzed chlorination system using the ACC-ethene model. Conversely, the ethanolic extract of *G. glabra*, another STW5 constituent, showed an antioxidant effect **(Schempp *et al.*, 2006)**. The current investigation suggests that the traditional use of licorice against inflammatory processes related with MPO could be favored when harvesting is in November.

Figure 40. Effect of *G. glabra* at different harvest times, liquiritin (1), and glycyrrhizin (2) on the myeloperoxidase (MPO)-chlorinating system. Flufenamic acid (FA) was used as a reference compound. Bars showing the same letter in the same subfigure are not significantly different (Tukey's test, $p < 0.05$, n=3 ± SD).

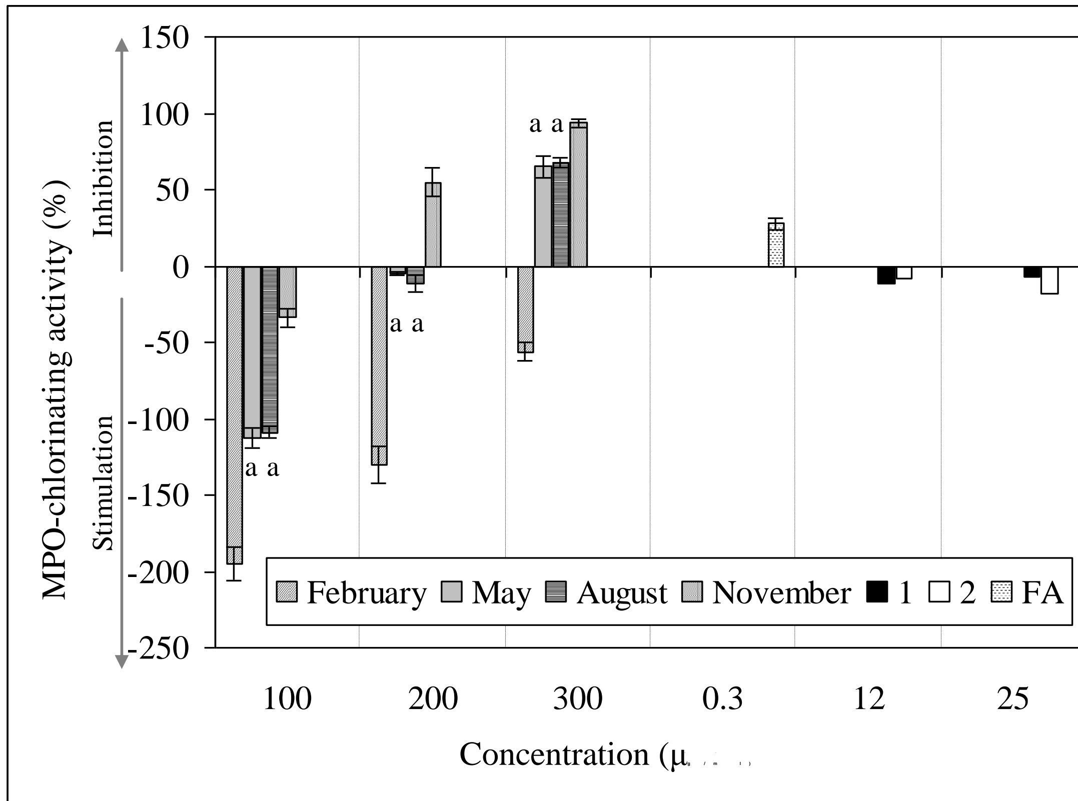
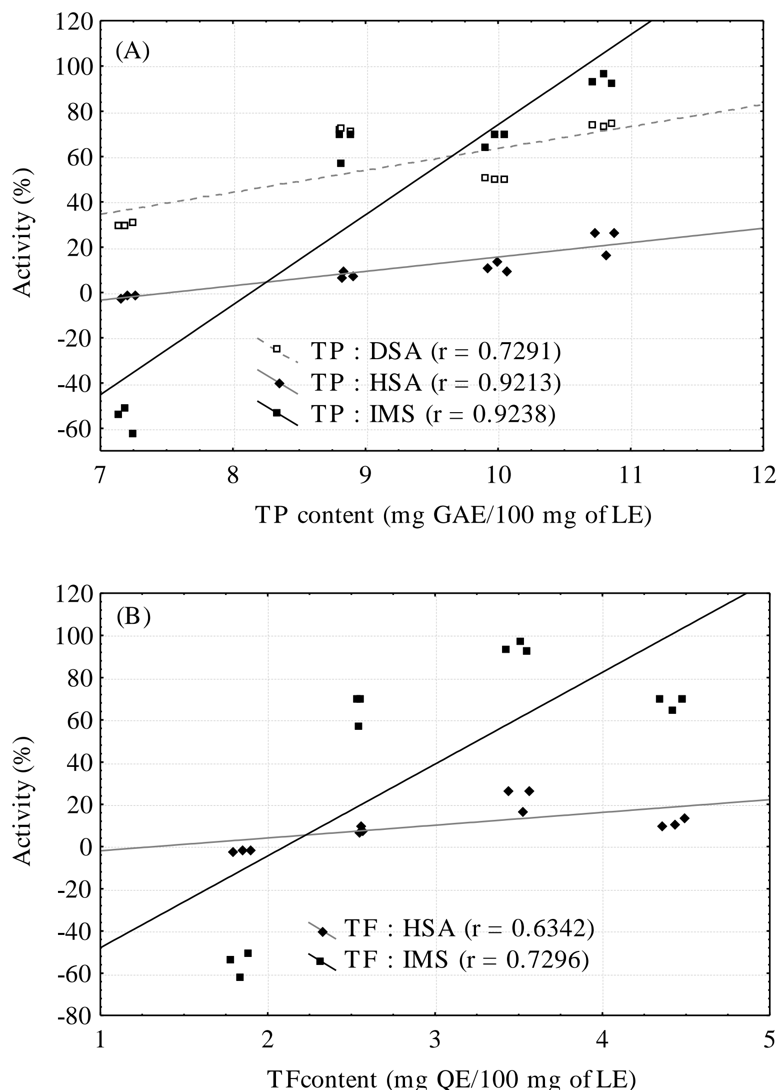
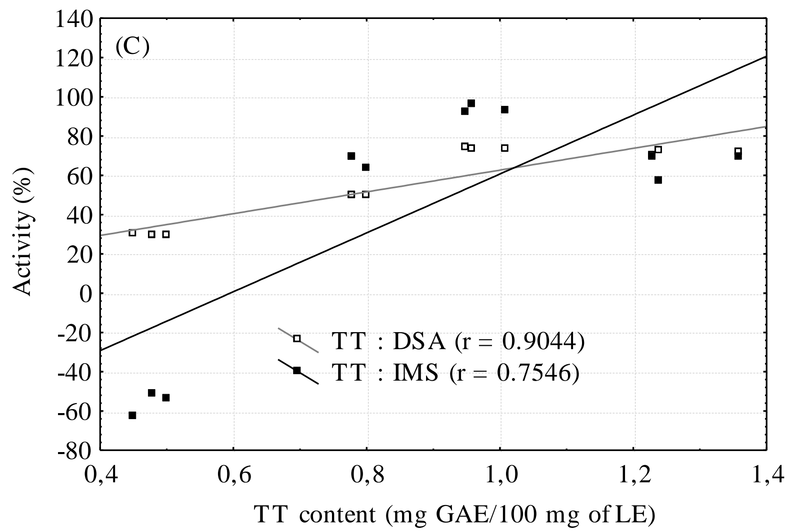
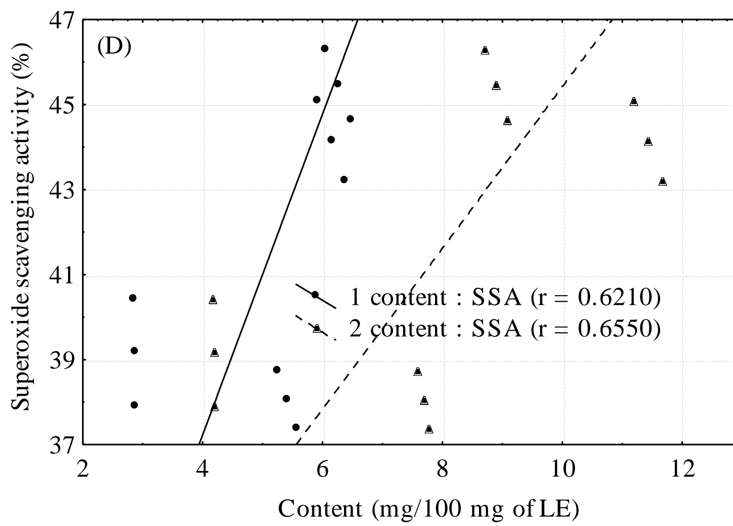
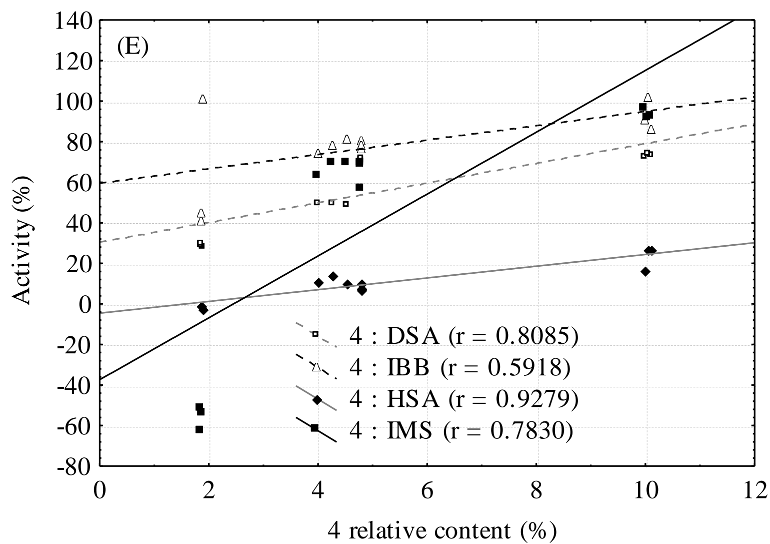


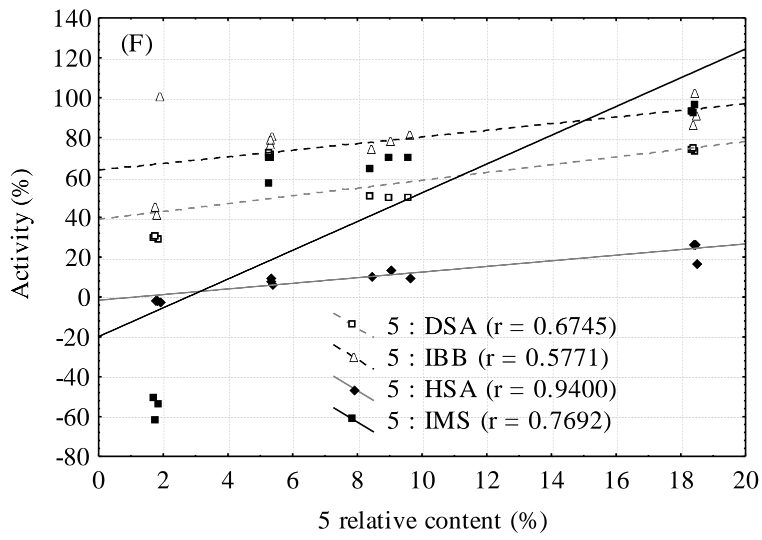
Figure 41. Correlation analysis between the chemical content and the biological activities of LE from *G. glabra* at different harvest times. TP, total phenolics; TF, total flavonoids; TT, total tannins; DSA, DPPH free radical scavenging activity; SSA, superoxide anion radical scavenging activity; IBB, inhibition of β-carotene-linoleate bleaching; HSA, hypochlorous acid scavenging activity; IMS, Inhibition of myeloperoxidase-chlorinating system; (1) liquiritin; (2) glycyrrhizin; (4) glabridin and (5) glabrene. The LE at 50 µg/ml was used for the correlation analysis with DSA, SSA and IBB, and LE at 300 µg/ml was used for the correlation analysis with HAS and IMS. Pearson's correlation coefficient (r), $p < 0.05$.




(C)
TT : DSA (r = 0.9044)
TT : IMS (r = 0.7546)
Activity (%)
TT content (mg GAE/100 mg of LE)


(D)
1 content : SSA (r = 0.6210)
2 content : SSA (r = 0.6550)
Superoxide scavenging activity (%)
Content (mg/100 mg of LE)


(E)
4 : DSA (r = 0.8085)
4 : IBB (r = 0.5918)
4 : HSA (r = 0.9279)
4 : IMS (r = 0.7830)
Activity (%)
4 relative content (%)


(F)
Activity (%)
5 relative content (%)
5 : DSA (r = 0.6745)
5 : IBB (r = 0.5771)
5 : HSA (r = 0.9400)
5 : IMS (r = 0.7692)
140
120
100
80
60
40
20
0
-20
-40
-60
-80
0
2
4
6
8
10
12
14
16
18
20

6. CONCLUSIONS

6.1. Phenolic content profiles and free radical-scavenging activity of roots of *Glycyrrhiza* species

The free radical scavenging activity of the extracts from *G. glabra*, *G. uralensis*, *G. echinata,* and *G. pallidiflora* roots was evaluated and could be related with phenolic groups. Although roots of *G. echinata* and *G. pallidiflora* have not been described as sources of the reputed licorice; nevertheless, they were found to represent valuable sources of superoxide radical scavengers. Tannins and flavonoids were observed to be the major contributors to the free radical scavenging activity of *Glycyrrhiza* extracts. Therefore, flavonoid, tannins and related metabolites may be a key parameter for food science and technology as well as nutritional studies of these plants. The results gained from these assays provide valuable data that make it possible to classify *Glycyrrhiza* species extracts with respect to their free radical scavenging potential and polyphenolic contents. This knowledge could be of interest for consumers to estimate the value of each *Glycyrrhiza* species.

6.2. Free radical-scavenging, antioxidant and immunostimulating effects of licorice infusion (*Glycyrrhiza glabra* L.)

The present *in vitro* study shows that licorice infusion (LI) has antioxidant, pro-oxidant, free radical-scavenging and immunostimulating activities, and its major constituents seem not to be involved in most of these bioactivities. This herbal preparation may have great relevance in the prevention of and therapies for diseases in which oxidants or free

radicals are implicated, and it might be a valuable alternative for mounting an effective non-specific immune response at the onset of viral, bacterial and parasitic infections. Further studies are needed to ascertain the *in vivo* effect of LI and to identify active constituents.

6.3. Variations in the chemical profile, free radical-scavenging and antioxidant activities of licorice (*Glycyrrhiza glabra* L.) as influenced by harvest time.

The content of individual components and grouped polyphenols of hydroalcoholic extract of licorice (LE) varied at different harvest times, and these changes were partially correlated with fluctuations in the antioxidant and free radical-scavenging activities. These variations might be attributed to seasonal changes in temperature, humidity and light, as well as different stage of plant metabolism. In general, the LE from May and November showed the most favourable antiradical and antioxidant effects. Glabridin and glabrene appeared to significantly contribute to the antioxidant activity of LE. It is noteworthy that the total content of polyphenols could only represent a general pattern in LE and do not precisely reveal the true patterns of individual phenols over harvest times. Variations of the content of individual compounds could be a better predictor of the variations in the bioactivities of licorice. The data emerging from the present study represents an approach to determine the best time for harvesting licorice with optimal chemical and pharmacological properties. Further studies are also recommended to perform greenhouse experiments at controlled growing conditions in order to compare the influence of light, temperature, and precipitation (rainfall).

7. ABSTRACT

7.1. Phenolic content profiles and free radical-scavenging activity of roots of *Glycyrrhiza* species

In this study, the free radical-scavenging activity of 80% methanolic extracts from *G. glabra*, *G. uralensis*, *G. echinata,* and *G. pallidiflora* roots was assessed using DPPH free radical and superoxide anion radical scavenging assays. The total contents of phenolics (TP), flavonoids (TF), and tannins (TT) were also measured. From the results, *G. glabra* showed the highest DPPH radical scavenging activity (DRSA), whereas *G. echinata* exhibited the lowest values of DRSA. Extracts from *G. uralensis*, *G. echinata,* and *G. pallidiflora* showed the highest superoxide radical scavenging activity (SRSA). The highest TP content was found out in *G. uralensis* and *G. echinata* (4.71 and 4.70 mg gallic acid equivalents/100 mg of extract, respectively). The highest TF content (1.84 mg quercetin equivalents (QE)/100 mg of extract) was observed in *G. glabra*, whereas the lowest value (0.53 mg QE/100 mg of extract) was measured in *G. pallidiflora.* Extracts from *G. uralensis* and *G. pallidiflora* showed the highest TT content. There were positive and significant correlations between TP and SRSA (r = 0.6881, $p < 0.05$) and between TT and SRSA (r = 0.7754, $p < 0.05$). The TF content was significantly correlated only with DRSA (r = 0.8804, $p < 0.05$). These results indicated that flavonoids and tannins were the major contributors to the free radical-scavenging capacity of these plants. Therefore, these phenolic groups could be important factors when determining the antiradical capacity of *Glycyrrhiza* spp. roots.

7.2. Free radical-scavenging, antioxidant and immunostimulating effects of licorice infusion (*Glycyrrhiza glabra* L.)

To contribute to the understanding of the mechanisms underlying the beneficial effects of licorice, the antioxidant, free radical-scavenging and immunostimulating effects of a licorice infusion (LI) were investigated, and its chemical profile was determined. From the results, two major components of LI were identified as (1) liquiritin and (2) glycyrrhizin. LI weakly scavenged DPPH and compounds 1 and 2 showed negligible effects. Both LI and 2 substantially scavenged superoxide radicals. The β-carotene bleaching was inhibited by LI, but compounds 1 and 2 showed no effect. The LI, 1, and 2 exhibited no meaningful activities against HOCl, and they showed pro-oxidant effects in the MPO-chlorinating system. Granulocytes and NK cells were markedly activated by LI, whereas 1 and 2 were inactive. The LI, 1, and 2 showed no effects on the lymphocyte cell cycle. These results support, in part, the traditional use of licorice to treat and prevent diseases in which oxidants or free radicals are implicated and suggest that LI could be used as a potential non-specific immune stimulator.

7.3. Variations in the chemical profile, free radical-scavenging and antioxidant activities of licorice (*Glycyrrhiza glabra* L.), as influenced by harvest time

This study investigated the variations in the chemical profile, free radical-scavenging and antioxidant activities of licorice extracts (LE) from plants harvested from February to November of 2008. Possible correlations between biological properties and the chemical composition of LE were also studied. From the results, the total contents of phenols, flavonoids and tannins in LE varied at different harvest times in the range of 7.20–10.79 mg GAE/100 mg of LE, 1.84–4.42 mg QE/100 mg of LE and 0.48–1.28 mg GAE/100 mg of LE, respectively. Liquiritin and glycyrrhizin, the major components of LE, varied in the range of 2.87–6.28 mg/100 mg of LE and 4.18–11.43 mg/ 100 mg of LE, respectively. The relative content of the other constituents, which were identified as a glycyrrhizin derivative (3), glabridin (4), glabrene (5) and a derivative of liquiritigenin (6), varied in the range of 0.88–11.38 %, 1.86–10.03 %, 1.80–18.40% and 5.53–16.31 %, respectively. Fluctuations in the chemical content correlated positively with the changes in the antioxidant and free radical scavenging activities of LE. In general, the best antiradical and antioxidant effects were observed in LE from plants harvested in May and November. The chemical profile of licorice quantitatively varied at different harvest times and these changes determined changes in the bioactivities. This data could pave the way to optimize harvesting protocols for licorice in relation with its health-promoting properties.

8. ABSTRAKT

8.1. Profily fenolického obsahu a antivolnoradikálová aktivita kořenů druhů *Glycyrrhiza*

V této studii byla určena antivolnoradikálová aktivita 80% metanolových extraktů kořenů z rostlin *G. glabra*, *G. uralensis*, *G. echinata* a *G. pallidiflora* za použití DPPH antivolnoradikálové a antisuperoxidové anionové zkoušky. Celkový obsah fenolů (TP), flavonoidů (TF) a taninů (TT) byl také změřen. Z výsledků je zřejmé, že *G. glabra* prokázala nejvyšší DPPH antivolnoradikálovou aktivitu (DRSA), zatímco *G. echinata* prokázala nejnižší hodnoty DRSA. Extrakty z *G. uralensis, G. echinata* a *G. pallidiflora* ukázaly nejvyšší superoxidovou antivolnoradikálovou aktivitu (SRSA). Nejvyšší TP obsah byl nalezen v *G. uralensis* a *G.echinata* (4.71 a 4.70 mg ekvivalentů kyseliny galové /100 mg extraktu, respektive). Nejvyšší obsah TF (1.84 mg kercetinových ekvivalentů (QE)/100 mg extraktu) byl pozorován v rostlině *G. glabra*, zatímco nejnižší hodnota (0.53 mg QE/100 mg extraktu) byla naměřena v rostlině *G. pallidiflora*. Extrakty z *G. uralensis* a *G. pallidiflora* prokázaly nejvyšší obsah TT. Byly zde pozitivní a významné korelace mezi TP a SRSA ($r = 0.6881$, $p < 0.05$) a mezi TT a SRSA ($r = 0.7754$, $p < 0.05$). Obsah TF byl významně korelován pouze s DRSA ($r = 0.8804$, $p < 0.05$). Tyto výsledky ukazují, že flavonoidy a taniny byly hlavními účastníky na antivolnoradikálové kapacitě těchto rostlin. Z tohoto důvodu, fenolické skupiny by mohly být důležitými faktory při určování antiradikálové kapacity kořenů druhu *Glycyrrhiza*.

8.2. Antivolnoradikálové, antioxidační a imunostimulační účinky nálevu z lékořice (*Glycyrrhiza glabra* L.)

Abychom porozuměli mechanizmům spočívajícím v blahodárných účincích lékořice, byly zkoumány antivolnoradikálové a imunostimulační účinky nálevu z lékořice (LI) a byl stanoven jeho chemický profil. Z výsledků je patrno, že hlavními komponenty LI byly identifikovány (1) liquiritin a (2) glycyrrhizin. LI slabě učinkoval proti DPPH a složky 1 a 2 prokázaly zanedbatelné účinky. Oba LI a 2 prokázaly značnou aktivitu proti superoxidovým radikálům. Bělení β-karotenu bylo potlačeno za pomoci LI, ale složky 1 a 2 neprokázaly žádný účinek. LI, 1 a 2 neprokázaly žádnou významnou aktivitu proti HOCL a prokázaly pro-oxidační účinky v MPO-chlórovacím systému. Granulocyty a NK buňky byly výrazně aktivovány za použití LI, zatímco 1 a 2 byly neaktivní. LI, 1 a 2 neprokázaly účinky na buněčný cyklus lymfocytů. Tyto výsledky částečně podporují tradiční používání lékořice k léčení a prevenci chorob, při kterých jsou obsaženy oxidanty a volné radikály a dávají podnět k tomu, že LI by mohl být použit jako potencionální nespecifický imunitní stimulátor.

8.3. Variace v chemickém profilu, antivolnoradikálové a antioxidační aktivity lékořice (*Glycyrrhiza glabra* L.), ovlivněné dobou sklizně

Tato studie zkoumala variace v chemickém profilu, antivolnoradikálové a antioxidační aktivity extraktů lékořice (LE) z rostlin sklizených od února do listopadu 2008. Možné korelace mezi biologickými vlastnostmi a chemickým složením LE byly také studovány. Z výsledků vyplývá, že celkový obsah fenolů, flavonoidů a taninů v LE se liší v různých dobách sklizně v rozsahu 7.20–10.79 mg GAE/100 mg LE, 1.84–4.42 mg QE/100 mg LE a 0.48–1.28 mg GAE/100 mg LE, respektive. Liquiritin a glycyrrhizin, hlavní komponenty LE, se lišily v rozsahu 2.87–6.28 mg/100 mg LE a 4.18–11.43 mg/ 100 mg LE, respektive. Relativní obsah dalších složek, které byly identifikovány jako deriváty glycyrrhizinu (3), glabridin (4), glabrene (5) a deriváty liquiritigeninu (6), se lišily v rozsahu 0.88–11.38 %, 1.86–10.03 %, 1.80–18.40% a 5.53–16.31%, respektive. Fluktuace v chemickém obsahu pozitivně korelovaly se změnami v antioxidačních a antivolnoradikálových aktivitách v LE. Obecně řečeno, nejlepší antiradikálové a antioxidační účinky byly zpozorovány v LE z rostlin sklizených v květnu a listopadu. Chemický profil lékořice se kvantitativně lišil v různých dobách sklizně a tyto změny určovaly změny v bioaktivitách. Tato data mohou vést k optimalizaci protokolů sklizně lékořice v souvislosti s jejími zdraví podporujícími vlastnostmi.

9. LIST OF ABBREVIATIONS

AI, activation index

APC, allophycocyanin

ACC, 1-aminocyclopropane-1-carboxylic acid

CHR, chalcone reductase

CHS, chalcone synthase

DTNB, dithiobisnitrobenzoic acid

DAD, diode-array detector

CD, cluster of differentiation

DMAPP, dimethylallyl diphosphate

ET, electron transfer

ESI-MS, electrospray ionization mass spectrometry

FSC, forward scatter

FITC, fluorescein isothiocyanate

HAT, hydrogen atom transfer

HPLC, high-pressure liquid chromatography

IPP, isopentenyl pyrophosphate

iNOS, inducible nitric oxide synthase

LPS, lipopolysaccharide

LDL, low-density lipoprotein

MS, mass spectrometry

MPO, myeloperoxidase

MIC, minimum inhibitory concentration

MFI, mean fluorescence intensity

PGE2, prostaglandin E2

PE, phycoerythrin

SSC, side scatter

THC, tetrahydroxychalcone

TNB, 2-nitro-5-thiobenzoate

UV, ultraviolet

XOD, xanthine oxidase

10. REFERENCES

Adams RP. (1987).Yields and seasonal variation of phytochemicals from *Juniperus* species of the United States. Biomass, 12, 129–139.

Afreen F, Zobayed SMA, Kozai T. (2005). Spectral quality and UV-B stress stimulate glycyrrhizin concentration of *Glycyrrhiza uralensis* in hydroponic and pot system. Plant Physiology and Biochemistry, 43, 1074–1081.

Aly AM, Al-Alousi L, Salem HA. (2005). Licorice: A possible anti-inflammatory and anti-ulcer drug. AAPS PharmSciTech, 6, E74–82.

Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA. (2004). Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chemistry, 84, 551–562.

Amić D, Davidović-Amić D, Bešlo D, Trinajstić N. (2003). Structure-Radical scavenging activity relationships of flavonoids. Croatica Chemica Acta, 76, 55–61.

Ammon HP, Kelber O, Okpanyi SN. (2006). Spasmolytic and tonic effect of Iberogast® (STW 5) in intestinal smooth muscle. Phytomedicine, 13, 67–74.

Ayabe SI, Uchiyama H, Aoki T, Akashi T. (2010). Plant Phenolics: Phenylpropanoids. Comprehensive Natural Products II, 929–976.

Bafna PA, Balaraman R. (2005). Anti-ulcer and anti-oxidant activity of Pepticare, a herbomineral formulation. Phytomedicine, 12, 264–270.

Belinky PA, Aviram M, Fuhrman B, Rosenblat M, Vaya J. (1998a). The antioxidative effects of the isoflavan glabridin on endogenous constituents of LDL during its oxidation. Atherosclerosis, 137, 49–61.

Belinky PA, Aviram M, Mahmood S, Vaya J. (1998b). Structural aspects of the inhibitory effect of glabridin on LDL oxidation. Free Radical Biology and Medicine, 24, 1419–1429.

Bennett A, Clark-Wibberley T, Stamford IF, Wright JE. (1980). Aspirin- induced gastric mucosal damage in rats: cimetidine and deglycyrrhizinated liquorice together give greater protection than low doses of either drug alone. Journal of Pharmacy and Pharmacology, 32, 151.

Bin-Hafeez B, Haque R, Parvez S, Pandey S, Sayeed I, Raisuddin S. (2003). Immunomodulatory effects of fenugreek (*Trigonella foenum* graecum L.) extract in mice. International Immunopharmacology, 3, 257–265.

Borges O, Borchard G, de Sousa A, Junginger HE, Cordeiro-da-Silva A. (2007). Induction of lymphocytes activated marker CD69 following exposure to chitosan and alginate biopolymers. International Journal of Pharmaceutics, 337, 254–264.

Bruneton J. (1995). Pharmacognosy, Phytochemistry, Medicinal Plants. Lavoisier Publishing INC, USA.

Brush J, Mendenhall E, Guggenheim A, Chan T, Connelly E, Soumyanath A, Buresh R, Barrett R, Zwickey H. (2006). The effect of *Echinacea purpurea*, *Astragalus membranaceus* and *Glycyrrhiza glabra* on CD69 expression and immune cell activation in humans. Phytotherapy Research, 20, 687–695.

Chang CC, Yang MY, Wen HM, Chern JC. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food and Drug Analysis, 10, 178–182.

Chung KT, Wong TY, Wei CI, Huang YW, Lin Y. (1998). Tannins and human health: A review. Critical Reviews in Food Science and Nutrition, 38, 421–464.

Chrtková A. (1995). Glycyrrhiza L. In: Slavík B. [ed.]: Květena České republiky, vol. 4. Academia, Praha.

Çyrak C, Radušienė J, Ivanauskas L, Janulis V. (2007). Variation of bioactive secondary metabolites in *Hypericum origanifolium* during its phenological cycle. Acta Physiologiae Plantarum, 29, 197–203.

Davis EA, Morris DJ. (1991). Medicinal uses of licorice through the millennia: the good and plenty of it. Molecular and Cellular Endocrinology, 78, 1–6.

Dehpour AR, Zolfaghari ME, Samadian T, Kobarfard F, Faizi M, Assari M. (1995). Antiulcer activities of liquorice and its derivatives in experimental gastric lesion induced by ibuprofen in rats. International Journal of Pharmaceutics, 119, 133–138.

Di Mambro VM, Fonseca MJV. (2005). Assays of physical stability and antioxidant activity of a topical formulation added with different plant extracts. Journal of Pharmaceutical and Biomedical Analysis, 37, 287–295.

Dong TTX, Cui XM, Song ZH, Zhao KJ, Ji ZN, Lo CK, Tsim KWK. (2003). Chemical assessment of roots of *Panax notoginseng* in China: Regional and seasonal variations in its active constituents. Journal of Agricultural and Food Chemistry, 51, 4617–4623.

Dorhoi A, Dobrean V, Zăhan M, Virag P. (2006). Modulatory effects of several herbal extracts on avian peripheral blood cell immune responses. Phytotherapy Research, 20, 352–358.

European Pharmacopoeia (2002). 4th ed. Deutscher Apotheker Verlag. Stuttgart.

Fenwick GR. (1990). Liquorice, *Glycyrrhiza glabra* L., composition, uses and analysis. Food Chemistry, 38, 119–143.

Fiore C, Eisenhut M, Krausse R, Ragazzi E, Pellati D, Armanini D, Bielenberg J. (2008). Antiviral effects of *Glycyrrhiza* species. Phytotherapy Research, 22, 141–148.

Fiore C, Eisenhut M, Ragazzi E, Zanchin G, Armanini D. (2005). A history of the therapeutic use of liquorice in Europe. Journal of Ethnopharmacology, 99, 317–324.

Fiore C, Salvi M, Palermo M, Sinigaglia G, Armanini D, Toninello A. (2004). On the mechanism of mitochondrial permeability transition induction by glycyrrhetinic acid. Biochimica and Biophysica Acta, 1658, 195–201.

Fox DA. (1997). The role of T cells in the immunopathogenesis of rheumatoid arthritis: new perspectives. Arthritis and Rheumatism, 40, 598–609.

Fuhrman B, Buch S, Vaya J, Belinky PA, Coleman R, Hayek T, Aviram M. (1997). Licorice ethanolic extract and its major polyphenol glabridin protect LDL against lipid peroxidation: *In vitro* and *ex-vivo* studies in humans and in the atherosclerotic apolipoprotein E deficient mice. American Journal of Clinical Nutrition, 66, 267–275.

Fuhrman B, Volkova N, Kaplan M, Presser D, Attias J, Hayek T, Aviram M. (2002). Antiatherosclerotic effects of licorice extract supplementation on hypercholesterolemic patients: Increased resistance of LDL to atherogenic modifications, reduced plasma lipid levels, and decreased systolic blood pressure. Nutrition, 18, 268–273.

Gupta VK, Fatima A, Faridi U, Negi AS, Shanker K, Kumar JK, Rahuja N, Luqman S, Sisodia BS, Saikia D, Darokar MP, Khanuja SP. (2008). Antimicrobial potential of *Glycyrrhiza glabra* roots. Journal of Ethnopharmacology, 116, 377–380.

Haraguchi H, Ishikawa H, Mizutani K, Tamura Y, Kinoshita T. (1998). Antioxidative and superoxide scavenging activities of retrochalcones in *Glycyrrhiza inflata*. Bioorganic and Medicinal Chemistry, 6, 339–347.

Haraguchi H, Yoshida N, Ishikawa H, Tamura Y, Mizutani K, Kinoshita T. (2000). Protection of mitochondrial functions against oxidative stresses by isoflavans from *Glycyrrhiza glabra*. Journal of Pharmacy and Pharmacology, 52, 219–223.

Haralampidis K, Trojanowska M, Osbourn AE. (2002). Biosynthesis of triterpenoid saponins in plants. Advances in Biochemical Engineering/Biotechnology, 75, 31–49.

Hatano T, Fukuda T, Liu YZ, Noro T, Okuda T. (1991). Phenolic constituents of licorice. IV. Correlation of phenolic constituents and licorice specimens from various sources, and inhibitory effects of licorice extracts on xanthine oxidase and monoamine oxidase. Yakugaku Zasshi, 111, 311–321.

Hatano T, Kagawa H, Yasuhara T, Okuda T. (1988). Two new flavonoids and other constituents in licorice root: Their relative astringency and radical scavenging effects. Chemical and Pharmaceutical Bulletin, 36, 2090–2097.

Hayashi H. (2009). Molecular biology of secondary metabolism: Case study for *Glycyrrhiza* plants. In: Kirakosyan A, Kaufman PB (Eds.), Recent Advances in Plant Biotechnology Springer Science+Business Media, LLC.

Hayashi H, Hiraoka N, Ikeshiro Y, Yamamoto H, Toshikawa T. (1998). Seasonal variation of glycyrrhizin and isoliquiritigenin glycosides in the root of *Glycyrrhiza glabra* L. Biological and Pharmaceutical Bulletin, 21, 987–989.

Hayashi H, Hosono N, Kondo M, Hiraoka N, Ikeshiro Y, Shibano M, Kusano G, Yamamoto H, Tanaka T, Inoue K. (2000). Phylogenetic relationship of six *Glycyrrhiza* species based on rbcL sequences and chemical constituents. Biological and Pharmaceutical Bulletin, 23, 602–606.

Hayashi H, Miwa E, Inoue K. (2005). Phylogenetic relationship of *Glycyrrhiza lepidota*, american licorice, in genus *Glycyrrhiza* based on rbcL sequences and chemical constituents. Biological and Pharmaceutical Bulletin, 28, 161–164.

Herrmann KM. (1995). The Shikimate pathway: Early steps in the biosynthesis of aromatic compounds. The Plant Cell, 7, 907–919.

Hertog MGL, Hollman PCH, Katan MB. (1992). Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. Journal of Agricultural and Food Chemistry, 40, 2379–2383.

Hu F, Lu R, Huang B, Ming L. (2004). Free radical scavenging activity of extracts prepared from fresh leaves of selected Chinese medicinal plants. Fitoterapia, 75, 14–23.

Huang D, Ou B, Prior RL. (2005). The Chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry, 53, 1841–1856.

Ibrahim RK, Anzellotti D. (2003). The Enzymatic basis of flavonoid biodiversity. Recent Advances in Phytochemistry, 37, 1–36.

Incerti A, Navari-Izzo F, Pardossi A, Izzo R. (2009). Seasonal variations in polyphenols and lipoic acid in fruits of tomato irrigated with sea water. Journal of the Science of Food and Agriculture, 89, 1326–1331.

Isbrucker RA, Burdock GA. (2006). Risk and safety assessment on the consumption of licorice root (*Glycyrrhiza* sp.), its extract and powder as a food ingredient, with emphasis on the pharmacology and toxicology of glycyrrhizin. Regulatory Toxicology and Pharmacology, 46, 167–192.

Ishii Y, Fujii Y. (1982). Effects of FM100, a fraction of licorice root, on serum gastrin concentration in rats and dogs. Japanese Journal of Pharmacology 32, 23–27.

Ishii T, Ishii N. (2006). Aging and age-related diseases caused by superoxide anion production from mitochondria. Seikagaku, 78, 201–207.

Jacobson MD, Weil M, Raff MC. (1997). Programmed cell death in animal development. Cell, 88, 347–354.

Jeong HG, You HJ, Park SJ, Moon AR, Chung YC, Kang SK, Chun HK. (2002). Hepatoprotective effects of 18*β*-glycyrrhetinic acid on carbon tetrachloride-induced liver injury: Inhibition of cytochrome P450 2E1 expression. Pharmacological Research, 46, 221–227.

Jeon YJ, Li MH, Lee KY, Kim JS, You HJ, Lee SK, Sohn HM, Choi SJ, Koh JW, Chang IY. (2006). KIOM-79 inhibits LPS-induced iNOS gene expression by blocking NF-kappaB/Rel and p38 kinase activation in murine macrophages. Journal of Ethnopharmacology, 108, 38–45.

Jo EH, Kim SH, Ra JC, Kim SR, Cho SD, Jung JW, Yang SR, Park JS, Hwang JW, Aruoma OI, Kim TY, Lee YS, Kang KS. (2005). Chemopreventive properties of the ethanol extract of chinese licorice (*Glycyrrhiza uralensis*) root: induction of apoptosis and G1 cell cycle arrest in MCF-7 human breast cancer cells. Cancer Letters, 230, 239–247.

Joung JY, Mangai Kasthuri G, Park JY, Kang WJ, Kim HS, Yoon BS, Joung H, Jeon JH. (2003). An overexpression of chalcone reductase of *Pueraria montana* var. lobata alters biosynthesis of anthocyanin and 5′-deoxyflavonoids in transgenic tobacco. Biochemical and Biophysical Research Communications, 303, 326–331.

Kagan VE, Kuzmenko AI, Shvedova AA, Kisin ER, Li R, Martin I, Quinn PJ, Tyurin VA, Tyurina YY, Yalowich JC. (2003). Direct evidence for recycling of myeloperoxidase-catalyzed phenoxyl radicals of a vitamin E homologue, 2,2,5,7,8-pentamethyl-6-hydroxy chromane, by ascorbate/dihydrolipoate in living HL-60 cells. Biochimica and Biophysica Acta (BBA), 1620, 72–84.

Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. Journal of Agricultural and Food Chemistry, 47, 3954–3962.

Kajiyama K, Hiraga Y, Takahashi K, Hirata S, Kobayashi S, Sankawa U, Kinoshita T. (1993). Flavonoids and isoflavonoids of chemotaxonomic significance from *Glycyrrhiza pallidiflora* (Leguminosae). Biochemical Systematics and Ecology, 21, 785–793.

Kato T, Horie N, Hashimoto K, Satoh K, Shimoyama T, Kaneko T, Kusama K, Sakagami H. (2008). Bimodal effect of glycyrrhizin on macrophage nitric oxide and prostaglandin E2 production. In Vivo, 22, 583–586.

Kim JK, Oh SM, Kwon HS, Oh YS, Lim SS, and Shin HK. (2006). Anti-inflammatory effect of roasted licorice extracts on lipopolysaccharide-induced inflammatory responses in murine macrophages. Biochemical and Biophysical Research Communications, 345, 1215–1223.

Kim YS, Jung DH, Kim NH, Lee YM, Jang DS, Song GY, Kim JS. (2007). KIOM-79 inhibits high glucose or AGEs-induced VEGF expression in human retinal pigment epithelial cells. Journal of Ethnopharmacology, 112, 166–172.

Kitagawa I, Chen WZ, Taniyama T, Harada E, Hori K, Kobayashi, M, Ren J. (1998). Quantitative determination of constituents in various licorice roots by means of high performance liquid chromatography. Yakugaku Zasshi, 118, 519–528.

Klebanoff SJ. (2005). Myeloperoxidase: Friend and foe. Journal of Leukocytes Biology, 77, 598–625.

Ko HC, Wei BL, Chiou WF. (2006). The effect of medicinal plants used in Chinese folk medicine on RANTES secretion by virus-infected human epithelial cells. Journal of Ethnopharmacology, 107, 205–210.

Kondo K, Shiba M, Nakamura R, Morota T, Shoyama Y. (2007). Constituent properties of licorices derived from *Glycyrrhiza uralensis*, *G. glabra*, or *G. inflata* identified by genetic information. Biological and Pharmaceutical Bulletin, 30, 1271–1277.

Krausse R, Bielenberg J, Blaschek W, Ullmann U. (2004). *In vitro* anti-*Helicobacter pylori* activity of *Extractum liquiritiae*, glycyrrhizin and its metabolites. Journal of Antimicrobial Chemotherapy, 54, 243–246.

Kuroda M, Mimaki Y, Sashida Y, Mae T, Kishida H, Nishiyama T, Tsukagawa M, Konishi E, Takahashi K, Kawada T, Nakagawa K, Kitahara M. (2003). Phenolics with PPAR-γ ligand-binding activity obtained from licorice (*Glycyrrhiza uralensis* roots) and ameliorative effects of glycyrin on genetically diabetic KK-A^y mice. Bioorganic and Medicinal Chemistry Letters, 13, 4267–4272.

Kuzuyama T. (2002). Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units. Bioscience, Biotechnology and Biochemistry, 66, 1619–1627.

Lai JH, Ho LJ, Kwan CY, Chang DM, Lee TC. (1999). Plant alkaloid tetrandrine and its analog block CD28-costimulated activities of human peripheral blood T cells: potential immunosuppressants in transplantation immunology. Transplantation, 68, 1383–1392.

Lee JR, Park SJ, Lee HS, Jee SY, Seo J, Kwon YK, Kwon TK, Kim SC. (2009). Hepatoprotective activity of licorice water extract against cadmium-induced toxicity in rats. Evidence-Based Complementary and Alternative Medicine, 6, 195–201.

Lee SE, Hwang HJ, Ha JS, Jeong HS, Kim, JH. (2003). Screening of medicinal plant extracts for antioxidant activity. Life Sciences, 73, 167–179.

Li HB, Wong CC, Cheng KW, Chen F. (2008). Antioxidant properties *in vitro* and total phenolic contents in methanol extracts from medicinal plants. LWT-Food Science and Technology, 41, 385–390.

Li W, Asada Y, Yoshikawa T. (2000). Flavonoid constituents from *Glycyrrhiza glabra* hairy root cultures. Phytochemistry, 55, 447–456.

Liu H, Qiu N, Ding H, Yao R. (2008). Polyphenols contents and antioxidant capacity of 68 Chinese herbals suitable for medical or food uses. Food Research International, 41, 363–370.

Luper S. (1999). A review of plants used in the treatment of liver disease: part two. Alternative Medicine Review, 4, 178–188.

Ma JY, Zhang J, Wang YF, Yao J. (2006). Extraction and analysis of total flavonoids from roots of cultivated *Glycyrrhiza pallidiflora*. Journal of Plant Resources and Environment, 15, 78–79.

Mae T, Kishida H, Nishiyama T, Tsukagawa M, Konishi E, Kuroda M, Mimaki Y, Sashida Y, Takahashi K, Kawada T, Nakagawa K, Kitahara M. (2003). A Licorice ethanolic extract with peroxisome proliferator-activated receptor-γ ligand-binding activity affects diabetes in KK-A^y mice, abdominal obesity in diet-induced obese C57BL mice and hypertension in spontaneously hypertensive rats. Journal of Nutrition, 133, 3369–3377.

Malenčić D, Maksimović Z, Popović M, Miladinović J. (2008). Polyphenol contents and antioxidant activity of soybean seed extracts. Bioresource Technology, 99, 6688–6691.

Melvold RW, Sticca RP. (2007). Basic and tumor immunology: A Review. Surgical Oncology Clinics of North America, 16, 711–735.

Nagata S. (1997). Apoptosis by death factor. Cell, 88, 355–365.

Nakagawa T, Yokozawa T, Kim YA, Kang KS, Tanaka T. (2005). Activity of Wen-Pi-Tang, and purified constituents of rhei rhizoma and *Glycyrrhizae radix* against glucose-mediated protein damage. American Journal of Chinese Medicine, 33, 817–829.

Nakagiri R, Oda H, Kamiya T. (2003). Small scale rat hepatocyte primary culture with applications for screening hepatoprotective substances. Bioscience Biotechnology and Biochemistry, 67, 1629–1635.

Nakamura R, Kase Y, Hashimoto K, Sakakibara I, Amagaya S, Aburada M. (2003). Elucidation of anti-gastric ulcer constituents of *Glycyrrhizae radix*. Journal of Natural Medicines, 57, 172–177.

Nassiri-Asl M, Hosseinzadeh H. (2008). Review of pharmacological effects of *Glycyrrhiza* sp. and its bioactive compounds. Phytotherapy Research, 22, 709–724.

Niizawa A, Kogure T, Hai LX, Fujinaga H, Takahashi K, Shimada Y, Terasawa K. (2003). Clinical and immunomodulatory effects of Fun-Boi, an herbal medicine, on collagen-induced arthritis *in vivo*. Clinical and Experimental Rheumatology, 21, 57–62.

Nomura T, Fukai T, Akiyama T. (2002). Chemistry of phenolic compounds of licorice (*Glycyrrhiza* species) and their estrogenic and cytotoxic activities. Pure and Applied Chemistry, 74, 1199–1206.

Nomura T, Fukai T. (1998). Phenolic constituents of licorice (*Glycyrrhiza* species) In: Herz W, Kirby GW, Moore RE, Steglich W, and Tamm CH. (Eds.), Progress in the chemistry of organic natural products. Springer-Verlag. Wien, Austria.

Nopp A, Stridh H, Grönneberg R, Lundahl J. (2002). Lower apoptosis rate and higher CD69 expression in neutrophils from atopic individuals. Inflammation Research, 51, 532–540.

Nordberg J, Arnér, ESJ. (2001). Reactive oxygen species, antioxidant, and the mammalian thioredoxin system. Free Radical Biology and Medicine, 31, 1287–1312.

Nose M, Terawaki K, Oguri K, Ogihara Y, Yoshimatsu K, Shimomura K. (1998). Activation of macrophages by crude polysaccharide fractions obtained from shoots of *Glycyrrhiza glabra* and hairy roots of *Glycyrrhiza uralensis.* Biological and Pharmaceutical Bulletin, 21, 1110–1112.

Obolentseva GV, Litvinenko VI, Ammosov AS, Popova TP, Sampiev AM. (1999). Pharmacological and therapeutic properties of licorice preparations (A review). Pharmaceutical Chemistry Journal, 33, 427–434.

Ohsako S, Elkon KB. (1999). Apoptosis in the effector phase of autoimmune diabetes, multiple sclerosis and thyroiditis. Cell Death and Differentiation, 6, 13–21.

Okada K, Tamura Y, Yamamoto M, Inoue Y, Takagaki R, Takahashi K, Demizu S, Kajiyama K, Hiraga Y, Kinoshita T. (1989). Identification of antimicrobial and antioxidant constituents from licorice of Russian and Xinjiang origin. Chemical and Pharmaceutical Bulletin, 37, 2528–2530.

Ozkan G, Baydar H, Erbas S. (2010). The influence of harvest time on essential oil composition, phenolic constituents and antioxidant properties of Turkish oregano (*Origanum onites* L.). Journal of the Science of Food and Agriculture, 90, 205–209.

Panayi GS, Corrigall VM, Pitzalis C. (2001). Pathogenesis of rheumatoid arthritis. The role of T cells and other beasts. Rheumatic Disease Clinics of North America, 27, 317–334.

Parr AJ, Bolwell GP. (2000). Phenols in the plant and in man. The potencial for possible nutricional enhancement of the diet by modifying the phenols content or profile. Journal of the Science of Food and Agriculture, 80, 985–1012.

Perez-Jimenez J, Arranz S, Tabernero M, Diaz- Rubio ME, Serrano J, Goni I, Saura-Calixto F. (2008). Updated methodology to determine antioxidant capacity in plant foods, oils and beverages: Extraction, measurement and expression of results. Food Research International, 41, 274–285.

Petyaev IM, Hunt JV. (1997). Micellar acceleration of oxygen-dependent reactions and its potential use in the study of human low density lipoprotein. Biochimica and Biophysica Acta, 1345, 293–305.

Phillips DR, Rasbery JM, Bartel B, Matsuda SP. (2006). Biosynthetic diversity in plant triterpene cyclization. Current Opinion in Plant Biology 9, 305–314.

Prior RL, Wu X, Schaich K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry, 53, 4290–4302.

Reddy M, Eirikis E, Davis C, Davis HM, Prabhakar U. (2004). Comparative analysis of lymphocyte activation marker expression and cytokine secretion profile in stimulated human peripheral blood mononuclear cell cultures: an in vitro model to monitor cellular immune function. Journal of Immunological Methods, 293, 127–142.

Revers FE. (1956). Clinical and pharmacological investigations on extract of licorice. Acta Medica Scandinavica, 154, 749–751.

Sa EH, Jin UH, Kim DS, Kang BS, Ha KT, Kim JK, Park WH, Kim CH. (2007). Herbal medicine Gamgungtang down-regulates autoimmunity through induction of TH2 cytokine production by lymphocytes in experimental thyroiditis model. Journal of Ethnopharmacology, 109, 472–479.

Salvi M, Fiore C, Armanini D, Toninello A. (2003) Glycyrrhetinic acid-induced permeability transition in rat liver mitochondria. Biochemical Pharmacology, 66, 2375–2379.

Salminen JP, Ossipov V, Haukioja E, Pihlaja K. (2001). Seasonal variation in the content of hydrolysable tannins in leaves of *Betula pubescens*. Phytochemistry, 57, 15–22.

Sato J, Goto K, Nanjo F, Kawai S, Murata K. (2000). Antifungal activity of plant extracts against *Arthrinium sacchari* and *Chaetomium funicola*. Journal of Bioscience and Bioengineering, 90, 442–446.

Scaccini C, Jialal I. (1994). LDL modification by activated polymorphonuclear leukocytes: A cellular model of mild oxidative stress. Free Radical Biology and Medicine, 16, 49–55.

Scheibmeir HD, Christensen K, Whitaker SH, Jegaethesan J, Clancy R, Pierce JD. (2005). A review of free radicals and antioxidants for critical care nurses. Intensive and Critical Care Nursing, 21, 24–28.

Schempp H, Weiser D, Kelber O, Elstner EF. (2006). Radical scavenging and anti-inflammatory properties of STW 5 (Iberogast®) and its components. Phytomedicine, 13, 36–44.

Schindhelm RK, Van Der Zwan LP, Teerlink T, Scheffer PG. (2009). Myeloperoxidase: A useful biomarker for cardiovascular disease risk stratification?. Clinical Chemistry, 55, 1462–1470.

Schmeda-Hirschman G, Rodríguez JA, Theoduloz C, Astudillo L, Feresin GE, Tapia A. (2003). Free radical scavengers and antioxidants from *Peumus boldus* Mol. (''Boldo''). Free Radical Research, 37, 447–452.

Sheela ML, Ramakrishna MK, Salimath BP. (2006). Angiogenic and proliferative effects of the cytokine VEGF in Ehrlich ascites tumor cells is inhibited by *Glycyrrhiza glabra*. International Immunopharmacology, 6, 494–498.

Shen XP, Xiao PG, Liu CX. (2007). Research and application of *Radix Glycyrrhizae*. Asian Journal of Pharmacodynamics and Pharmacokinetics, 7, 181–200.

Shibata SA. (2000). A drug over the millennia: pharmacognosy, chemistry, and pharmacology of licorice. Yakugaku Zasshi, 120, 849–862.

Simms PE, Ellis TM. (1996). Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. Clinical and Diagnostic Laboratory Immunology, 3, 301–304.

Simons R, Vincken JP, Bakx EJ, Verbruggen MA, Gruppen H. (2009). A rapid screening method for prenylated flavonoids with ultra-high-performance liquid chromatography/electrospray ionisation mass spectrometry in licorice root extracts. Rapid Communications in Mass Spectrometry, 23, 3083–3093.

Singleton VL, Orthofer R, Lamuela-Raventos RM. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Methods in Enzymology, 299, 152–178.

Slimestad R, Verheul B. (2009). Review of flavonoids and other phenolics from fruits of different tomato (*Lycopersicon esculentum* Mill.) cultivars. Journal of the Science of Food and Agriculture 89, 1255–1270.

Statti GA, Tundis R, Sacchetti G, Muzzoli M, Bianchi A, Menichini F. (2004). Variability in the content of active constituents and biological activity of *Glycyrrhiza glabra*. Fitoterapia 75, 371–374.

Suja KP, Jayalekshmy A, Arumughan C. (2005). Antioxidant activity of sesame cake extract. Food Chemistry, 91, 213–219.

Sun HX, Pan HJ. (2006). Immunological adjuvant effect of *Glycyrrhiza uralensis* saponins on the immune responses to ovalbumin in mice. Vaccine, 24, 1914–1920.

Tang SY, Whiteman M, Peng ZF, Jenner A, Yong EL, Halliwell B. (2004). Characterization of antioxidant and antiglycation properties and isolation of active ingredients from traditional chinese medicines. Free Radical Biology and Medicine, 36, 1575–1587.

Toor RK, Savage GP, Lister CE. (2006). Seasonal variation in the antioxidant composition of greenhouse grown tomatoes. Journal of Food Composition and Analysis, 19, 1–10.

Tseng SH, Lee HH, Chen LG, Wu CH, Wang CC. (2006). Effects of three purgative decoctions on inflammatory mediators. Journal of Ethnopharmacology, 105, 118–24.

Valentão P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos, ML. (2001). Antioxidant activity of *Centaurium erythraea* infusion evidenced by its superoxide radical scavenging and xanthine oxidase inhibitory activity. Journal of Agricultural and Food Chemistry, 49, 3476–3479.

Van Antwerpen P, Moreau P, Zouaoui-Boudjeltia K, Babar S, Dufrasne F, Moguilevsky N, Vanhaeverbeek M, Ducobu J, Nève J. (2008b). Development and validation of a screening procedure for the assessment of inhibition using a recombinant enzyme. Talanta, 75, 503–510.

Van Antwerpen P, Prévost M, Zouaoui-Boudjeltia K, Babar S, Legssyer I, Moreau P, Moguilevsky N, Vanhaeverbeek M, Ducobu J, Nève J, Dufrasne F. (2008a). Conception of myeloperoxidase inhibitors derived from flufenamic acid by computational docking and structure modification. Bioorganic and Medicinal Chemistry, 16, 1702–1720.

Vaya J, Belinky PA, Aviram M. (1997). Antioxidant constituents from licorice roots: Isolation, structure elucidation and antioxidative capacity toward LDL oxidation. Free Radical Biology and Medicine, 23, 302–313.

Wagner H, Jurcic K. (2002). Immunological studies of Revitonil®, a phytopharmaceutical containing *Echinacea purpurea* and *Glycyrrhiza glabra* root extract. Phytomedicine, 9, 390–397.

Weiss SJ. (1989). Tissue destruction by neutrophils. The New England Journal of Medicine, 320, 365–376.

Witztum JL, Steinberg D. (1991). Role of oxidized low density lipoprotein in atherogenesis. Journal of Clinical Investigation, 88, 1785–1792.

Wojdyło A, Oszmiański J, Czemerys R. (2007). Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chemistry, 105, 940–949.

Wong CC, Li HB, Cheng KW, Chen F. (2006). A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. Food Chemistry, 97, 705–711.

Wu RT, Lin WJ, Chiang HC, Horng LY, Chung YM. (1990). Modulation of experimental autoimmune uveitis with formosanin-C in guinea pigs. Journal of Ocular Pharmacology, 6, 301–311.

Wu YT, Shen C, Yin J, Yu JP, Meng Q. (2006). Azathioprine hepatotoxicity and the protective effect of liquorice and glycyrrhizic acid. Phytotherapy Research, 20, 640–645.

Xu L, Liu Y, He X. (2005). Inhibitory effects of berberine on the activation and cell cycle progression of human peripheral lymphocytes. Cellular and Molecular Immunology, 2, 295–300.

Yamamoto Y, Tani T. (2006). Brief survey of *Glycyrrhiza* plant resources in Xinjiang, China. Journal of Traditional Medicines, 23, 27–35.

Yao L, Caffin N, D'arcy B, Jiang Y, Shi J, Singanusong R, Liu X, Datta N, Kakuda Y, Xu Y. (2005). Seasonal variations of phenolic compounds in Australia-Grown tea (*Camellia sinensis*). Journal of Agricultural and Food Chemistry, 53, 6477–6483.

Yesil-Celiktas O, Girgin G, Orhan H, Wichers HJ, Bedir E, Vardar-Sukan F. (2007). Screening of free radical scavenging capacity and antioxidant activities of *Rosmarinus officinalis* extracts with focus on location and harvesting times. European Food Research and Technology, 224, 443–451.

Yoon G, Jung YD, Cheon SH. (2005). Cytotoxic allyl retrochalcone from the roots of *Glycyrrhiza inflata*. Chemical and Pharmaceutical Bulletin, 53, 694–695.

Yu O, McGonigle B. (2005). Metabolic engineering of isoflavone biosynthesis. Advances in Agronomy, 86, 147–190.

Zeng L, Zhang RY, Meng T, Lou ZC. (1990). Determination of nine flavonoids and coumarins in licorice root by high-performance liquid chromatography. Journal of Chromatography A, 513, 247–254.

Zhang R, Brennan ML, Shen Z, MacPherson JC, Schmitt D, Molenda CE. (2002). Myeloperoxidase functions as a major enzymatic catalyst for initiation of lipid peroxidation at sites of inflammation. The Journal of Biological Chemistry, 277, 46116–46122.

Zhang Q, Ye M. (2009). Chemical analysis of the Chinese herbal medicine Gan-Cao (licorice). Journal of Chromatography A, 1216, 1954–1969.

Ziegler SF, Ramsdell F, Alderson MR. (1994) The activation antigen CD69. Stem Cells, 12, 456–465.

Zwickey H, Brush J, Iacullo CM, Connelly E, Gregory WL, Soumyanath A, Buresh R. (2007). The effect of *Echinacea purpurea*, *Astragalus membranaceus* and *Glycyrrhiza glabra* on CD25 expression in humans: a pilot study. Phytotherapy Research, 21, 1109–1112.

11. PUBLICATIONS AND CONGRESS PRESENTATIONS

JOURNAL ARTICLES

Cheel J, Van Antwerpen P, Nève J, Areche C, San Martin A, Tůmová L, Neugebauerová J, Vokřál I, Wsól V. (2010). Effect of harvest time on the chemical profile, free radical scavenging, antioxidant and gastroprotective activities of *Glycyrrhiza glabra* (Submitted to the Journal of the Science of Food and Agriculture). (IF = 1.386).

Cheel J, Van Antwerpen P, Tůmová L, Onofre G, Vokurková D, Zouaoui-Boudjeltia K, Vanhaeverbeek M, Nève J. (2010). Free radical scavenging, antioxidant and immunostimulating effects of a licorice infusion (*Glycyrrhiza glabra* L.). Food Chemistry 122, 508-517. (IF = 3.146).

Cheel J, Onofre G, Vokurková D, Tůmová L, Neugebauerová J. (2010). Licorice Infusion: Chemical profile and effects on the activation and the cell cycle progression of human lymphocytes. Pharmacognosy Magazine 6, 26-33.

Saud G, Carbone F, Perrotta G, Moya M, Figueroa C, Herrera R, Retamales J, Carrasco B, **Cheel J**, Schmeda-Hirschman G, Caligari Peter DS. (2009). Transcript profilig suggests transcriptional repression of the flavonoid pathway in the white-fruited Chilean strawberry, *Fragaria chiloensis* (L.) Mill. Genetic Resources and Crop Evolution 56, 895-903. (IF = 1.238).

Orrego R, Leiva L, **Cheel J**. (2009). Inhibiting Effect of three *C*-glycosylflavonoids from *Cymbopogon citratus* (lemongrass) on human low density lipoprotein oxidation. Molecules 14, 3906-3913. (IF = 1.738).

Cheel J, Tůmová L, Neugebauerová J, Dušek J. (2008). Free radical scavenging activity of *Glycyrrhiza* species. Folia Pharm. Univ. Carol. XXXVII, 41-47.

INTERNATIONAL BOOK CHAPTER

Schmeda-Hirschmann G, Simirgiotis M, **Cheel J**. (2010). Chemistry of the Chilean Strawberry (*Fragaria chiloensis* spp. chiloensis). Manuscript accepted for publication as a Book Chapter in a special issue of Genomics, Transgenics, Molecular Breeding and Biotechnology of Strawberry. Global Science Books.

CONGRESS PRESENTATIONS

Cheel J, Areche, C., San Martin, A., Tůmová, L.: Gastroprotective action of a hydroalcoholic extract of licorice (*Glycyrrhiza glabra* L. roots) and its major constituents: Assessment of its mode of action. 60. Česko-Slovenské farmakologické dny. Hradec Králové, Czech Republic, September 15-17, 2010. In: Acta Medica 2010, 53 (3), 168-169.

Cheel J, Onofre G, Areche C, Vokurkova D, San Martin A, Tůmová L.: Studies on the immunomodulating effects of three merodιterpenoids from the marine algae

Stypopodium flabelliforme. 60. Česko-Slovenské farmakologické dny. Hradec Králové, Czech Republic, September 15-17, 2010. In: Acta Medica 2010, 53 (3), 168.

Areche C, **Cheel J**, Sepulveda B, San Martin A, Tůmová L.: Studies on the mode of gastroprotective action in mice of epitaondiol and sargaol: two meroditerpenoids from *Stypopodium flabelliforme*. 60. Česko-Slovenské farmakologické dny. Hradec Králové, Czech Republic, September 15-17, 2010. In: Acta Medica 2010, 53 (3), 161-162.

Cheel J, Tůmová L, Neugebauerová J, Van Antwerpen P, Nève J, Areche C, San-Martín A, Zouaoui-Boudjeltia K, Vanhaeverbeek M, Dušek J.: Seasonal dynamics of the chemical composition and biological properties of licorice (*Glycyrrhiza glabra* L.). 6th Conference on Aromatic and Medicinal Plants of Southeast European Countries (6th CMAPSEEC). Antalya-Turkey, April 18-22, 2010. In: Pharmacognosy Magazine 2010, 6 (22) Suppl. pp. 69.

Cheel J, Orrego R, Leiva O, Tůmová L, Dušek J.: Antioxidant properties of three *C*-glycosylflavonoids from *Cymbopogon citratus* on human low density lipoprotein oxidation. 6th Conference on Aromatic and Medicinal Plants of Southeast European Countries (6th CMAPSEEC). Antalya-Turkey, April 18-22, 2010. In: Pharmacognosy Magazine 2010, 6 (22) Suppl. pp. 68.

Cheel J, Onofre G, Tůmová L, Van Antwerpen P, Vokurková V, Nève J.: Antiradical, antioxidant and immunostimulating effects of a licorice infusion (*Glycyrrhiza glabra* L.). 4th International Young Scientists' Biology Conference. Kharkiv, Ukraine, 2009, pp. 97-98.

Cheel J, Onofre G, Vokurková D, Tůmová, L.: Licorice infusion: HPLC chemical profile and effect on the activation and cell cycle progression of lymphocytes. 4th International Young Scientists' Biology Conference. Kharkiv, Ukraine, 2009, pp. 98-99.

Cheel J, Tůmová L, Tuma J, Neugebauerová J, Dušek J.: Free radical scavenging activity and phenolic content of *Glycyrrhiza* species. Fifth Conference on Medicinal and Aromatic Plants of Southeast European Countries (5th CMAPSEEC). Brno, Czech Republic, 2008, pp. 132-132.

Tůmová L, Tuma J, **Cheel J**, Neugebauerová J, Dušek J.: Oxidative stress and flavonoid production by *Ononis arvensis* L. culture in vitro. Fifth Conference on Medicinal and Aromatic Plants of Southeast European Countries. Brno, Czech Republic, 2008, pp. 24-24.

Tůmová L, Pešlová, Tůma J, **Cheel J**, Dušek J.: Fotooxidační stres a produkce flavonolignanu kulturou *Silybum marianum* in vitro. In: Proceedings of the I. Plant scientific workshop; Prague, Česká zemědělská univerzita, Czech Republic, 2008, pp. 132-135.

ORAL PRESENTATION

Cheel J. Free radical scavenging activity and phenolic content of *Glycyrrhiza* species. Fifth Conference on Medicinal and Aromatic Plants of Southeast European Countries (CMAPSEEC). Brno, Czech Republic, 2-5 September 2008.

12. ATTENDANCE AT SEMINARS AND SCIENTIFIC MEETINGS

Journée des Doctorants en Sciences Pharmaceutiques. Institute of Pharmacy, Université Libre de Bruxelles, Belgium. 7th May 2009.

Seminar: Sample Preparation and Chromatographic Analysis. Sigma-Aldrich, Prague, 8th April 2008.

13. GRANTS AND HONOURS

International grant (Bourses d'excellence – research grant IN.WBI) awarded by the Wallonia-Brussels Internacional (IN.WBI) Program, Belgium. The awarded proyect was entitled: *"Effect of licorice (Glycyrrhiza glabra L.) on hypochlorous acid and the myeloperoxidase-chlorinating system: Influence of harvest times".* The research activities were carried out in the Laboratory of Pharmaceutical Chemistry, Institute of Pharmacy, Université Libre de Bruxelles, Brussels, Belgium. 2009.

Member of the reviewing committee (referee) of the Journal of Ethnopharmacology (Elsevier). List of reviewers in: Journal of Ethnopharmacology, 126, (2009), 1–4.