COMPOSITION OF HAWTHORN (*Crataegus* spp.) FRUITS AND LEAVES AND EMBLIC LEAFFLOWER (*Phyllanthus emblica*) FRUITS

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ABSTRACT

Hawthorn (*Crataegus* sp.) is widely distributed in the northern hemisphere (Asia, Europe and North America). It has been used as a medicinal material and food for hundreds of years both in Europe and in China. Clinical investigations and other research suggest that extracts of hawthorn fruits and leaves have multiple health effects including hypolipidaemic, anti-atherosclerotic, hypotensive, cardioprotective and blood vessel relaxing activities. Hawthorn fruit extracts have also displayed antioxidant and radical scavenging activities.

Emblic leafflower fruit (*Phyllanthus emblica*) is widely used in Chinese and Indian traditional medicine. It has been found to have anti-cancer, hypoglycaemic and hypolipidaemic activities as well as cardioprotective effects and antioxidant activity. The fruit is currently used as a functional food targeted at obese people in China.

Phenolic compounds, procyanidins (PCs), flavonols and *C*-glycosyl flavones in hawthorn and hydrolysable tannins in emblic leafflower fruits are considered among the major bioactive compounds in these berries. Moreover, hawthorn and emblic leafflower fruits are rich in vitamin C, triterpenoids, fruit acids, sugar alcohols and some other components with beneficial effects on the health of human beings.

The aim of the thesis work was to characterise the major phenolic compounds in hawthorn fruits and leaves and emblic leafflower fruits as well as other components contributing to the nutritional profile and sensory properties of hawthorn fruits. Differences in the content and compositional profile of the major phenolic compounds, sugars, acids and sugar alcohols within various origins and species of hawthorn were also investigated.

Acids, sugars and sugar alcohols in the fruits of different origins/cultivars belonging to three species (*C. pinnatifida, C. brettschneideri* and *C. scabrifolia*) of hawthorn were analysed by gas chromatography (GC-FID) and mass spectrometry (Publication I). Citric acid, quinic acid, malic acid, fructose, glucose, sorbitol and *myo*-inositol were found in all the subspecies. Sucrose was present only in *C. scabrifolia* and three cultivars of *C. pinnatifida* var. *major*.

Forty-two phenolic compounds were identified/tentatively identified in fruits of *C. pinnatifida* var. *major* by polyamide column chromatography combined with high-performance liquid chromatograph-electrospray ionisation mass spectrometry (HPLC-ESI-MS) (Publication II). Ideain, chlorogenic acid, procyanidin (PC) B2, (-)-epicatechin, hyperoside and isoquercitrin were the major phenolic components identified. In addition, 35 phenolic compounds were tentatively identified based on UV and mass spectra. Eleven major phenolic compounds (hyperoside, isoquercitrin, chlorogenic acid,

ideain, (-)-epicatechin, two PC dimers, three PC trimers and a PC dimer-hexoside) were quantified in the fruits of 22 cultivars/origins of three species of Chinese hawthorn by HPLC-ESI-MS with single ion recording function (SIR) (Publication III).

The fruits of the hawthorn cultivars/origins investigated fell into two groups, one rich in sugars and flavonols, the other rich in acids and procyanidins. Based on the compositional features, different biological activities and sensory properties may be expected between cultivars/origins of the two groups. The results suggest that the contents of phenolic compounds, acids, sugars and sugar alcohols may be used as chemotaxonomic information distinguishing the hawthorn species from each other.

Phenolic compounds in fruits and leaves of *C. grayana* and their changes during fruit ripening/harvesting were investigated using HPLC-UV-ESI-MS (Publication IV). (-)-Epicatechin, PC B2 and C1, hyperoside and a quercetin-pentoside were the major phenolic compounds in both fruits and leaves. Three *C*-glycosyl flavones (a luteolin-*C*-hexoside, a methyl luteolin-*C*-hexoside and an apigenin-C-hexoside) were present in leaves in abundance, but only at trace levels in fruits. Ideain and 5-*O*-caffeoylquinic acid were found in fruits only. Additionally, eleven phenolic compounds were identified/tentatively identified in both leaves and fruits (three B-type PC trimers, two B-type PC tetramers, a quercetin-rhamnosylhexoside, a quercetin-pentoside, a methoxykaempferol-methylpentosylhexoside, a quercetin-hexoside acetate, a methoxykaempferol-pentoside, chlorogenic acid and an unknown hydroxycinnamic acid derivative). The total content of phenolic compounds reached the highest level by the end of August in fruits and by the end of September in leaves. The compositional profiles of phenolic compounds in fruits and leaves of *C. grayana* were different from those of *C. pinnatifida, C. brettschneideri*, *C. scabrifolia*, *C. pinnatifida*. var. *major*, *C. monogyna*, *C. laevigata* and *C. pentagyna*.

Phenolic compounds in emblic leafflower fruits were characterised by Sephadex LH-20 column chromatography combined with HPLC-ESI-MS (Publication V). A mucic acid gallate, three isomers of mucic acid lactone gallate, a galloylglucose, gallic acid, a digalloylglucose, putranjivain A, a galloyl-HHDP-glucose, elaeocarpusin and chebulagic acid represented the major phenolic compounds in fruits of emblic leafflower.

In conclusion, results of this study significantly increase the current knowledge on the key bioactive and nutritional components of hawthorn and emblic leafflower fruits. These results provide important information for research on the mechanism responsible for the health benefits of these fruits.

ABBREVIATIONS

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
BW	body weight
CA	chromosome aberrations
CAT	catalase
COX-2	cyclooxygenase 2
DAD	diode array detector
DM	dry mass
DP	degree of polymerisation
DPPH	2,2-diphenyl-picrylhydrazyl
FABMS	fast atom bombardment mass spectrometry
FID	flame ionisation detector
ESI	electronic spray ionisation
EMB	emblic leafflower fruits
γ-IFN	interferon-γ
GC	gas chromatography
GPx	glutathione peroxidase
GSH	reduced glutathione
GST	glutathione S-transferase
HDL	high density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	high performance liquid chromatography
IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthase
IRI	ischaemia-reperfusion injury
LDL	low density lipoprotein
LPO	lipid peroxidation
MDA	malondialdehyde
NDEA	*N*-nitrosodiethylamine
NMR	nuclear magnetic resonance
NO	nitric oxide
PC	procyanidin
PCA	principle component analysis
PE	*Phyllanthus emblica*
PMN	polymorphonuclear leukocyte
ROS	reactive oxygen species
SIR	single ion recording
SOD	superoxide dismutase
STZ	streptozotocin
TBARS	thiobarbituric acid-reactive substances
TLC	thin layer chromatography
TNF-α	tumour necrosis factor-α
TGF-β1	transforming growth factor- β1

LIST OF ORIGINAL PUBLICATIONS

1. Liu, P.Z., Kallio, H., Lü, D.G, Zhou, C.S., Ou, S.Y., and Yang B.R. Acids, sugars, and sugar alcohols in Chinese hawthorn (*Crataegus* spp.) fruits. *Journal of Agricultural and Food Chemistry*, 2010, 58, 1012–1019.

2. Liu, P.Z., Yang, B.R., and Kallio, H. Characterization of phenolic compounds in Chinese hawthorn (*Crataegus pinnatifida* Bge. var. *major*) fruit by high-performance liquid chromatography-electrospray ionization mass spectrometry. *Food Chemistry*, 2010, 121, 1188–1197.

3. Liu, P.Z., Kallio, H., Lü, D.G, Zhou, C.S., and Yang B.R. Quantitative analysis of phenolic compounds in Chinese hawthorn (*Crataegus* spp.) fruits by high performance liquid chromatography-electrospray ionization mass spectrometry. *Food Chemistry*, 2011, 127, 1370–1377.

4. Liu, P.Z., Kallio, H., and Yang B.R. Phenolic compounds in hawthorn (*Crataegus grayana*) fruits and leaves and changes during fruit ripening. *Journal of Agricultural and Food Chemistry,* 2011, 59, 11141–11149.

5. Yang, B.R., Kortesniemi, M., Liu, P.Z. and Salminen, J-P. Characterization of phenolic compounds in emblic leafflower (*Phyllanthus emblica* L.) fruits by high performance liquid chromatograph-electrospray ionisation mass spectrometry. *Journal of Agricultural and Food Chemistry* (Submitted).

1. INTRODUCTION

Hawthorn (*Crataegus* spp.) and emblic leafflower fruits (*Phyllanthus emblica* L.) have used in Asia and Europe as food and medicine for a long period of time. In the traditional medicine systems of China, India and some European countries, the health effects and functional properties of different parts of these plants have been well-documented.

Hawthorn is the common name for *Crataegus* species in *Rosaceae* family. There are over 1000 species of *Crataegus* distributed primarily in Asia, Europe and North America (Zhao and Tian, 1996). Different species dominate and different parts of the plant are used in these regions. In China, *Crataegus pinnatifida* Bge. is the most important wild species. The majority of hawthorns currently cultivated in China belong to cultivars of *Crataegus pinnatifida* var. *major* N.E.Br. Hawthorn fruits are consumed fresh or processed into jams, jellies, soft drinks, candies and canned fruits. The Chinese Pharmacopoeia describes dried fruits of *C. pinnatifida* and *C. pinnatifida* var. *major* N.E.Br. as used for stimulating digestion, improving circulation and for treating hypertension and hyperlipidaemia (China Pharmacopoeia Committee, 2005). In Europe, the major species are *Crataegus laevigata* Poir. (syn: *Crataegus oxyacantha* L.), *Crataegus monogyna* Jacq., *Crataegus pentagyna* Waldst., *Crataegus nigra* Waldst. and *Crataegus azarolus* L. Various parts (fruits, leaves, flowers and flowering tops) of different *Crataegus* species are included in the European pharmacopeia as well as in the German, British, French and Swiss pharmacopeias as antispasmodic, cardiotonic, diuretic, hypotensive and anti-atherosclerotic agents (Chang et al., 2002). In Europe and the US, aqueous ethanol extracts of hawthorn leaves and flowers (flowering tops) are clinically used in complimentary therapy for class I-II heart failure according to the classification of the New York Heart Association (NYHA).

Phyllanthus emblica L. (syn: *Emblica officinalis* Gaertn.), commonly known as emblic leafflower fruit, Indian gooseberry or Amla, belongs to the genus *Phyllanthus* in the family *Euphorbiaceae*. The species is naturally distributed in the tropical and subtropical areas of Asia, such as southern China and India. The fruits have been consumed as food and used as traditional medicinal materials for a long time in China, India and southeast Asian countries (Baliga and Dsouza, 2011).

In vitro and *in vivo* studies have shown that the extracts of the fruits have strong antioxidative and radical scavenging activities against DPPH, $O_2^{\bullet-}$, $OH^{\bullet}$ and NO radicals (Nampoothiri et al., 2011; Pozharitskaya et al., 2007; Reddy et al., 2010). Moreover, preclinical and clinical studies carried out in recent decades have shown that fruits of emblic leafflower possess antibacterial, antidiabetic, hypolipidaemic, anticancer, anti-inflammatory, immunomodulatory, antiatherogenic, antihypercholesterolaemia,

gastroprotective, hepatoprotective, cardiovascular protective and neuroprotective properties (Khan, 2009; Krishnaveni and Mirunalini, 2010; Nampoothiri et al., 2011; Sabu and Kuttan, 2002; Baliga et al., 2011; Bhattacharya et al., 2007).

Phenolic compounds in combination with vitamin C are considered to be the major antioxidant and bioactive components in hawthorn and emblic leafflower fruits (Suryanarayana et al. 2007). Although some scientists have suggested that antioxidative activities cannot be considered a key mechanism in the biological activities of phenolic compounds (Arts and Hollmann, 2005), most reports suggest that the health effects of hawthorn and emblic leafflower fruits can be attributed to the antioxidative activities of the fruits.

The aim of the current review is to summarise the literature on the composition, biological activities and physiological effects of bioactive compounds in plants of *Crataegus* species and *Phyllanthu emblica*. A comparison of the profile and content of phenolic compounds in different species/origins as well as changes in the composition of fruits and leaves during the developmental and ripening period of hawthorn fruits has also been reviewed.

2. REVIEW OF THE LITERATURE

2.1. Composition of *Crataegus* spp.

2.1.1. Phenolic compounds in hawthorn of different origins

Several groups of phenolic compounds, including procyanidins, flavanols, flavonols, *C*-glycosyl flavones, phenolic acids, anthocyanins and lignans have been reported in different parts of hawthorn plants (**Table 1**). In fruits, oligomeric procyanidins and their glycosides are the major phenolic compounds, whereas flavonols, flavonol glycosides and *C*-glycosyl flavones dominate in leaves (Liu et al., 2011a).

2.1.1.1. Procyanidins

Procyanidins in hawthorn consists primarily of (-)-epicatechin as the flavan-3-ol unit. Only few reports have mentioned the existence of catechin in *Crataegus* spp., either in the form of monomer or as a constituent unit in oligomeric and polymeric procyanidins (Svedström et al. 2002a). The procyanidins identified so far are exclusively of the B-type (flavan-3-ol linked through single C-4/C-8 or C-4/C-6 interflavanol linkages) (Svedström et al., 2002b). More than thirty oligomeric procyanidins have been reported in the fruits and leaves of *Crataegus* spp. Different plant parts and different species contain the same compounds as the major procyanidins, whereas both the proportions and the contents of individual compounds vary considerably (Liu et al., 2010b; Svedström et al., 2002a). **Figure 1** shows the common structures of the major procyanidins in hawthorn.

Procyanidins (PC) B2 and B5 were identified as the major PC dimers and procyanidin C1 the major trimer in fruits of *C. pinnatifida* var. *major*. In addition, aglycons and glycosides of oligomeric procyanidins with a degree of polymerisation (DP) 2-6 were tentatively identified (Cui et al., 2006a; Cui et al., 2006b; Liu et al, 2010b; Liu et al., 2011b). Similar profiles of procyanidin aglycons were found in fruits of *Crataegus scabrifolia*, *C. pinnatifida* and *Crataegus brettschneideri*, whereas the content of procyanidin glycosides varied among species (Cui et al., 2006a; Cui et al., 2006b; Liu et al, 2010b; Liu et al., 2011b). The content and profile of procyanidin glycosides may be used as a chemotaxonomic marker to distinguish among different *Crataegus* species (Liu et al., 2011b).

Significant correlations between the contents of individual procyanidins in fruits of *C. pinnatifida* var. *major*, *C. pinnatifida*, *C. scabrifolia* and *C. brettschneideri* have been found (Cui et al., 2006a; Liu et al., 2011b).

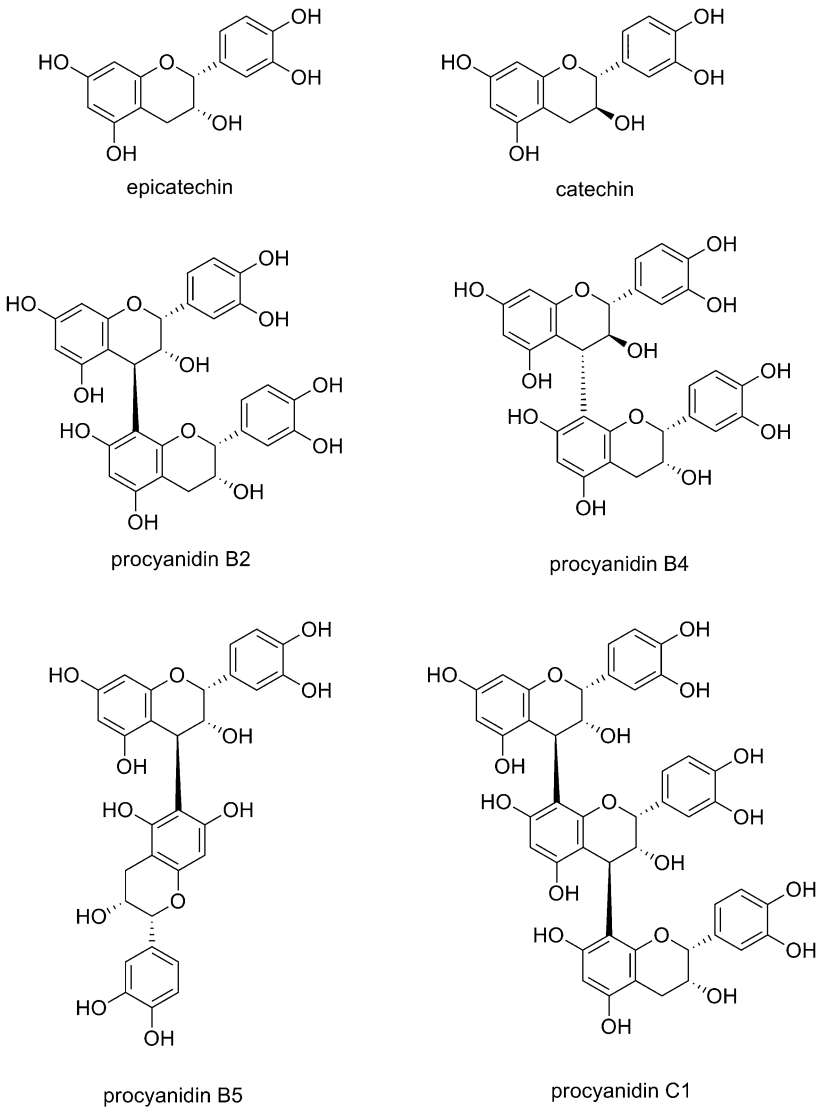


Figure 1. Procyanidins in hawthorn

(-)-Epicatechin, PC dimers B2, B4 and B5, PC timer C1, (-)-epicatechin-(4β→6)-(-)-epicatechin-(4β→8)-(-)-epicatechin and (-)-epicatechin-(4β→8)-(-)-epicatechin-(4β→6)-(-)-epicatechin, PC tetramer D1 and PC pentamer E1 are the major procyanidins both in leaves and in flowers of *C. laevigata* (Svedström et al., 2002a; Svedström et al. 2002b). A PC hexamer has also been found (Rohr, et al., 2000; Svedström et al., 2002a). The profile of procyanidins in leaves of *C. grayana* was similar as that in leaves of *C. laevigata* (Liu et al., 2011a). (-)-Epicatechin and PC B2 were also reported in flowers of two *C. azarolus* varieties (*C. a.* var. *aronia* and *C. a.* var. *eu-azarolus*) growing in Tunisia (Bahri-Sahloul et al., 2009). Procyanidin-containing (+)-catechin units (procyanidin B4,

(+)-catechin-(4β→8)-(-)-epicatechin) were reported in *C. laevigata* but not in Chinese hawthorn (Cui et al., 2006a; Liu et al., 2010a; Rohr et al., 2000; Svedström et al., 2002b).

Procyanidins of DP>6 were found in the leaves and flowers of *C. laevigata* (Svedström et al., 2006), but the separation of these compounds by HPLC was insufficient. So far, only limited research has been carried out on the phenolic compounds in the leaves of Chinese hawthorn. Cui and colleagues did not find procyanidins in the leaves of *C. pinnatifida* var. *major*, probably due to the relatively low content (Cui et al., 2006a).

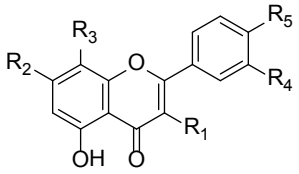
Only several structures of oligo-procyanidins in hawthorn have been determined in detail. It is still challenging work to identify all the procyanidins because 1) the contents of most of these compounds in hawthorn are quite low; 2) purifying and isolating these compounds are difficult; 3) the absence of commercially available reference compounds; 4) the absence of a reliable structural analysis method.

2.1.1.2. Flavonols and flavonol glycosides

Quercetin, kaempferol and sexangularetin (8-methoxykaempferol) are the major flavonol aglycons reported in hawthorn. Hyperoside, isoquercitrin and rutin are often reported as the major flavonol glycosides. Rutin was not found in the fruits of some cultivars of *C. pinnatifida* var. *major*, and isoquercitrin and rutin were not found in fruits and leaves of *C. grayana* (Liu et al., 2011a; 2010b; 2011b). Petereit and Nahstedt summarised the aglycons and glycosides of flavonols in *C. monogyna*, *C. laevigata*, *C. pentagyna*, *C. nigra*, and *C. azarolus* (Petereit and Nahstedt, 2005). In addition to the aglycons mentioned above, they reported flavonol glycosides kaempferol-3-*O*-neohesperidoside, hyperoside (quercetin-3-*O*-galactoside), crataegide (quercetin-3'-*O*-arabinoside), spiraeoside (quercetin-4'-*O*-glucoside), rutin (quercetin-3-*O*-rutinoside), quercetin-3-*O*-rhamnosylgalactoside, sexangularetin-3-*O*-glucoside, sexangularetin-3-*O*-neohesperidoside and sexangularetin-3-*O*-(6″-*O*-malonyl)-β-D-glucoside (Petereit and Nahstedt, 2005). The structure of these flavonols and flavonol glycosides are presented in **Figure 2**.

Quercetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside was identified in the leaves of *C. pinnatifida* var. *psilosa* by NMR (Oh et al., 1994). A compound of the same molecular weight was found in the fruits of *C. pinnatifida* var. *major* and in the leaves and fruits of *C. grayana*; this was tentatively identified as quercetin-rhamnosylhexoside (Liu et al., 2011b; Liu et al., 2010b). Two quercetin-pentosides were found in the leaves and fruits of *C. grayana* and a quercetin-(di-rhamnosyl)hexoside was tentatively identified in the fruits of *C. pinnatifida* var. *major* (Liu et al., 2011a; Liu et al., 2010a). Derivatives of quercetin glycosides such as quercetin hexoside acetate were also reported in the fruits and leaves of *C. grayana* (Liu et al., 2011a).

Glycosides of kaempferol and sexangularetin are often present as minor components in hawthorn. Sexangularetin, sexangularetin-3-glucoside, sexangularetin-3-neohesperidoside and kaempferol-3-neohesperidoside were found in the pollen of *C. monogyna* (Dauguet et al., 1993). In the leaves and fruits of *C. grayana*, a methoxykaempferol-methylpentosylhexoside and a methoxykaempferol-pentoside were found (Liu et al., 2011a). These compounds were probably glycosides of sexangularetin.



	R_1	R_2	R_3	R_4	R_5
Kaempferol	OH	OH	H	H	OH
Kaempferol-3-*O*-neohesperidoside	O-β-D-Glu-(2→1)-α-L-Rha	OH	H	H	OH
Quercetin	OH	OH	H	OH	OH
Hyperoside	O-β-D-Gal	OH	H	OH	OH
Isoquercitrin	O-β-D-Glu	OH	H	OH	OH
Crataegide	OH	OH	H	O-β-L-Ara	OH
Spiraeoside	OH	OH	H	OH	O-β-D-Glu
Rutin	O-β-D-Glu-(6→1)-α-L-Rha	OH	H	OH	OH
Quercetin-3-*O*-rhamnosylgalactoside	O-β-D-Gal-(6→1)-α-L-Rha	OH	H	OH	OH
Sexangularetin	OH	OH	OCH_3	H	OH
Sexangularetin-3-*O*-glucoside	O-β-D-Glu	OH	OCH_3	H	OH
Sexangularetin-3-*O*-neohesperidoside	O-β-D-Glu-(2→1)-α-L-Rha	OH	OCH_3	H	OH
Sexangularetin-3-*O*-(6"-*O*-malonyl)-β-D-glucoside	O-(6"-*O*-malonyl)-β-D-Glu	OH	OCH_3	H	OH
Lueeolin-7-*O*-glucoside	H	O-β-D-Glu	H	OH	OH
Luteolin-7-*O*-β-D-glucuronide	H	O-β-D-glucuronide	H	OH	OH

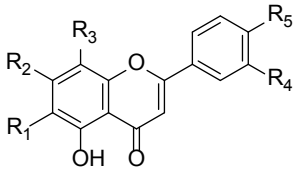
Figure 2. Flavonols and *O*-glycosides of flavonols and flavones in hawthorn. Glu, glucose; Rha, rhamnose; Gal, galactose; Ara, arabinose.

2.1.1.3. *C*-glycosyl flavones

Most *C*-glycosyl flavones in hawthorn are derivatives of apigenin and luteolin (**Table 1** and **Figure 3**). Vitexin (apigenin-8-*C*-glucoside) and its derivatives such as vitexin-2″-*O*-rhamnoside are the most common *C*-glycosyl flavones found in the species of hawthorn investigated so far (**Figure 3**) (Petereit and Nahstedt, 2005; Oh et al., 1994, Ringl et al., 2007). Vitexin and vitexin-2″-*O*-rhamnoside were identified in the flowering tops of *C. laevigata*, *Crataegus rhipidopylla* and their hybrid *Crataegus x macrocarpa* with TLC and HPLC (Ringl et al., 2007). Oh et al. reported vitexin-2″-rhamnoside in the leaves of *C. pinnatifida* var. *psilosa* (Oh et al., 1994). Six *C*-glycosyl flavones were isolated from the leaves of *C. pinnatifida* var. *major*. These compounds were identified as vitexin, 6″-*O*-acetylvitexin, 2″-*O*-acetylvitexin, 2″-*O*-rhamosylvitexin, 8-*C*-β-D-(2″-

O-acetyl)-glucofuranosyl-apigenin and 3″-*O*-acetylvitexin by NMR (Zhang and Xu, 2001a; Zhang and Xu, 2003a).

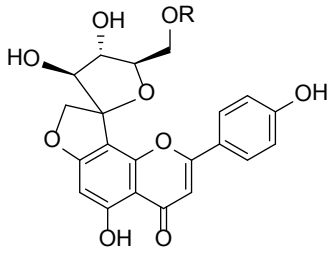
The profile of the *C*-glycosyl flavones in hawthorn may be one important chemotaxonomic marker of differentiating species of hawthorn. Vitexin-2″-*O*-rhamnoside, vitexin and isovitexin were found in the flowering tops of *C. monogyna*, *C. pentagyna,* and *C. laevigata* (Prinz et al., 2007, Ringl et al., 2007). Isoorientin and orientin were the major *C*-glycosyl flavones, and isoorientin-2″-*O*-rhamnoside, orientin-2″-*O*-rhamnoside and isovitexin-2″-*O*-rhamnoside the minor ones in the flowering tops of *C. pentagyna*. These compounds were not found in *C. monogyna* and *C. laevigata*. Vitexin-2″-*O*-(4‴-*O*-acetyl)-rhamnoside was a dominating compound in *C. monogyna* but was not detected in *C. laevigata* and *C. pentagyna* (Prinz et al., 2007, Ringl et al., 2007).



	R_1	R_2	R_3	R_4	R_5
Vitexin	H	OH	β-D-Glu	H	OH
Isovitexin	β-D-Glu	OH	H	H	OH
8-*C*-β-D-(2"-*O*-acetyl)-glucofuranosyl-apigenin	H	OH	2-O-Ac-β-D-Glufuranose	H	OH
2"-*O*-acetylveitexin	H	OH	2-O-Ac-β-D-Glu	H	OH
3"-*O*-acetylveitexin	H	OH	3-O-Ac-β-D-Glu	H	OH
6"-*O*-acetylveitexin	H	OH	6-O-Ac-β-D-Glu	H	OH
Vitexin-2"-*O*-rhamnoside	H	OH	β-D-Glu-(2→1)-α-L-Rha	H	OH
Vitexin-2"-*O*-(4'"-*O*-acetyl)-rhamnoside	H	OH	β-D-Glu-(2→1)-4-O-Ac-α-L-Rha	H	OH
Isovitexin-2"-*O*-rhamnoside	β-D-Glu-(2→1)-α-L-Rha	OH	H	H	OH
Vincenin 1	Xyl	OH	Glu	H	OH
Vincenin 2	Glu	OH	Glu	H	OH
Vincenin 3	Glu	OH	Xyl	H	OH
Schaftoside	β-D-Glu	OH	α-L-Ara	H	OH
Isoschaftoside	α-L-Ara	OH	β-D-Glu	H	OH
Neoschaftoside	β-D-Glu	OH	β-L-Ara	H	OH
Neoisoschaftoside	β-L-Ara	OH	β-D-Glu	H	OH
Vitexin-4'-*O*-rhamosylglucoside	H	OH	β-D-Glu	H	O-β-D-Glu-(2→1)-α-L-Rha
Vitexin-4',7-di-*O*-glucoside	H	O-β-D-Glu	β-D-Glu	O-β-D-Glu	OH
Orientin	H	OH	β-D-Glu	OH	OH
Isoorientin	β-D-Glu	OH	H	OH	OH
Acetylorientin	H	OH	2-O-Ac-β-D-Glu	OH	OH
Acetylisoorientin	2-O-Ac-β-D-Glu	OH	H	OH	OH
Orientin-2"-*O*-rhamnoside	H	OH	β-D-Glu-(2→1)-α-L-Rha	OH	OH
Isoorientin-2"-*O*-rhamnoside	β-D-Glu-(2→1)-α-L-Rha	OH	H	OH	OH

Figure 3. *C*-glycosides of flavones reported in hawthorn. Glu, glucose; Rha, rhamnose; Gal, galactose; Ara, arabinose; Ac, acetyl; Xyl, xylose.

Pinnatifida A, B, C and D are *C*-glycosyl flavones isolated for the first time from *C. pinnatifida* var. *major* (**Figure 4**) (Zhang and Xu, 2001b; Zhang et al., 2001c). The presence of two bonds (a C_8-$C_{1''}$ and a C_7-*O*-$C_{2''}$) between the apigenin and the fructose units is a common feature of the structures of these compounds.



	Sugar unit	R
Pinnatifida A	α-D-fructofuranose	H
Pinnatifida B	α-D-fructofuranose	Ac
Pinnatifida C	β-D-fructofuranose	H
Pinnatifida D	β-D-fructofuranose	Ac

Figure 4. Structures of pinnatifida A, B, C, and D. Ac, acetyl

2.1.1.4. Other phenolic compounds

Luteolin-7-*O*-glucoside was reported by Petereit and Nahstedt (2005). Luteolin-7-*O*-β-D-glucuronide and eriodictyol-7-*O*-glucuronide were reported in *C. rhipidopylla* and *C. x macrocarpa* (Prinz et al., 2007)

Chlorogenic acid was found both in the fruits and leaves of all hawthorn species investigated so far. However, its isomer 5-*O*-caffeoylquinic acid (neochlorogenic acid) was reported only in the fruits of *C. grayana* (Liu et al., 2011a). Caffeic acid was detected in the leaves of *C. laevigata* (Svedström et al., 2006), gallic acid and hydroxybenzoic acid in the leaves of *Crataegus cuneata* (Ma and Gu, 2003; Ma et al., 2007), and protocatechuic acid in the fruits of *C. pinnatifida* (Zhang et al., 2001d). Six lignan glycosides were found in the leaves of *C. pinnatifida* (Gao et al., 2010). The compounds were (-)-2α-*O*-(β-D-glucopyranosyl)-lyoniresinol, tortoside A, erythro-1-(4-*O*-β-D-glucopyranosyl-3-methoxyphenyl)-2-[4-(3-hyroxypropyl)-2,6-dimethoxyphenoxy]-1,3-propanediol, (7S,8R)-urolignoside, (7S,8R)-5-methoxydihydrodehy drodiconiferyl alohol-4-*O*-β-D-glucopyranoside and acernikol-4″-*O*-β-D-glucopyranoside (**Table 1**) (Gao et al., 2010).

2.1.1.5. Contents of phenolic compounds in hawthorn

Colorimetric methods and HPLC combined with UV detector or mass spectrometry have been used to determine the total content of phenolics. A capillary electrophoretic

method has been used for the analysis of flavonoids in ethanolic extracts of single-styled hawthorn (*C. monogyna*)(Urbonaviciūte et al., 2006).

The content of flavonoids varied in the range of 3-19 g kg^{-1} dry mass (average 12 g kg^{-1}) in the flowering tops (flowers and leaves) of 150 samples of wild European hawthorn belonging to *C. monogyna*, *C. laevigata* and their hybrids (Peschel et al., 2008). Froehlicher et al. compared the total phenolics in different parts/organs of *C. monogyna* by the Folin-Ciocalteu reagent method (Froehlicher et al., 2009). Dried flowering tops and flower buds had higher contents of phenolic compounds (56 and 49 g gallic acid equivalents kg^{-1} dry mass [DM]) than dried and fresh fruits (12 g gallic acid equivalents kg^{-1} DM). The phenolic content was also determined in two cell lines selected from callus-initiated flowering buds. The level in red cells was 123 g kg^{-1} DM and that in yellow cells was 1 g kg^{-1} DM (Froehlicher et al., 2009).

The total contents of flavonoids determined by HPLC-UV were 84 and 45 g kg^{-1} DM, respectively, in the fruits and leaves of *C. scabrifolia* and 31 and 66 g kg^{-1} DM, respectively, in the fruits and leaves of *C. pinnatifida* (Gao and Feng, 1994). Ten major phenolic compounds were quantified in the fruits of *C. pinnatifida* var. *major* using an HPLC-UV method (Cui et al., 2006a). The content of (-)-epicatechin was 8 g kg^{-1} DM. The content of procyanidin dimers (sum of B2 and B5), procyanidin trimers (C1, II and III), tetramers and pentamers was 9, 4, 2 and 1 g kg^{-1} DM, respectively. The total procyanidin level was 23 g kg^{-1} DM, accounting for 92% of the total content of phenolic compounds. The mean degree of polymerisation (mDP) of procyanidins was 1.65. Lower contents of oligomeric procyanidins were reported in the flowers (12 g kg^{-1} DM), leaves (16 g kg^{-1} DM) and fruits (2 g kg^{-1} DM) of *C. laevigata* (Svedström et al., 2002a).

The contents of procyanidins were determined in the fruits and leaves of eight Chinese hawthorn species by a gravimetric method after acetone/water extraction and acidic precipitation (Gao et al., 1995). *C. scabrifolia* contained the highest level of procyanidins (101 g kg^{-1} DM) in fruits among the species studied. The contents of procyanidins in the fruits of *C. pinnatifida*, *C. pinnatifida* var. *major*, *C. cuneata*, *Crataegus hupehensis* and *Crataegus kansuensis* were 18, 61, 27, 52 and 40 g kg^{-1} DM, respectively. The procyanidin content in the fruits of *Crataegus wilsonii* and *Crataegus auranitia* was lower than the detection limit of the method, whereas the leaves of these species contained the highest levels of procyanidins (122 and 134 g kg^{-1} DM, respectively) among all these species. The content of procyanidins in the leaves of *C. scabrifolia* was 106 g kg^{-1} DM, higher than the levels in the rest of the species studied (22 to 53 g kg^{-1} DM). The procyanidin contents reported in this study were rough estimations based on precipitation and gravimetric measurements, and the values were significantly higher

and less accurate than the levels determined by HPLC-UV methods (Cui et al., 2006a; Gao and Feng, 1994).

Currently, only the fruits of *C. pinnatifida* and *C. pinnatifida* var. *major* are accepted in the Chinese Pharmacopoeia. Fruits and leaves of *C. scabrifolia* contain higher levels of phenolic compounds than *C. pinnatifida* and may be potential new raw materials for food and health care products.

Eleven major phenolic compounds (hyperoside, isoquercitrin, chlorogenic acid, ideain, (-)-epicatechin, two procyanidin dimers, three procyanidin trimers and a procyanidin dimer-hexoside) were quantified in the fruits of 22 cultivars/origins of three species of the Chinese hawthorn (*Crataegus* spp.) by HPLC-ESI-MS-SIR (Liu et al., 2011b). The total contents of procyanidins in fruits of *C. pinnatifida* var. *major* and *C. scabrifolia* were 18 and 20 g kg^{-1} DM, respectively, significantly higher than those in *C. brettschneideri* (8 g kg^{-1} DM) and *C. pinnatifida* (9 g kg^{-1} DM). The fruits of *C. scabrifolia* contained the highest level of procyanidin dimer-hexoside (1 g kg^{-1} DM), which was present in trace amounts in the fruits of *C. pinnatifida* and *C. brettschneideri*. The content of this compound in fruits of *C. pinnatifida* var. *major* was 0.2 g kg^{-1} DM. Hyperoside (1 g kg^{-1} DM), isoquercitrin (less than 1 g kg^{-1} DM) were the major flavonol glycosides, and chlorogenic acid (up to 2 g kg^{-1} DM) the major phenolic acid. The level of chlorogenic acid was lower than the average content (200 mg kg^{-1} fresh fruits) in 37 cultivars of *C. pinnatifida* var. *major* reported previously (Cui et al., 2006a). Ideain (0–700 mg kg^{-1} DM) was found in all the samples except *C. scabrifolia* (Liu et al., 2011b).The sensory properties of fruits and berries are significantly influenced by the content and composition of sugars, acids and phenolic compounds. Principle component analyses (PCA) were carried out using the data on sugars, acids and phenolic compounds of these samples (Liu et al., 2010a; Liu et al., 2011b). The PCA-biplot is presented in **Figure 5**. The cultivars of *C. pinnatifida* var. *major* fell into two groups, one rich in procyanidins and fruit acids, the other in flavonols and sugars. In contrast, the cultivars of *C. brettschneideri* were more homogenous, belonging mostly to the flavonol- and sugar-rich group (**Figure 5**).

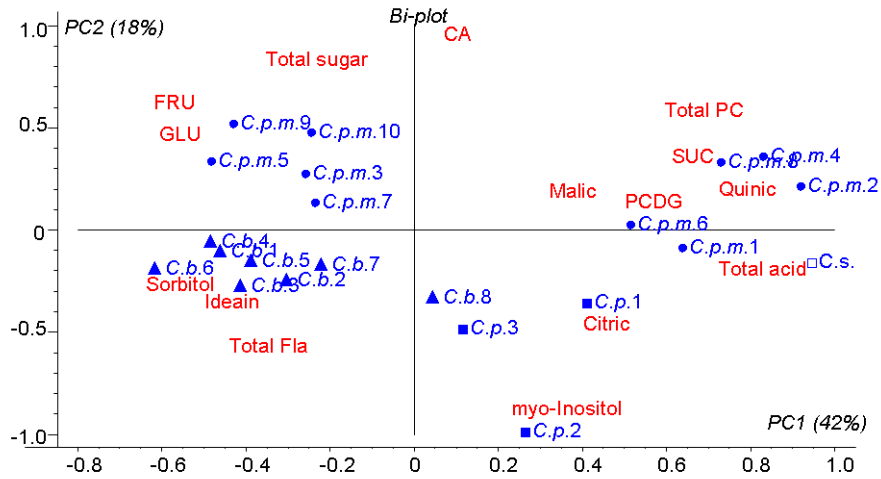


Figure 5. PCA plot showing the correlation between flavonols, procyanidins, sugars and acids in hawthorn fruits of different origins. *C. p. m.*, *C. pinnatifida* var. *major*; *C. b.*, *C. brettschneideri*; *C. p.*, *C. pinnatifida* Bge.; *C. s.*, *C. scabrifolia*; CA, chlorogenic acid; Citric, citric acid; Malic, malic acid; Quinic, quinic acid; Glu, glucose; FRU, fructose; SUC, sucrose; Total Fla, total flavonol; Total PC, total procyanidin; PCDG, procyanidin dimer-glycoside.

Rehwald et al. analysed the flavonoid content in the dried leaves of *C. monogyna* collected at different plant development stages (Rehwald et al., 1994). The flavonoid content in the leaves of *C. monogyna* reached the highest level in the pre-flowering and flowering periods (Rehwald et al., 1994). After this point, the flavonoid content in the leaves decreased and remained at low levels for the rest of the growth season. In the fruits of *C. pinnatifida* var. *major*, procyanidins were synthesised rapidly in the early growth stage and reached the highest level in the fruits (14 g kg^{-1} of fresh weight) by 61 days after blooming (average weight of fruit, 4.4 g). Thereafter, the procyanidin content in fruits decreased gradually, reaching a level around 4 g kg^{-1} of fresh weight at the optimal maturity stage (average weight of fruit, 11.6 g) (Cui et al., 2006a). Liu et al. followed the changes in the content of phenolic compounds the fruits and leaves of in *C. grayana* from early August to early October. The contents of phenolic compounds in the fruits was the highest around mid-August and early September (22 g kg^{-1} DM), and in leaves around late September and early October (62 g kg^{-1} DM) (Liu et al., 2011a).

Bahri-Shloul et al. investigated the polyphenol contents in flower buds and open flowers of two *C. azarolus* L. varieties (*C. a.* var. *aronia* and *C. a.* var. *eu-azarolus*) grown in Tunisia (Bahri-Sahloul et al., 2009). Chlorogenic acid, hyperoside, rutin, spiraeoside, (-)-epicatechin, quercetin and procyanidin B2 were found in all samples. The buds contained higher levels of phenolics than the opened flowers. The buds of *C. a.* var. *aronia* (164 mg gallic acid equivalents kg^{-1} DM) had higher levels of

polyphenols than those of *C. a.* var. *eu-azarolus* (142 mg gallic acid equivalents kg^{-1} DM).

Although many studies have been done on the phenolic contents in hawthorn, systematic investigations based on reliable methods are still limited. In earlier stages, most of the studies just reported the total contents of phenolic compounds using colorimetric methods. These results were easily affected by other compounds in the extract with similar absorption wavelengths as phenolic compounds. Moreover, the compositional profile of individual phenolic compounds was also missing. With the development of HPLC and other modern methods, it has been easier to determine the contents of individual phenolics in hawthorn. However, the absence of commercial reference compounds has limited the accuracy of more recent results. New analytical methods to evaluate phenolic compounds are still required for future research.

Table 1. Phenolic compounds reported in hawthorn of different species/origins.

Species/varieties (Site of collection)	Organs	Procyanidins	Flavonols	*C*-glycosyl flavones	Other phenolics
C. pinnatifida (China)	Fruits	(-)-Epicatechin; Dimer: B2, B5; Trimer: C1 and two unknowns; Tetramer: D1.	Hyperoside; Isoquercitrin; Rutin; Quercetin	Vitexin	Chlorogenic acid; Ideain
	Leaves	(-)-Epicatechin.	Hyperoside; Rutin; Quercetin	Vitexin	Six lignans *
C. pinnatifid. var. *major* (China)	Fruits	(-)-Epicatechin; Dimer: B2, B5, two unknowns; Trimer: C1 and two unknowns; Tetramer: D1 and 7 unknowns; Pentamers: 4 unknowns; Hexamers: 2 unknowns; Glycoside: 2 hexosides of monomers, 7 hexosides of dimers, 1 hexoside of trimer, 2 hexosides of tetramers, 1 hexoside of pentamer; An unknown PA derivative	Hyperoside; Isoquercitrin; Quercetin; Quercetin-di-(rhamnosyl) hexoside; Quercetin-rhamnosyhexoside; Rutin	Vitexin	Chlorogenic acid; Ideain; Protocatechuic acid
	Leaves	(-)-Epicatechin; Dimer: B2; Trimer, C1.	Hyperoside; Isoquercitrin; Rutin; 4‴-O-rhamnosylrutin	2″-O-acetylvitexin; 3″-O-acetylveitexin; 6″-O--acetylvitexin; 4‴-O-glucosylvitexin; Pinnatifida A, B, C, D; Vitexin; Vitexin-2″-rhamnoside; Vitexin-4″-O-glucosides 8-C-β-D-(2″-O-acetyl)-glucofuranosyl-apigenin	Chlorogenic acid
C. brettschneideri (China)	Fruits	(-)-Epicatechin; Dimer: B2, B5; Trimer: C1 and two unknowns; Tetramer: D1; A PA dimer hexoside	Hyperoside; Isoquercitrin		Chlorogenic acid; Ideain.
C. scabrifolia (China)	Fruits	(-)-Epicatechin; Dimer: B2, B5; Trimer: C1 and two unknowns; Tetramer: D1; A PA dimer hexoside.	Hyperoside; Isoquercitrin; Rutin	Vitexin.	Chlorogenic acid.
	Leaves		Hyperoside; Isoquercitrin; Rutin 4‴-O-rhamnosylrutin	4‴-O-glucosylvitexin; Vitexin; Vitexin-2″-O-rhamnoside; Vitexin-4″-O-glucosides	
C. cuneata (China)	Fruits		Hyperoside.		
	Leaves		Hyperoside; Isoquercitrin; Rutin; 4‴-O-rhamnosylrutin.	4‴-O-glucosylvitexin; Vitexin; Vitexin-2″-O-rhamnoside; Vitexin-4″-O-glucosides.	Gallic acid; Hydroxybenzoic acid; protocatechuic aldehyde.
C. pinnatifida var. *psilosa* (China, Korea)	Fruits		Hyperoside; Isoquercitrin.	Vitexin.	
	Leaves		Hyperoside; Quercetin-3-O-rhamnosylgalactoside	Vitexin; Vitexin-2″-O-rhamnoside	

Species/varieties (Site of collection)	Organs	Procyanidins	Flavonols	*C*-glycosyl flavones	Other phenolics
C. hupehensisi (China)	Fruits		Hyperoside; Rutin	Vitexin	
	Leaves		Hyperoside; Rutin.		Chlorogenic acid
C. sanguinea (China)	Fruits		Hyperoside; Rutin.	Vitexin.	
C. maximowiczii (China)	Fruits		Hyperoside; Rutin.	Vitexin.	
C. wilsonii (China)	Leaves		Hyperoside; Rutin.		Chlorogenic acid.
C. aurantia (China)	Leaves		Hyperoside; Rutin.		Chlorogenic acid.
C. kansuensis (China)	Fruits	(-)-Epicatechin	Hyperoside		
	Leaves			Rutin	Chlorogenic acid
C. grayana (Finland)	Fruits	(-)-Epicatechin; Dimer: B2, B5; Trimer: C1 and two unknowns Tetramer: D1.	Hyperoside; Two quercetin-pentosides; Methoxykaempferol-methylpentosylhexoside; Quercetin-hexoside acetate; Quercetin-rhamnosyhexoside.	Luteolin-C-hexoside; Methyl luteolin-C-hexoside.	Neochlorogenic acid Chlorogenic acid Ideain..
	Leaves	(-)-Epicatechin; Dimer: B2, B5; Trimer: C1 and two unknowns Tetramer: D1	Hyperoside; Two quercetin-pentosides; Methoxykaempferol-methylpentosylhexoside; Quercetin-hexoside acetate; Methoxykaempferol-pentoside; Quercetin-rhamnosyhexoside.	Apigenin-C-hexoside; Luteolin-C-hexoside; Methyl luteolin-C-hexoside.	Chlorogenic acid.
C. monogyna (Europe)	Leaves		Hyperoside; Isoquercitrin; Rutin; 4‴-O-rhamnosylrutin., Sexangularetin-3-O -glucoside; Kaempferol-3-O-glucoside.	4″-acetylvitexin-2″-O- rhamnoside; 4‴-O-glucosylvitexin Isovitexin; Vitexin; Vitexin-2″-O-rhamnoside; Vitexin-4″-O-glucosides.	Chlorogenic acid.
	Pollen		Sexangularetin; Sexangularetin-3-glucoside; Sexangularetin-3-neohesperidoside; Kaempferol-3-neohesperidoside		

Table 1. Phenolic compounds reported in hawthorn of different species/origins (continue).

Species/varieties (Site of collection)	Organs	Procyanidins	Flavonols	*C*-glycosyl flavones	Other phenolics
C. laevigata (Europe)	Fruits	(-)-Epicatechin; Dimer: B2, B4 and B5; Timers: C1, epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin, epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin; Tetramer: D1; Pentamer: (-)-epicatechin units liked through C-4 β/C-8 bonds.			
	Leaves	(-)-Epicatechin; Dimer: B2, B4 and B5; Timers: C1, epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin, epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin; Tetramer: D1; Pentamer: (-)-epicatechin units liked through C-4 β/C-8 bonds.	Hyperoside; Isoquercitrin; Rutin.	Acetylvitexin-2″-O-rhamnoside; Isovitexin; Vitexin; Vitexin-2″-O-rhamnoside.	Chlorogenic acid; Caffeic acid.
	Flowers	(-)-Epicatechin; Dimer: B2, B4 and B5; Timers: C1, epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin, epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin; Tetramer: D1; Pentamer: (-)-epicatechin units liked through C-4 β/C-8 bonds.	Hyperoside; Isoquercitrin; Rutin.		
C.x macrocarpa (Europe)	Leaves		Hyperoside; Isoquercitrin; Rutin.	Vitexin; Vitexin-2″-O-rhamnoside; (S)- and (R)- eriodictyol-7-O-glucuronide; Luteolin-7-O-β-D-glucuronide	Chlorogenic acid.
C. azarolus. var. *aronia* (Tunisia)	Flowers	(-)-Epicatechin; Procyanidin B2	Hyperoside; Isoquercitrin; Rutin; Spiraeoside; Quercetin.		Chlorogenic acid.
C. azarolus. var. *eu-azarolus* (Tunisia)	Flowers	(-)-Epicatechin; Procyanidin B2	Hyperoside; Isoquercitrin; Rutin; Spiraeoside; Quercetin.		Chlorogenic acid.
C. rhipidophylla (Europe)	Leaves		Hyperoside; Isoquercitrin; Rutin.	Isovitexin; Vitexin; Vitexin-2″-O-rhamnoside; (S)- and (R)- eriodictyol-7-O-glucuronide; Luteolin-7-O-β-D-glucuronide	Chlorogenic acid.
C. pentagyna (Europe)	Leaves		Hyperoside; Isoquercitrin; Rutin; Sexangularetin-3-O -glucoside.	Isoorientin; Isoorientin-2″-O- rhamnoside; Isovitexin; Orientin; Orientin-2″-O- rhamnoside; Vitexin; Vitexin-2″-O- rhamnoside.	

* Six lignans include (-)-2α-O-(β-D-glucopyranosyl)-lyoniresinol, tortoside A, (7S,8R)-urolignoside, acernikol-4"-O-β-D-glucopyranoside, (7S,8R)-5-methoxydihydrodehy drodiconiferyl alohol-4-O-β-D-glucopyranoside and erythro-1-(4-O-β-D-glucopyranosyl-3-methoxyphenyl)-2-[4-(3-hyroxypropyl)-2,6-dimethoxyphenoxy]-1,3-propanediol.

2.1.2. Triterpene acids

Triterpene acids have been suggested to be another group of bioactive compounds in hawthorn species. Evidence has shown that triterpene acids have beneficial effects such as anti-cancer activity (Hsu et al., 1997; Liu, 1995).

Oleanolic acid and ursolic acid are the major triterpene acids and have been detected in the fruits of hawthorn (**Figure 6**) (Zou and Chen, 2006; Cui et al, 2006a). However, only oleanolic acid was reported in the leaves of hawthorn (Zou and Chen, 2006; Gu et al., 2006; Cui et al, 2006a).

Zou and Chen (2006) determined the contents of oleanolic acid and ursolic acid in the fruits of *C. cuneata* by HPLC-DAD, yielding 24 g kg^{-1} and 2.2 g kg^{-1}, respectively. Cui et al. (2006a) analysed the contents of oleanolic acid and ursolic acid in 37 cultivars of *C. pinnatifida* var. *major*. The average contents were 0.95 and 0.15 g kg^{-1} fresh fruit, respectively.

Ren et al. carried out the quantification of ursolic acid in the leaves of *C. pinnatifida*, demonstrating a content of 2.6 g kg^{-1} DM (Ren et al., 2007)

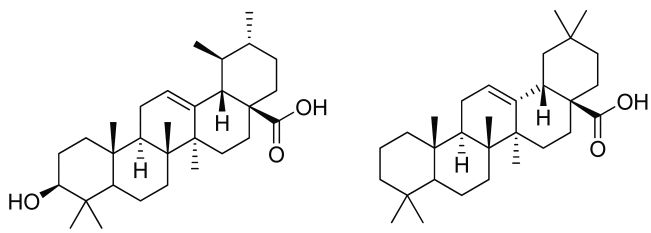


Figure 6. Triterpene acids in hawthorn.

2.1.3. Other compounds

Meng and Wang (2005) reported the nutritional compounds in the fruits of 18 cultivars of *C. pinnatifida* var. *major*. The contents of total soluble sugar, total acid, protein and vitamin C were 90, 20, 6 and 0.7 g kg^{-1} DM, respectively. The vitamin C content was higher compared to the levels commonly reported in various fruits (Meng and Wang , 2005).

The contents and profiles of acids, sugars and sugar alcohols in 18 cultivars of *C. pinnatifida* var. major and *C. brettschneideri* commonly cultivated in China and four samples of the species *C. pinnatifida* and *C scabrifolia* of natural origin were reported by Liu et al. (2010). Citric acid (20–84 g kg^{-1} DM]), quinic acid (5–56 g kg^{-1} DM),

malic acid (3–11 g kg^{-1} DM), fructose (55–184 g kg^{-1} DM), glucose (53–166 g kg^{-1} DM), sorbitol (30–157 g kg^{-1} DM) and *myo*-inositol (1–3 g kg^{-1} DM) were found in all the samples. Sucrose was present only in *C. scabrifolia* (152 g kg^{-1} DM) and three cultivars of *C. pinnatifinda* var. *major* (110–237 g kg^{-1} DM). *C. scabrifolia* contained a significantly higher level of quinic acid and a lower level of sorbitol than the rest of the samples studied ($P < 0.01$). The content of sorbitol in *C. brettschneideri* was typical higher compared to those in *C. pinnatifida* var. *major*, *C. scabrifolia* and *C. pinnatifida* ($P < 0.01$). The samples of *C. pinnatifida* var. *major* differed from *C. pinnatifida* by its high sugar content ($P < 0.01$). The hawthorn samples analysed fell into two groups: those rich in sugars and those rich in acids (Liu et al., 2010).

2.2. Health effects of the phenolic compounds of hawthorn

The biological activities and physiological effects of phenolic compounds in hawthorn have been investigated with phenolic extracts prepared from hawthorn fruits, leaves and flowering tops (leaves and flowers) by extraction with ethanol, methanol, aqueous ethanol and aqueous methanol of different concentrations. Some of these are commercial extracts of standardised composition, whereas others have been prepared at the laboratory scale. Fractions of ethanol extracts and phenolic compounds purified from hawthorn have been tested to study the biological activities of different groups or individual compounds.

2.2.1. Effects on the heart and vascular system

In Europe and the US, hawthorn extracts are used as an adjuvant therapy for patients with congestive heart failure. The species used most frequently for the development of standardised extracts and in clinical trials are *C. laevigata* and *C. monogyna*. The plant parts commonly used are leaves with flowers, often referred to as flowering tops. Recently fresh and dried fruits are also used. Two standardised extracts from the leaves and flowers of *C. laevigata* have been often used in clinical trials, in the compounds WS1442 containing 188 g kg^{-1} oligomeric procyanidins and LI123 containing 22 g kg^{-1} flavonoids. Several reviews have been published on the clinical use of hawthorn extracts as a complimentary treatment for chronic heart failure (Fong and Bauman, 2002; Furey and Tassell, 2008; Guo et al., 2009; Pittler et al., 2003; Rigelsky and Sweet, 2002).

A meta-analysis was carried out with data from ten clinical trials including 855 patients with chronic heart failure (New York Heart Association classes I to III), of which seven trials used WS1442 and three used LI132 (Guo et al., 2009). The daily dosage of the extracts ranged from 160 mg to 1800 mg. Hawthorn extracts increased

the maximal workload and exercise tolerance of the patients. Fatigue and shortness of breath were also improved by the hawthorn extracts. Overall, the results of the meta-analysis showed an improvement in heart function and alleviation of the symptoms of heart failure by the extracts, suggesting beneficial effects of the extracts when used in combination with conventional treatment for heart failure such as triamterene, hydrochlorothiazide and ACE inhibitors (Guo et al., 2009). An eight-week treatment with an aqueous ethanol (49%) extract from a mixture of fresh berries of *C. laevigata* and *C. monogyna* with a daily dosage corresponding to 6.4 mg oligomeric procyanidins and 12.7 mg total phenolic compounds significantly improved the exercise tolerance of patients with congestive heart failure of New York Heart Association class II (Degenring et al., 2003).

In vitro and animal studies have supported the cardio-protective effects of hawthorn extracts. *C. monogyna* fruit extract (by 70% aqueous ethanol) and the main phenolic compounds in the extract caused partial uncoupling of oxidative phosphorylation and suppression of H_2O_2 production in isolated rat heart mitochondria. This suggests that the potential cardio-protective effect of hawthorn extract occurs through inhibiting the generation of free radicals within mitochondria (Bernatoniene et al., 2009). Aqueous ethanol (50%) extract of dried fruits of *C. laevigata* showed radical scavenging and cardio-protective activities *in vitro* (Swaminathan et al., 2010). The extract scavenged superoxide, hydroxyl and peroxyl radicals improved the recovery of cardiac contractile function, reduced the size of the myocardial infarct, and decreased the activities of creatine kinase and lactate dehydrogenase in an experimental model of ischaemia/reperfusion injury. The extract may have played a role in the activation of anti-apoptotic pathways and suppression of pro-apoptotic pathways (Swaminathan et al., 2010).

Cardiac remodelling in response to pressure overload refers changes in heart structure in order to maintain adequate delivery of blood to the body during stress (Frey and Olson, 2003). Early remodelling is an adaptive response to increased load on the heart and/or the loss of contractile components in order to maintain pumping capacity. Chronic long-term overload results in an enlarged left ventricle, a progressive deterioration in pump function and impaired diastolic and systolic function of the left ventricle (Hwang et al., 2008; Hwang et al., 2009). Three weeks of treatment with a hawthorn extract (WS1442) attenuated systolic dysfunction of the left ventricle and modified cardiac remodelling induced by one month of aortic constriction in rats (Hwang et al., 2008).

An aqueous ethanol (50%) extract of the leaves and stems as well as the berries of *C. laevigata* showed negative chronotropic effects on spontaneously beating neonatal murine cardiomyocytes *in vitro* (Long, et al., 2006). The extracts from the leaves,

stems and fruits showed anti-arrhythmic effects and induced rhythmicity in quiescent cardiomyocytes (Long, et al., 2006). However, different chromatographic fractions of the raw extracts showed opposite chronotropic effects *in vitro* (Long, et al., 2006). Chloroform, ethyl acetate and aqueous ethanol (70%) extracts of the flowering tops of *Crataegus meyeri* A. Pojark reduced arrhythmia induced by myocardial ischaemia in open-chest anaesthetised male rats. The anti-arrhythmic effect of the aqueous ethanol extract was clearly stronger than those of the chloroform and ethyl acetate extracts (Garjani et al., 2000).

Endothelial dysfunction and inflammation play pivotal roles in the development of atherosclerosis and cardiovascular diseases. Hawthorn phenolic extracts may protect endothelial cells by regulating the expression of apoptosis-associated genes (Ling et al., 2008). Restenosis may result from the biological response of the arterial wall to local injury caused by angioplasty. Neointima formation is a crucial process in restenosis (Weintraub, 2007). Oral administration of a hawthorn phenolic extract reduced neointima formation after balloon catheter dilatation of the carotid artery in rats (Furst et al., 2010). Vessel injury induces release of mediators such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF), promoting the migration and proliferation of vascular smooth muscle cells (VSMCs) and neointimal hyperplasia (Levitzki, 2005). Hawthorn extract decreased serum-induced VSMC migration and proliferation in rat carotid artery model *in vitro* (Furst et al., 2010). The extract inhibited VSMC DNA synthesis induced by PDGF but not that by basic fibroblast growth factor (bFGF) and EGF. The extract decreased the activation of PDGF receptor-β and ERK signalling in VSMCs (Furst et al., 2010). Therefore, the hawthorn extract may inhibit the proliferation and migration of VSMCs without retarding endothelialisation, a major healing mechanism in recovery from vessel injury.

2.2.2. Hypolipidaemic effects

The ethanol/aqueous ethanol extracts of fruits of *C. laevigata* (Rajendran et al., 1996) and *C. pinnatifida* (Kuo et al., 2009; Ye et al., 2010; Zhang et al., 2002a) as well as the fruit powder of *C. pinnatifida* (Kwok et al., 2010) have shown lipid-lowering effects in animals fed with high fat diets. The extracts lowered the level of plasma total and LDL cholesterol and increased the level of HDL cholesterol. In addition, *C. pinnatifida* fruit extract decreased the plasma level of triacylglycerols (Kuo et al., 2009; Ye et al., 2010). The mechanism of the hypolipidaemic effects may have involved the following aspects: 1) The extracts may have reduced the absorption of dietary cholesterol by downregulating the activity of intestinal acyl CoA:cholesterol acyltransferase (ACAT) (Zhang et al., 2002a). 2) The extracts may have enhanced the clearance of circulating LDL through increased expression of hepatic LDL receptor

(Rajendran et al., 1996). 3) The extracts may have reduced the biosynthesis of cholesterol *via* inhibition of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), a rate-limiting enzyme in cholesterol biosynthesis (Ye et al., 2010). 4) The extracts may have increased the metabolism of cholesterol into bile acids by upregulation of hepatic cholesterol 7α-hydroxylase activity (Rajendran et al., 1996; Zhang et al., 2002a). Quercetin, hyperoside, rutin and chlorogenic acid are likely among the major bioactive compounds responsible for the hypolipidaemic effects of hawthorn extracts (Ye et al., 2010). These compounds have shown synergy in inhibiting the activity of HMG-CoA reductase *in vitro*. The inhibitory effects on HMG-CoA reductase may be positively associated with the hydrophobicity of the compounds (Ye et al., 2010).

Gavage administration of an aqueous ethanol (80%) extract of *C. pinnatifida* fruits (flavonoid content of25 g kg^{-1} in the extract), at 200 mg kg^{-1} body weight (BW) for seven days, reduced food intake, body weight, weight of adipose tissue and plasma triacylglycerol level in male Syrian golden hamsters fed with a high fat diet (Kuo et al., 2009). The extract reduced the accumulation of fat droplets in 3T3-L1 adipocytes in a dose-dependent manner *in vitro*. The hypolipidaemic effects of the hawthorn phenolic extract were associated with activation of PPARα in the adipose tissue of the hamsters and were reversed by the treatment with MK886, a PPARα antagonist (Kuo et al., 2009). The hypolipidaemic effects of the phenolic extract of hawthorn may be partially mediated by regulation of the activity lipoprotein lipase (LPL) in muscle and in adipose tissue (Fan et al., 2006).

2.2.3. Hypotensive effects

The hypotensive potential of phenolic fractions of hawthorn has been investigated *in vitro*, in animal models and in clinical studies. Hawthorn extracts have been shown to induce endothelium-dependent NO-mediated vasorelaxation (Brixius et al., 2006; Chen et al., 1998) and inhibition of Ca^{2+} influx to the smooth muscle (Chen et al., 1998).

An ethanol extract from the fruits of *Crataegus* spp. counteracted vasoconstriction in U46619- precontracted rat mesenteric arteries (Chen et al., 1998). The activity of the extract was endothelium-dependent and was significantly reduced by pretreatment of the arteries with nitric oxide synthase (NOS) inhibitors [N^{ω} nitro-L-arginine methyl ester (L-NAME) or methylene blue], suggesting an increased production of NO by the endothelial cells as the key mechanism of the vasodilating effects (Chen et al., 1998). An aqueous extract of *Crataegus tanacetifolia* leaves (100 mg kg^{-1} BW per day) and a hyperoside-rich fraction of the extract (6 mg kg^{-1} BW per day) were given to N^{ω} nitro-L-arginine methyl ester (L-NAME)-induced hypertensive rats for 4 weeks by gavage.

The hyperoside-rich fraction significantly attenuated the increase in mean arterial blood pressure. Both the leaf extract and the hyperoside-rich fraction reduced the thickening of the media layer of the coronary blood vessels caused by N^{ω} nitro-L-arginine methyl ester (L-NAME) treatment (Koçyildiz et al., 2006). The addition of *C. pinnatifida* fruit powder to the high fat diet of Sprague-Dawley rats improved acetylcholine-induced relaxation of isolated aortas pretreated with phenylephrine (Kwok et al., 2010). Bolus injection of an aqueous methanol (70%) extract of the flowering tops of *C. meyeri* caused a dose-dependent reduction in mean arterial blood pressure in male Wistar rats (Garjani et al., 2000).

In a double-blind, randomised, placebo-controlled clinical trial, supplementation with an extract of the flowering tops of *C. laevigata*, 1200 mg daily for 16 weeks, reduced diastolic blood pressure compared with placebo in hypertensive type 2 diabetes patients (Walker et al., 2006). The extract contained 22 g kg^{-1} flavonoids (Walker et al., 2006). Consumption of an aqueous ethanol extract of leaves and flowers of *C. curvisepala* for three months decreased both the systolic and diastolic blood pressures in subjects with primary mild hypertension (Asgary et al., 2004).

2.2.4. Antioxidant and radical scavenging activities

The antioxidant and radical scavenging activities of extracts of different *Crataegus* species have been investigated in various *in vitro* assays. The pulp and peel of hawthorn of undefined species showed the highest activity in ferric reducing/antioxidant power (FRAP) assay among 28 fruits studied, the ferric-reducing activity of the peel being twice as high as that of the pulp (Guo et al., 2003). Methanol and aqueous extracts of *C. pentagyna* subsp. *elburensis* showed 2,2-diphenyl-picrylhydrazyl (DPPH) radical-scavenging, nitric oxide radical scavenging and Fe^{2+} chelating activities. Higher Fe^{2+} chelating activity was seen in the methanol extract than in the aqueous extract (Ebrahimzadeh and Bahramian, 2009).

Ethyl acetate extracts prepared from the floral buds and open flowers of *C. azarolus* (Bahri-Sahloul et al., 2009) and fruit extracts of *C. monogyna* prepared with ethanol and water (Bernatoniene et al., 2008) were effective in scavenging $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radicals. An ethanol/water (3/2, v/v) extract of flower-bearing branches of *C. laevigata* showed antioxidant potential in the ABTS assay (Periera da Silva et al., 2000). Chlorogenic acid, hyperoside, rutin, spiraeoside, isoquercitrin, quercetin, (-)-epicatechin and procyanidin B2 were suggested to be the compounds with strong radical-scavenging activities in floral bud extracts of *C. azarolus* (Bahri-Sahloul et al., 2009). (-)-epicatechin and (+)-catechin showed the highest radical scavenging activity among the phenolic compounds studied. Clear synergistic effects were observed among different phenolic compounds (Bernatoniene et

al., 2008). The ethanol extract of *C. monogyna* fruits contained higher levels of phenolic compounds and showed greater radical scavenging activities than the aqueous extract of the fruits (Bernatoniene et al., 2008).

Extracts prepared with methanol/acetone/H_2O (7/7/3, v/v/v) from the dried flowers and flowering tops of *C. monogyna* contained higher levels of phenolic compounds than those from fresh and dried fruits (as gallic acid equivalents, 50 g kg^{-1} *vs*. 10 g kg^{-1} DM) (Froehlicher et al., 2009). The extract from dried flowers contained the highest content of procyanidins (as cyanidin equivalent, 17 g kg^{-1} DM). The extracts from flowering tops and flowers showed higher antioxidant/radical scavenging activities than those from fresh and dried fruits in the ABTS, DPPH and Cu^{2+}-induced LDL-oxidation assays (Froehlicher et al., 2009). Corresponding extracts were prepared from cell lines selected from the calli from flowering buds of the same species. The extract from a red cell line contained a higher level of total phenolic compounds and showed higher antioxidative and radical scavenging activities than the flower extract, indicating the potential of cell culture as an alternative method for the production of phenolic compounds of hawthorn (Froehlicher et al., 2009). The reference compounds quercetin, procyanidin B2 and (-)-epicatechin showed higher activities than those of chlorogenic acid, hyperoside and rutin in these assays (Froehlicher et al., 2009).

An aqueous extract from a concoction of leaves and unripe fruits of *C. aronia* syn. *azarolus* L. inhibited the oxidation of β-carotene in the coupled oxidation of β-carotene and linoleic acid, and also inhibited 2,2´-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced lipid oxidation in human plasma and Fe^{2+}-induced lipid peroxidation in rat liver homogenates (Ljubuncic et al., 2005). The extract scavenged superoxide radicals in the nitro-blue tetrazolium reduction assay and increased intracellular glutathione levels in cultured Hep G2 cells. A clear dose-dependent effect was seen in the effects of the extract in most assays (Ljubuncic et al., 2005).

The oxidation of LDL plays a key role in the early stage of atherogenesis. Both Cu^{2+}-induced and macrophage-mediated LDL oxidation may be involved in the process. Antioxidants protecting LDL from oxidative stress may be beneficial in lowering the incidence of atherosclerosis and cardiovascular diseases. A hot water extract of the dried fruits of *C. pinnatifida* reduced LDL oxidation in a Cu^{2+}-induced cell-free system as well as in sodium nitroprusside (SNP)-treated RAW 264.7 macrophage cells (Chu et al., 2003). The ethyl acetate fraction of the ethanol extract of *C. pinnatifida* fruits protected LDL from Cu^{2+}-induced oxidation *in vitro* (Zhang et al., 2001d). Protocatechuic acid, chlorogenic acid, quercetin, hyperoside, isoquercitrin, rutin and (-)-epicatechin purified from the ethyl acetate extract reduced the production of thiobarbituric acid-reactive substances (TBARS) in Cu^{2+}-induced LDL-oxidation and inhibited peroxy radical-induced oxidation of α-tocopherol and LDL in the 2,2´-azobis(2-amidinopropane) dihydrochloride (AAPH)

assay. The effects were dose-dependent. Quercetin, isoquercitrin and hyperoside showed stronger effects than the other phenolic compounds. Supplementation of hawthorn fruit powder (2% of the fodder) to rats fed with a canola oil-rich diet for 6 weeks increased the serum α-tocopherol level by 20% compared to the control group without hawthorn fruit supplementation (Zhang et al., 2001d). The procyanidin fraction (DP 1-3) of the fruits of *C. pinnatifida* var. *major* scavenged superoxide and hydroxyl radicals and inhibited lipid peroxidation *in vitro* (Jin and Liu, 2007).

Prolyl endopeptidase is a serine protease that specifically degrades proline-containing peptides, such as neuropeptides involved in the process of learning and memory. Abnormally high activity of prolyl endopeptidase is implicated in Alzheimer's disease (Toide et al., 1998). The ethanol extract of the fruits of *C. pinnatifida* var. *major* (three extractions with 95%, 80% and 80% ethanol overnight) contained 25 g kg^{-1} polyphenols. The ethanol extract was further extracted with ethyl acetate. The ethyl acetate fraction inhibited the activity of prolyl endopeptidase and showed $O_2^{\cdot-}$ and $^{\cdot}OH$ scavenging activities (Cui et al., 2006b). The ethyl acetate fraction also inhibited the activities of tyrosinase and lipoxygenase. Tyrosinase is a key enzyme in the biosynthesis of melanin from tyrosine induced by UV irradiation. Hawthorn phenolic extract may have potential as a functional ingredient for skin care products with skin lightening effects. Lipoxygenase catalyses conversion of arachidonic acid to series-4 leukotrienes, a group of eicosanoids with pro-inflammatory and vasoconstricting activities. The ethyl acetate fraction contained 214 g kg^{-1} polyphenols, consisting of procyanidins (197 g kg^{-1}, DP 1-5), chlorogenic acid (12 g kg^{-1}) and flavonoids (5 g kg^{-1}). The composition of the ethyl acetate fraction differed from that of the ethanol extract by a significantly higher (about 10 times higher) content of (-)-epicatechin and procyanidin B2. The ethyl acetate fraction showed greater radical scavenging activity and inhibitory effects on tyrosinase compared with the ethanol extract (Cui et al., 2006b). A commercial hawthorn leaf extract rich in vitexin-2″-rhamnoside (200 g kg^{-1} DM) showed radical scavenging activities and inhibitory effects on tyrosinase, lipoxygenase and prolyl endopeptidase comparable to those observed in the ethyl acetate fraction from the fruits. In addition, the leaf extract showed an inhibitory effect on elastase, an activity not seen in the fruit extracts (Cui et al., 2006b).

Ionising radiation of living tissues generates free radicals, which induce DNA damage and lead to mutagenesis and carcinogenesis. A *Crataegus microphylla* fruit extract (prepared with 75% aqueous methanol) showed radioprotective effects against genotoxicity induced by gamma irradiation in mouse bone marrow cells (Hosseinimehr et al., 2007). Antioxidative and radical scavenging activities are likely among the key mechanisms of the radio-protective effect of the hawthorn extract (Hosseinimehr et al., 2007).

2.2.5. Anti-inflammatory activities

A flavonoid extract from the dried fruits of *C. pinnatifida* decreased the production of prostaglandin E_2 (PGE_2) and NO induced by lipopolysaccharide (LPS) in macrophage RAW 264.7 cells *in vitro* (Kao et al., 2005). Pretreatment of rats with the flavonoid extract (50-200 mg kg^{-1} BW per day by gavage) for 5 days significantly attenuated the increase in the activities of hepatic alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) induced by lipopolysaccharide. The extract also attenuated neutrophil infiltration and liver necrosis induced by lipopolysaccharide. The extract decreased the hepatic expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) induced by lipopolysaccharide in rats (Kao et al., 2005). iNOS expression can be induced in various cells such as macrophages, smooth muscle cells and hepatocytes by exposure to endogenous and exogenous stimulants. Increased NO production by the expression of iNOS is involved in the process of inflammation (Duval et al., 1996). Inhibitors of iNOS activity might offer protection from inflammation and hepatic damage induced by endogenous and exogenous toxins (Zhang et al., 2000a). Pretreatment with a topical application of a hot water extract from the fruits of *C. pinnatifia* reduced 12-*O*-tetradecanoylpohorbol-13-acetate (TPA)-induced mouse ear oedema (Kao et al., 2007). The extract downregulated the expression of COX-2 and iNOS and inhibited the activation of ornithine decarboxylase in the skin treated with TPA (Kao et al., 2007). In addition, the extract inhibited the generation of reactive oxygen species and benzo[*a*]pyrene/TPA-induced skin tumour formation as well as TPA-induced transformation of JB6 P^+ cells (Kao et al., 2007).

A 70% aqueous ethanol extract of the mixed fruits of *C. monogyna* and *C. oxycantha* (1:1) contained 35 g kg^{-1} phenolic compounds (Tadić et al., 2008). The extract showed dose-dependent scavenging activity on $DPPH^{\cdot}$ radicals *in vitro*. Administered by gavage, the extract reduced carrageenan-induced paw oedema and protected the gastric mucosa from ethanol-induced ulcers in rats (Tadić et al., 2008). Moderate bactericidal activity of the extract was observed against the Gram-positive bacteria *Micrococcus flavus, Bacillus subtilis* and *Listeria monocytogenes* (Tadić et al., 2008).

2.3. Composition of *Phyllanthus emblica*

2.3.1. Phenolic compounds

Phenolic compounds including hydrolysable tannins, proanthocyanidins, flavanols, flavonols and compounds belonging to other phenolic groups have been reported in different parts of emblic leafflower (*Phyllanthus emblica*) plants (Table 2).

2.3.1.1. Hydrolysable tannins

Hydrolysable tannins are derivatives of gallic acid (3,4,5-trihydroxy benzoic acid). Gallic acid is esterified to a core polyol, and the galloyl groups may be further esterified or oxidatively crosslinked to yield more complex hydrolysable tannins (Hagerman, 2002).

Tannins are considered the major bioactive components of the fruits of *Phyllanthus emblica* (*PE*) (Ghosal et al., 2006). Jacob et al. determined the tannin content of *PE* as 4% of the fresh fruit weight (Jacob et al., 1988). In a study by Wu and Zhou, the tannin content of *PE* fruit powder was 350 g kg^{-1} DM (Wu and Zhou, 1996). In the ripening period of the fruits, the highest content of tannins was detected at the early growth stage of the fruits. The level was as high as 40 g kg^{-1} (Chen et al., 1995). In different parts of the plant of *P. emblica*, both simple esters of gallic acid with other acids and hydrolysable tannins were found. The structures are presented in **Figures 7–11**.

Although many investigations have been carried out on hydrolysable tannins, there is still debate as to the compositional profiles of hydrolysable tannins in *PE*. For instance, Ghosal et al. reported that the fruits of *PE* contain hydrolysable tannins, emblicanin A (2,3-di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-2-keto-gluconolactone) and emblicanin B (2,3,4,6-bis-(*S*)-hexahydroxydiphenoyl-2-keto-gluconolactone), along with pedunculagin and punigluconin (**Figure 7**) (Ghosal et al., 2006). However, Majeed et al. suggested that emblicanin A and B did not exist in the fruits of *PE*. The compounds identified as emblicanin A and B by Ghosal were shown to be 1(*β*)-*O*-galloylglucose (**Figure 9**) and mucic acid 1,4-lactone 5-*O*-gallate (**Figure 8**) according to their NMR spectra, respectively. In addition, mucic acid 2-*O*-gallate (**Figure 8**) and its two isomers were identified (Majeed et al., 2009).

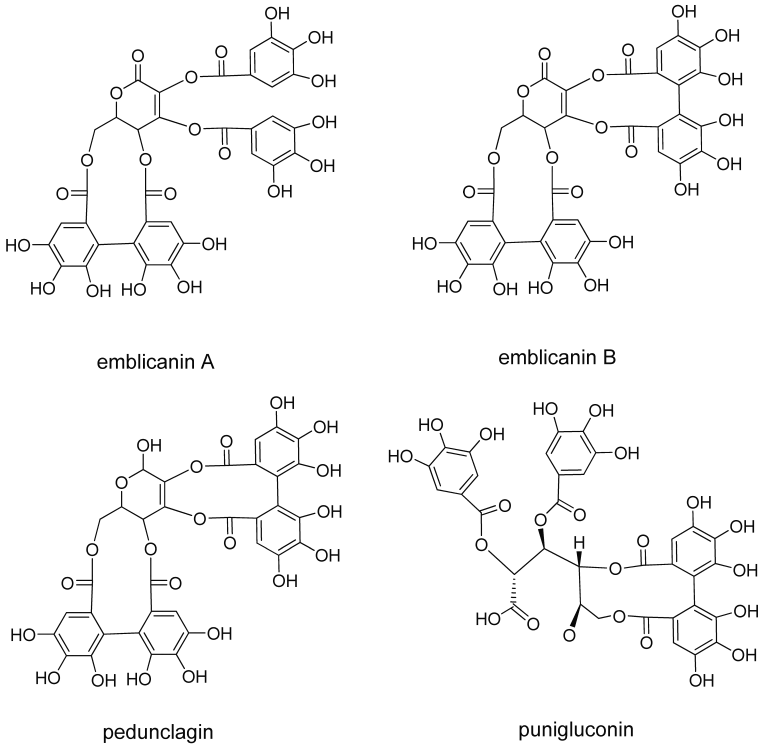


Figure 7. Hydrolysable tannins in *P. emblica* (1)

The team of Zhang carried out a series of studies to investigate the compositional profiles of different parts of *PE* (Zhang, et al., 2000b; 2000c; 2001e; 2001f; 2001g).

In the juice of *PE*, fourteen phenolic constituents were isolated and identified by Zhang et al. (2011e). Most of them were simple esters of gallic acid (**Figure 8**). Three known compounds, gallic acid, chebulic acid (**Figure 11**) and 1(*β*)-*O*-galloylglucose, were identified by comparison with the physical and spectral data of reference compounds. Six new esters of gallic acid including L-malic acid 2-*O*-, mucic acid 2-*O*-, mucic acid 1,4-lactone 2-*O*- , 5-*O*-, 3-*O*- and 3,5-di-*O*-gallates were isolated and their structures were determined by spectral and chemical methods. Methyl esters of these compounds were also found and identified. However, the authors suggested that most of the methyl esters were generated during sample preparation. Mucic acid 2-*O*-gallate, mucic acid 1,4-lactone 2-*O*-gallate and mucic acid 5-*O*-gallate were the major phenolic constituents of the juice together with 1(*β*)-*O*-galloylglucose (Zhang et al., 2001e).

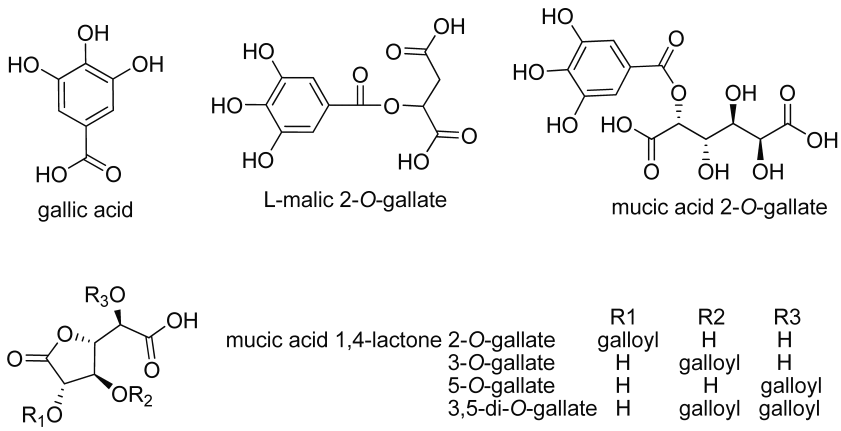


Figure 8. Simple esters of gallic acid

In another study, the 60% aqueous acetone extract of the fruits of *PE* was purified using MCI-gel CHP 20P, Sephadex LH-20, MCI-gel CHP 20P, Chromatorex ODS and Toyopearl HW-40F column chromatography. A new ellagitannin, phyllanemblinin A and eleven known hydrolysable tannins were isolated and identified by spectral and chemical methods. Phyllanemblinin A was confirmed to be ellagitannin derived from furosin. The eleven known hydrolysable tannins were identified as 1(*β*)-*O*-, 1(*β*),6-di-*O*- and 1(*β*),2,3,6-tetra-*O*-galloylglucose, corilagin, chebulanin, chebulagic acid, elaeocarpusin, punicafolin, tercatain, mallonin and putranjivain A. Structures of some of these compounds are presented in **Figures 9–11** (Zhang et al., 2001f).

In the ethanol extract of the fresh leaves and branches of *PE*, Zhang et al. isolated and identified five new ellagitannins named as phyllanemblinins B-F (**Figure 10**). An additional 25 known tannins and related compounds were isolated and identified. Phyllanemblinin B was identified as an ellagitannin having a hexahydroxydiphenoyl group derived from furosin. Phyllanemblinin C is structurally related to chebulagic acid. Phyllanemblinins D, E and F were found to be positional isomers of neochebuloyl-1-*β*-*O*-galloylglucose. The known tannins were 1(*β*)-*O*-, 1(*β*),4-di-*O*- and 3,6-di-*O*-galloylglucose, corilagin, furosin, chebulanin, chebulagic acid, mallonin, putranjivains A and B, neochebulagic acid, carpinusnin, geraniin, gallic acid 3-*O*-*β*-D-glucoside, gallic acid 3-*O*-(6′-*O*-galloyl)-*β*-D-glucoside, flavogallonic acid bislactone and chebulic acid (Zhang et al., 2001f).

In a subsequent study by Zhang et al., the ethanol extract of the fresh leaves and branches of *PE* was suspended in water and then extracted with diethyl ether. The diethyl ether layer was further partitioned between hexane and MeOH, and the MeOH layer was chromatographed successively over Sephadex LH-20, silica gel, MCI-gel CHP 20P and Chromatorex ODS columns to yield 19 known compounds. Of these

compounds, four tannins, 1(*β*),2,3,6-tetra-*O*-, 1(*β*)-,2,4,6-tetra-*O*- and 1(*β*)-,2,3,4,5-penta-*O*-galloylglucose as well as decarboxyellagic acid were identified by comparison with the physical and spectral data in the literature. The water layer was purified by the same method, followed by additional silica gel column chromatography to afford seven flavonoids and two other compounds (Zhang et al., 2002b).

Another study carried out by Zhang's group investigated the compositional profile of the 60% aqueous acetone extract of the air-dried roots of *PE*. The crude extract was partitioned between ethyl ester and water. In the ethyl ester phase, 13 compounds were identified as gallic acid, pyrogallol, protocatechuic acid, corilagin, 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose, 1,2,3,4,6-penta-*O*-galloyl-*β*–D-glucose, (±)-gallocatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin 3-*O*-gallate, (-)-epicatechin 3-*O*-gallate and (-)-epicatechin-(4*β*→8)-epigallocatechin 3-*O*-gallate, respectively (Zhang et al., 2000b).

Zhang et al. isolated six compounds from the 70% acetone extract of the dry fruit powder of *PE*. Their structures were investigated by UV, IR, MS and NMR spectrometry. Two new tannins, 3-ethoxy-gallic acid and isostrictinin, were found in fruits of *PE* for the first time (Zhang et al., 2003b).

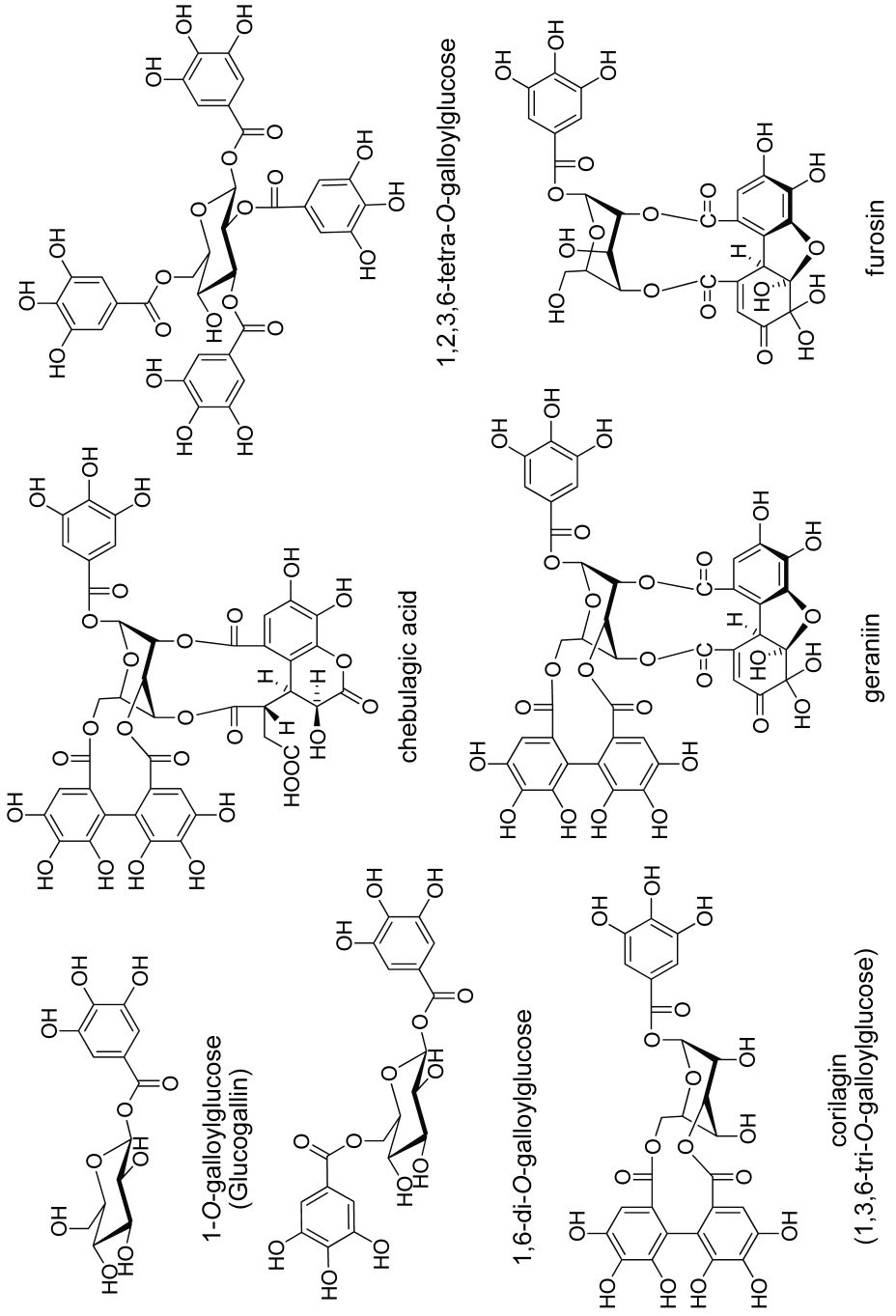


Figure 9. Hydrolysable tannins in *P. emblica* (2).

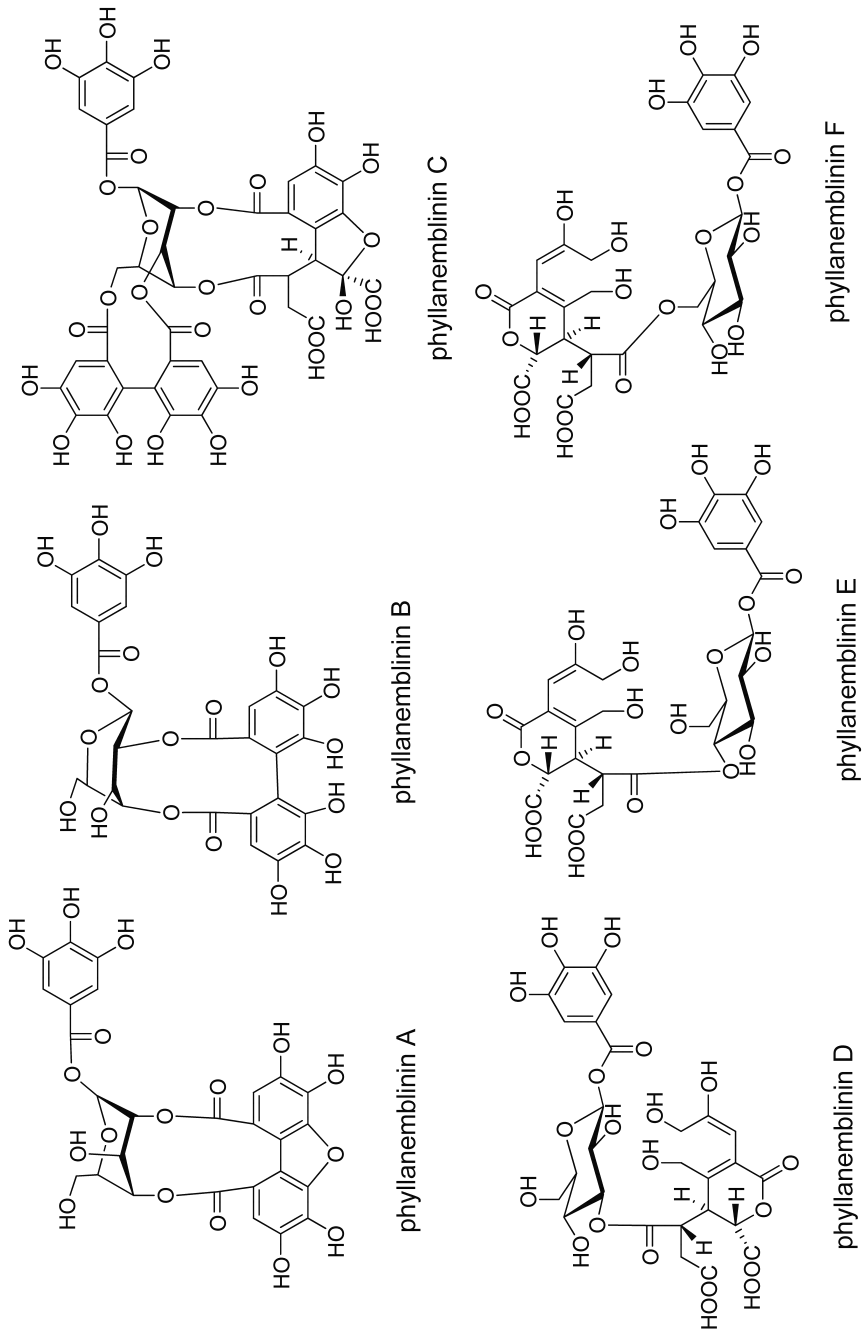


Figure 10. Hydrolysable tannins in *P. emblica* (3).

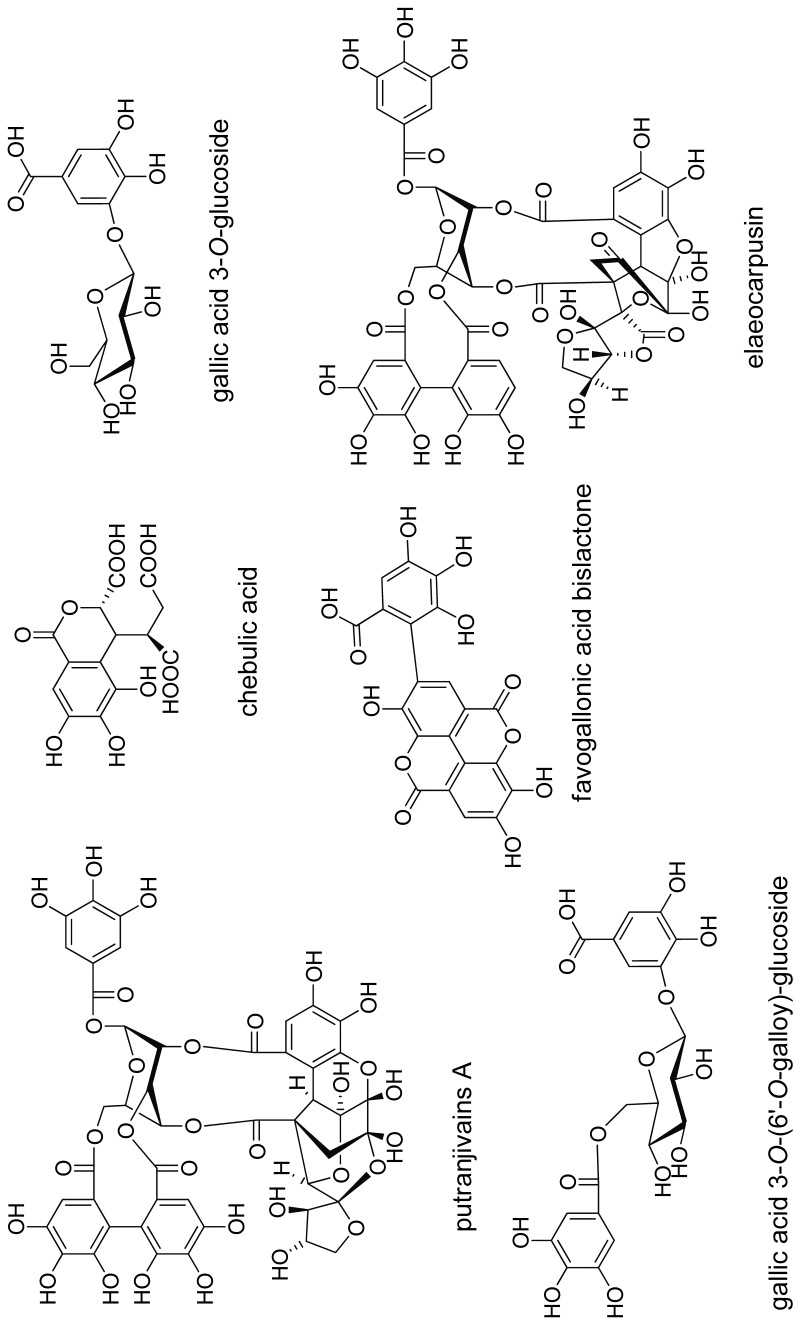


Figure 11. Hydrolysable tannins in *P. emblica* (4).

2.3.1.2. Flavonoids and phenolic acids

Flavonoids form the other major phenolic group found in *PE*. Compounds belonging to the flavanols, proanthocyanidins, flavonols, flavanonols and flavanones as well as their derivatives have been reported. **Figures 12** and **13** show the structures of the flavonoids detected in *PE*.

Zhang et al. reported the existence of flavanols and their oligomers (proanthocyanidins) (**Figure 12**) in *PE*. (-)-Epiafzelechin, (+)-gallocatechin, (-)-epigallocatechin, (-)-epicatechin, prodelphinidin B1 and B2, (-)-epicatechin-(4*β*→8)-epigallocatechin and prodelphinidin B2 3′-*O*-gallate were isolated from an ethanol extract of the fresh leaves and branches of *PE* (Zhang et al., 2001f). In extracts of the roots of *PE*, two new compounds were characterised as epigallocatechin-(2*β*→7, 4*β*→8)-gallocatechin (prodelphinidin A1) and (-)-epicatechin-(4*β*→8)-gallocatechin by negative-ion FABMS and ^{1}H-NMR. The structure of (-)-epicatechin-(4*β*→8)-gallocatechin was also confirmed by thiolysis (mercaptoethanol-HCl), yielding gallocatechin and (-)-epicatechin-4-(2-hydroxy)ethylthio ether (Zhang et al., 2000b). In the same fraction, they also found (±)-gallocatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin 3-*O*-gallate, (-)-epicatechin 3-*O*-gallate and (-)-epicatechin-(4*β*→8)-epigallocatechin 3-*O*-gallate (Zhang et al., 2000b).

In a study by Zhang et al., fourteen flavonoids, including naringenin (**Figure 13**), eriodictyol (**Figure 13**), kaempferol (**Figure 13**), dihydrokaempferol, quercetin (**Figure 13**), naringenin 7-*O*-glucoside (prunin), naringenin 7-*O*-(6″-*O*-galloyl)-glucoside, naringenin 7-*O*-(6″-*O*-*trans*-*p*-coumaroyl)-glucoside, kaempferol 3-*O*-rhamnoside, quercetin 3-*O*-rhamnoside, myricetin 3-*O*-rhamnoside and (-)-epigallocatechin 3-*O*-gallate, were identified in the Et_2O fraction of the ethanol extract of *PE* roots by comparison of the physical and spectral data with literature values. In addition, two new flavonoids were identified as (*S*)-eriodictyol 7-*O*-(6″-*O*-*trans*-*p*-coumaroyl)-β-D-glucopyranoside and (*S*)-eriodictyol 7-*O*-(6″-*O*-galloyl)-β-D-glucopyranoside by NMR in the same fraction. Seven flavonoids, including eriodictyol 7-*O*-glucoside, kaempferol 3-*O*-rhamnoside, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-glucoside, myricetin 3-*O*-rhamnoside, rutin and 3-*O*-methylellagic acid 4′-*O*-α-L-rhamnopyranoside were identified in the water layer (Zhang et al., 2002b). Most of these compounds were flavonols, flavanonols and flavanones. **Figure 13** shows structure the aglycones.

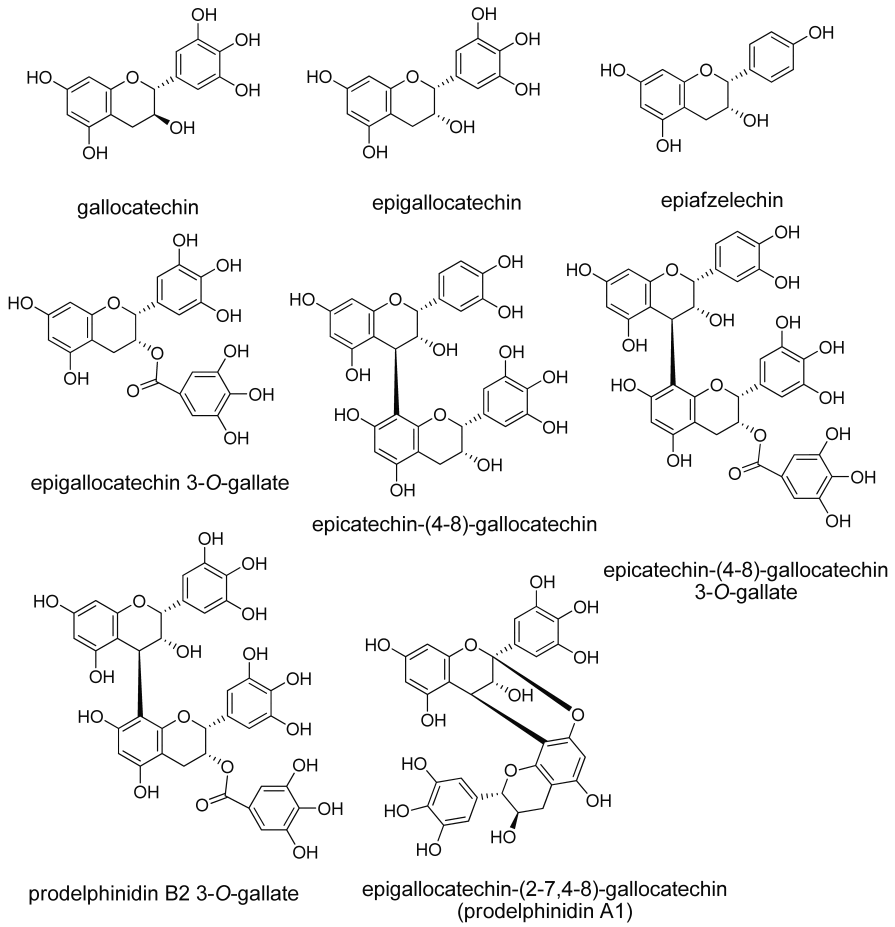


Figure 12. Flavanols and proanthocyanidins in *P. emblica*.

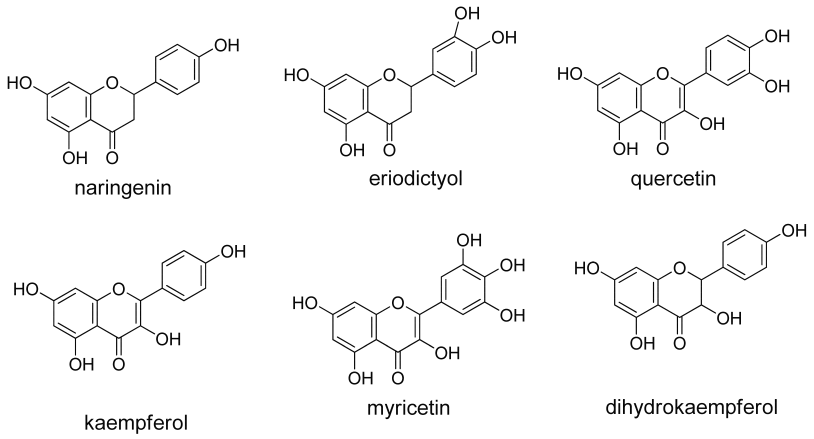


Figure 13. Aglycones of the flavonols, flavanonols and flavanones in *P. emblica*.

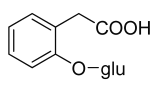
Luo et al. isolated six compounds from the 95% ethanol extract of the dry fruit of *PE*. They were identified as cinnamic acid, quercetin, 5-hydroxymethylfurfural, gallic acid, *β*-daucosterol and ellagic acid using mass spectrometry and NMR. Cinnamic acid and 5-hydroxymethylfurfural were identified as components of *PE* fruit for the first time (Luo et al., 2009).

Habib-ur-Rehman et al. investigated the ethanol extract of the shoots and leaves of *PE* with UV, IR, MS and 2D-NMR. Two derivatives of kaempferol, i.e. kaempferol-3-*O*-α-L-(6″-methyl)-rhamnopyranoside and kaempferol-3-*O*-α-L-(6″-ethyl)-rhamnopyranoside were isolated and identified (Habib-ur-Rehman et al., 2007).

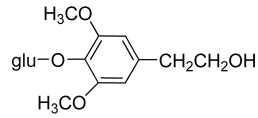
El-Desouky et al. isolated a new acylated apigenin glucoside [apigenin-7-O-(6″-butyryl)-β-glucopyranoside] from the methanol extract of the leaves of *PE* together with the known compounds gallic acid, methyl gallate, 1,2,3,4,6-penta-*O*-galloylglucose and luteolin-4′-*O*-neohesperiodoside. Their chemical structures were elucidated on the basis of NMR spectroscopic studies (^{1}H NMR, ^{13}C NMR) (El-Desouky et al., 2008).

2.3.1.3. Other phenolics

Two new phenolic glycosides, 2-carboxylmethylphenol 1-*O*-*β*-D-glucopyranoside and 2,6-dimethoxy-4-(2-hydroxyethyl)phenol 1-*O*-*β*-D-glucopyranoside (**Figure 14**), were isolated from 60% aqueous acetone extract of the air-dried roots of *PE* and identified by FABMS and NMR (Zhang et al., 2001g).



2-carboxylmethylphenol
1-*O*-glucoside



2,6-dimethoxy-4-(2-hydroxyethyl)phenol
1-*O*-glucoside

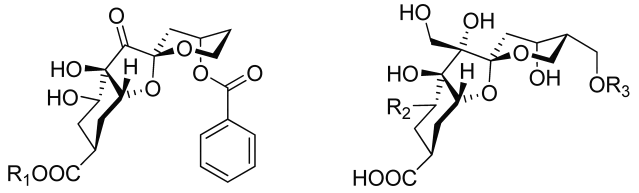
Figure 14. Two new phenolic glycosides found in roots of *P. emblica*.

Table 2. Phenolic compounds found in plant of *P. emblica*.

Plant parts	Hydrolysable tannins	Flavonoids	Other phenolics
Fruits	chebulanin, chebulagic acid, chebulic acid, corilagin, elaeocarpusin, emblicanin A&B, ellagic acid, gallic acid, methyl gallate, 3-ethoxy-gallic acid, L-malic acid 2-O-gallates, mucic acid 2-O-gallates, mucic acid 1,4-lactone 2-O- , 5-O-, 3-O-, and 3,5-di-O-gallates, 1(β)-O- , 1(β),6-di-O-, and 1(β),2,3,6-tetra-O-galloylglucose, isostrictinin, mallonin, pedunculagin, phyllanemblinin A, punicafolin, putranjivain A, punigluconin, tercatain	quercetin	cinnamic acid
Leaves & branches	carpinusnin, chebulanin, chebulagic acid, chebulic acid, corilagin, flavogallonic acid bislactone, furosin, gallic acid 3-O-β-D-glucoside, gallic acid 3-O-(6′-O-galloyl)-β-D-glucoside, 1(β)-O-, 1(β),4-di-O-, and 3,6-di-O-galloylglucose, geraniin, mallonin, neochebulagic acid, phyllanemblinins B-F, putranjivains A & B	Flavanols: (-)-epiafzelechin, (+)-gallocatechin, (-)-epigallocatechin, (-)-epicatechin Proanthocyanidins: prodelphinidins B1 & B2, (-)-epicatechin-(4 β →8)-epigallocatechin, prodelphinidin B2 3′-O-gallate Flavones: apigenin-7-O-(6″-butyryl)-β-glucopyranoside, luteolin-4′-O-neohesperiodoside	
Roots	corilagin, decarboxyellagic acid, gallic acid, 1(β),2,3,6-tetra-O-galloylglucose, 1(β),2,3,4,6-penta-O- galloylglucose, 1(β),2,4,6-tetra-O-galloylglucose, 1(β),2,3,4,5-penta-O- galloylglucose, 3-O-methylellagic acid 4′-O-α-L-rhamnopyranoside, pyrogallol, protocatechuic acid	Flavanols: (±)-gallocatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin 3-O-gallate, (-)-epicatechin 3-O-gallate Proanthocyanidins: (-)-epicatechin-(4β→8)-gallocatechin, epigallocatechin-(2β→7, 4β→8)-gallocatechin (prodelphinidin A1), (-)-epicatechin-(4β→8)-epigallocatechin 3-O-gallate Flavanones: naringenin, naringenin 7-O-glucoside, naringenin 7-O-(6″-O-galloyl)-glucoside, naringenin 7-O-(6″-O-trans-p-coumaroyl)-glucoside, eriodictyol, eriodictyol 7-O-glucoside, (S)-eriodictyol 7-O-(6″-O-trans-pcoumaroyl)-β-D-glucopyranoside, (S)-eriodictyol 7-O-(6″-O-galloyl)-β-D-glucopyranoside; Flavonols: myricetin 3-O-rhamnoside, kaempferol, kaempferol 3-O-rhamnoside, quercetin, quercetin 3-O-rhamnoside, quercetin 3-O-glucoside, rutin; Flavanonol: dihydrokaempferol	2-carboxylmethylphenol 1-O-β-D-glucopyranoside 2,6-dimethoxy-4-(2-hydroxyethyl)phenol 1-O-β-D-glucopyranoside

2.3.2. Norsesquiterpenoids and sesquiterpenoids

Zhang et al. reported a novel highly oxygenated norbisabolane from the roots of *PE* and named it phyllaemblic acid (**Figure 15**). The compound was isolated from an 60% aqueous acetone extract of the air-dried roots of *PE*. The structure of the compound was fully characterised by 1D- and 2D-NMR and FABMS (Zhang et al., 2000f).



Norsesquiterpenoids Sesquiterpenoids

	R_1	R_2	R_3
Phyllaemblic acid	H	-	-
Phyllaemblicin A	β-D-Glu	-	-
Phyllaemblicin B	β-D-Glu-(2→1)-β-D-Glu	-	-
Phyllaemblicin C	β-D-Glu-(2→1)-β-D-Glu-(2→1)-α-L-Ara	-	-
Phyllaemblic acid methyl ester	CH_3	-	-
Phyllaemblic acid B	-	OH	H
Phyllaemblic acid C	-	H	H
Phyllaemblicin D	-	H	Glu

Figure 15. Norsesquiterpenoids and sesquiterpenoids found in *P. emblica*.

Zhang et al. investigated the norsesquiterpenoids and sesquiterpenoids in a 60% aqueous acetone extract of the air-dried roots of *PE*. The crude extract was partitioned between ethyl ester and water. In the organic fraction, phyllaemblic acid and one of its methyl esters and three novel glycosides, phyllaemblicins A, B and C were isolated and identified. The structures of these compounds were established by negative-ion FABMS and ^{1}H NMR (Zhang et al., 2000b). In another study carried out by Zhang et al., the crude 60% aqueous acetone extract of the air-dried roots of *PE* was partitioned successively with ethyl acetate and 1-butanol. Three novel bisabolane-type sesquiterpenoids, phyllaemblic acids B and C and phyllaemblicin D, were isolated from the 1-butanol layer and identified by FABMS and NMR (Zhang et al., 2001g).

Figure 15 presents the structures of the norsesquiterpenoids and sesquiterpenoids found in *PE*. The nosesquiterpenoid has a basic tricyclic skeleton consisting of thirteen carbon atoms; the basic tricyclic skeleton of sesquiterpenoids consists of fifteen carbon atoms.

2.3.3. Vitamin C and other vitamins

The therapeutic potential of the *PE* fruits was attributed to their ascorbic acid content for many years. Researchers believed that the fruits were rich in vitamin C, and because of the presence of tannins, the ascorbic acid was not easily oxidised, even in the dried fruit (Kapoor, 1990). However, Ghosal et al. (1996) used HPLC to show that *PE* fruits do not contain ascorbic acid, either in the free or in the conjugated form. Instead, two hydrolysable tannins of low molecular weight, namely, emblicanin A and emblicanin B were found, together with other tannins such as punigluconin and pedunclagin. The two emblicanins were shown to exhibit very strong antioxidative activities. Majeed et al. (2006) suggested that there was ascorbic acid in the juice of *PE*, but the concentration was lower than the levels previously reported and indicated that earlier methods of quantification of ascorbic acid did not resolve coeluting mucic acid gallates.

Some reports have shown that the fruits of *PE* are good source of vitamin C. Jacob et al. (1988) investigated the ascorbic acid content in fruits of *PE* and its changes during storage. They found the content of ascorbic acid in fruits of *PE* was 5.7 g kg^{-1} and decreased significantly after 7 days of storage in a refrigerator or at room temperature.

Scartezzini et al. (2006) developed an HPLC-DAD method to evaluate the level of vitamin C in *PE* fruits. They found that the fruit contained 4 g kg^{-1} DM ascorbic acid. Raghu et al. (2007) investigated the vitamin C contents in fresh and dried *PE* fruits (sun-dried and shade-dried) using different methods (2,4-dinitrophenylhydrazine [DNPH], indophenol-xylene and enzymatic methods, as well as HPLC coupled to a fluorescence detector). In fresh fruits, the results were 469, 207, 214 and 236 g kg^{-1}, respectively, using these methods. Only about 18% of the amount originally present in the fresh fruit was retained after drying in the sun for 2 days or in the shade for 4 days. They concluded that the vitamin C content in *PE* fruits was over-estimated because the analysis methods (colourimetric methods) were not accurate and that vitamin C was oxidised during the drying process. However, the fruits of *PE* (fresh and dried) are still a good source of vitamin C (Raghu et al., 2007).

Chen et al. (1995) followed changes in fruit composition during ripening. The vitamin C content reached its highest level (4 g kg^{-1}) in March of the next year, at which point it was three times higher than the level in the middle of October.

In Khan's (2009) study, the vitamin C content in the juice of *PE* fruits was 4.8 mg ml^{-1}. Wu and Zhou (1996) determined the vitamin contents in dry fruit powder. Their results showed that the contents of vitamin B1, vitamin B2, vitamin C and carotene were 4.9 mg kg^{-1}, 3.1 mg kg^{-1}, 31.80 g kg^{-1} and 6.9 mg kg^{-1} respectively.

2.3.4. Other compounds

Soman and Pillay (1962) suggested that the fruit of *PE* was the richest natural source of mucic acid (D-galactaric acid). The dry fruits contained 4–9% mucic acid in free or bound form.

Some reports have shown that the fruit of *PE* is a good source of dietary fibre. A study by Ramulu and Rao showed that the total (TDF), insoluble (IDF) and soluble (SDF) dietary fibre contents of *PE* were 73, 58 and 15 g kg^{-1}, respectively (Ramulu and Rao, 2003). A study by Jacob et al. showed that the pectin content of *PE* fruits was 20 g kg^{-1} (Jacob et al., 1998).

Barthakur and Arnold (1991) studied the contents of nutrients in the fruits of *PE*. Sixteen free amino acids were found in the fruits with a total content of 4.5 g kg^{-1} fresh weight. The protein content was 0.69% fresh weight and moisture 79.8%.

Wu et al. (2003) analysed the fatty acid composition of *PE* seeds by GC-MS. Tributyl phosphate (0.03%), myristic acid (14:0, 0.10%), diethyl phthalate (0.02%), palmitoleic acid (16:1n-7, 0.02%), palmitic acid (16:0, 13.75%), margaric acid (17:0, 0.12%), oleic acid (18:1n-9, 15.95%), stearic acid (18:0, 6.30%), *trans*-9,12-octadecadienoic acid (*trans*-linoleic acid, 19.74%), *trans*-9, *trans*-12, *trans*-15-octadecatrienoic acid (all *trans*-linoleinic acid, 43.16%), *trans*-11-eicosenoic acid (0.30%), arachidic acid (20:0, 0.32%), behenic acid (22:0, 0.052%) and lignoceric acid (24:0, 0.057%) were found in the ether extract of seeds. The authors reported that the fatty acids in *trans*-form comprised 79.8% of the total content. However, trans-fatty acids are rarely found in natural resources. These results should be verified by further studies.

In addition, Zhang et al. (2002b) identified tuberonic (12-OH jasmonic) acid glucoside, 2-(2-methylbutyryl)-phloroglucinol 1-*O*-β-D-glucopyranoside and 2-(2-methylbutyryl) phloroglucinol 1-*O*-(6″-*O*-β-D-apiofuranosyl)-β-D-glucoside in an extract of *PE* root.

2.4. Health effects of *Phyllanthus emblica*

The fruit of *PE* has been used as a medical and food material in traditional Asian medicine in Siddha, Sri Lanka, India and China (Poltanov et al., 2009). During the Ming Dynasty of China, the famous Chinese pharmacopoeia, Bencao Gangmu, recorded that anmole (one of the common names of the fruits of *P. emblica*) had health effects in terms of reducing obesity and modulating blood lipid levels. In traditional Indian medicine (Ayurveda), a number of medicinal properties are ascribed to the fruit of *PE*. The fruit of *PE* is a necessary constituent of many Ayurvedic polyherbal formulations which are still commonly used to treat various ailments including diarrhoea, jaundice, inflammation, cerebral and intestinal disorders, diabetes mellitus, coronary heart disease, cancer,

rheumatic pain, diseases of the eye and genitalia, gonorrhoea, constipation, asthma, biliousness and tonic hair (Baliga and Dsouza, 2011; Scartezzini and Speroni, 2000; Rao and Siddiqui, 1964; Aslokar et al., 1992; Satyavai et al., 1976; Perry, 1980).

In recent decades, preclinical and clinical studies have shown that the fruits of emblic leafflower possess antibacterial, antidiabetic, hypolipidaemic, anticancer, anti-inflammatory, immunomodulatory, antiatherogenic, antihypercholesterolaemia, gastroprotective, hepatoprotective, cardiovascular protective and neuroprotective properties (Khan, 2009; Krishnaveni and Mirunalini, 2010; Nampoothiri et al., 2011; Sabu and Kuttan, 2002; Baliga and Dsouza, 2011; Bhattacharya et al., 2007).

The effects of some polyherbal formulations, e.g. *Triphala*, in which *PE* is one of the major components, have also been investigated. However, these formulations consisted of several different herbs. The mechanism of their health effects are more difficult to explain.

2.4.1. Antioxidant, radical scavenging and anti-aging activities

The excess generation of free radicals is linked to many human diseases e.g. chronic inflammation, cancer, cardiovascular diseases, ischaemia/reperfusion injury, rheumatoid arthritis, diabetes and neurological disorders. Moreover, the process of aging is also linked with the generation of free radicals (Devasagayam et al., 2004; Valko et al., 2007; Reuter et al., 2010). Reactive oxygen species [ROS, superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2)] and reactive nitrogen species [RNS, nitric oxide (NO) and peroxynitrite ($ONOO^-$)], respectively, cause oxidative and nitrosative stress. Free radicals generated by the actions of these species are highly reactive and cause damage to membrane lipids, proteins and DNA (Devasagayam et al., 2004).

PE is rich in antioxidant compounds such as phenolic compounds and vitamin C, which are believed to contribute to most of the health effects of the fruit. **Table 3** summarises the literature on the antioxidant and radical scavenging activities of *PE*.

The free radical-scavenging activity of different solvent extracts and individual compounds in the extracts of *PE* were evaluated in several *in vitro* studies (Nampoothiri et al., 2011; Pozharitskaya et al., 2007; Kumaran and Karunakaran, 2006; Kumar et al., 2006). Nampoothiri et al. found that a methanol extract exhibited the highest scavenging activity against DPPH, $O_2^{\bullet-}$, $OH^{\bullet}$ and NO radicals. A methanol extract of *PE* fruits also significantly inhibited the oxidation of low density lipoprotein (LDL) *in vitro* (Nampoothiri et al., 2011). The free radical-scavenging activity of individual compounds in an aqueous extract of *PE* fruits was evaluated by a combination of HPTLC-DAD and postchromatographic $DPPH^{\bullet}$ radical derivatisation assay. Gallic acid, ellagic acid and

emblicanins A and B were found in the *PE* extract with scavenging activities against DPPH$^{\bullet}$ radicals. The DPPH$^{\bullet}$ scavenging activity of emblicanins A and B was 7.86 and 11.20 times higher, respectively, than that of ascorbic acid and 1.25 and 1.78 times higher, respectively, than that of gallic acid (Pozharitskaya et al., 2007).

Kumaran and Karunakaran (2006) found that the ethyl acetate fraction of a methanolic extract of *PE* fruits showed strong NO scavenging activity *in vitro.* Gallic acid, methyl gallate, corilagin, furosin and geraniin were isolated and identified from the fraction; gallic acid was found to be the major compound. Geraniin showed the highest NO scavenging activity among the isolated compounds (Kumaran and Karunakaran, 2006). Kumar et al. (2006) reported that gallic acid and tannic acid were the major antioxidant components in phenolic fractions of *PE* by both free radical scavenging and reducing power assays. Further, the extracts of *PE* also exhibited significant protection of DNA against oxidative damage as evidenced by migration of DNA on an agarose gel (Kumar et al., 2006).

A series of studies carried out by Reddy et al. showed the ameliorative effects of a *PE* fruit extract (an aqueous fruit extract dry powder, including 49.5% tannins) against alcohol-induced oxidative changes in rats. Tannins present in *PE* fruit extract were suggested to contribute antioxidant as well as NO scavenging properties (Reddy et al., 2009a; 2009b; 2010a; 2010b; and 2011). In their studies, the administration of a *PE* fruit extract resulted in a significant ($p<0.05$) reduction in NO levels, lipid peroxidation (LPO), the cholesterol/phospholipid (C/P) ratio, the activities of Na^{+}/K^{+} and Mg^{2+} ATPases and fluorescent anisotropic values in erythrocyte membranes in alcohol-treated rats (Reddy et al., 2009a). By following changes in the plasma biochemical profile in rats fed with alcohol, the administration of *PE* fruit (250 mg kg^{-1} BW per day) extract was found to significantly modify plasma lipid and lipoprotein patterns, and also decreased NO_x, total bilirubin and creatinine levels in alcohol-administered rats. *PE* fruit extract administration to alcohol-treated rats also significantly increased plasma total protein, the A/G ratio (Albumin/Globulin ratio) and uric acid levels in alcohol-treated rats ($P<0.05$) (Reddy et al., 2010a). The hepatoprotective effects of the *PE* fruit extract were also investigated in alcohol-administered rats (Reddy et al., 2009b; Reddy et al., 2010b). Administration of the *PE* fruit extract to alcohol-treated rats offered hepatoprotective effects and also significantly reduced lipid peroxide levels and protein carbonyls and restored levels of enzymatic and non-enzymatic antioxidants (Reddy et al., 2009b; Reddy et al., 2010b). This observation was supplemented by a histopathological examination of the liver (Reddy et al., 2009b; Reddy et al., 2010b). A similar ameliorative effect of a *PE* aqueous extract on ochratoxin-induced LPO in the kidney and liver of mice was found by Chakraborty and Verma et al. (2010).

Table 3. Antioxidant, radical scavenging and anti-aging activities of *PE*.

Subject and method	Plant parts	Dose	Result	Reference
In vitro	Methanol extract		The methanol extract of *PE* fruit exhibited the highest scavenging activity against DPPH, $O_2^{\bullet-}$, $OH^{\bullet}$ and NO radicals. The methanol extract also significantly inhibited the oxidation of low density lipoprotein (LDL) *in vitro*.	Nampoothiri et al., 2011
In vitro, combination of HPTLC-DAD and postchromatographic $DPPH^{\bullet}$ radical derivatisation assay.	Gallic acid, ellagic acids, emblicanins A and B		All compounds showed activities in scavenging of $DPPH^{\bullet}$ radicals. Emblicanins A and B showed highest activities.	Pozharitskaya et al., 2007
In vitro	Methanolic extract of *PE* fruit		The ethyl acetate fraction of methanolic extract of *PE* fruit showed strong NO scavenging activity in vitro. Gallic acid was found to be a major compound in the ethyl acetate fraction and geraniin showed highest NO scavenging activity among the isolated compounds from the ethyl acetate fraction.	Kumaran and Karunakaran, 2006
In vitro	Free and bound phenolics of *PE*		Positive correlation between antioxidant activity and phenolic content was found (R = 0.74). Gallic acid and tannic acid were identified as the major antioxidant components in phenolic fractions of *PE*. The extracts of *PE* also exhibited significant protection to DNA against oxidative damage.	Kumar et al., 2006
Alcohol-induced oxidative changes in rats, *in vivo*.	*PE* fruit extract (an aqueous fruit extract dry powder, including 49.5% tannins)	250 mg kg^{-1} BW per day for 60 days	*PE* fruit extract showed ameliorative effects against alcohol-induced oxidative changes in different organs of rats (erythrocyte membrane, plasma, liver, brain). The tannins present in PE fruit extract with antioxidant as well as NO scavenging properties were suggested to contribute to these effects	Reddy et al., 2009a; 2009b; 2010a; 2010b; and 2011
Ochratoxin-induced LPO in the kidney and liver of mice, *in vivo*.	*PE* aqueous extract	2 mg per animal per day for 45 days	Significant reduction in the ochratoxin-induced LPO in liver and kidney of mouse were found.	Chakraborty and Verma, 2010
Oxidative stress during aging process in rats, *in vivo*.	SunAmla/ ethyl acetate extract of *PE* fruit	40/10 mg kg^{-1} BW per day for 100 days	Extract of *PE* fruit could attenuate age-related renal dysfunction and prevent age-related hyperlipidaemia through attenuating oxidative stress in the aging process.	Yokozawa et al., 2007a; 2007b

The beneficial effects of a *PE* fruit extract on alcohol-induced brain mitochondrial dysfunction in rats were also reported (Reddy et al., 2011). Administration of the *PE* fruit extract to alcohol-treated rats lowered the levels of NO, protein carbonyls and lipid peroxide levels and elevated the activities of the antioxidant enzymes succinate dehydrogenase (SDH), nicotinamide adenine dinucleotide (NADH) dehydrogenase and cytochrome *c* oxidase as well as the content of cytochromes in the brain (Reddy et al., 2011).

Yokozawa et al. (2007a; 2007b) found that an extract of *PE* fruit could attenuate age-related renal dysfunction and prevent age-related hyperlipidaemia by attenuating oxidative stress in the aging process in rats. Animals were administered SunAmla (an enzymatic *PE* fruit juice extract, Taiyo Kagaku Co., Ltd., Japan) or a polyphenol-rich ethyl acetate extract of *PE* fruit; both interventions counteracted the elevation in serum creatinine and urea nitrogen levels in aged rats. In addition, hypotensive and hypolipidaemic effects were found (Yokozawa et al., 2007a; 2007b). Oral administration of the *PE* fruit extract also significantly increased the hepatic peroxisome proliferator activated receptor α (PPARα) protein level and inhibited the serum and hepatic mitochondrial TBARS levels in aged rats (2007b). Furthermore, oral administration of SunAmla or ethyl acetate extract of *PE* fruit significantly reduced TBARS levels in the kidney in aged rats (Yokozawa et al., 2007a; Yokozawa et al., 2007b).

2.4.2. Effects on diabetes and diabetic complications

Diabetes mellitus is a heterogeneous metabolic disorder characterised by high levels of blood glucose. The effects of *PE* and its extract on diabetes mellitus and diabetic complications have been studied recently, mostly in animal models. However, some studies have investigated the effects on human beings (**Table 4**).

2.4.2.1. Effects on type 1 and type 2 diabetes

The anti-diabetic activities of *PE* and its extract have been studied in animal models and in humans. A combined methanolic (75%) extract of *Terminalia chebula*, *T. belerica*, *PE* called '*Triphala*' (an equal proportion of the abovementioned three plant extracts) was found to significantly reduce blood sugar levels in normal rats and in alloxan-induced type 1 diabetic rats within 4 h of oral administration with a dose of 100 mg kg^{-1} BW. Continuous, daily administration of the drug produced a sustained effect (Sabu and Kuttan, 2002). Krishnaveni et al. (2010) found that oral administration of an ethanol extract of *PE* fruit (200 mg kg^{-1} BW for 45 days) resulted in a significant reduction in blood glucose and a significant increase in plasma insulin in streptozotocin (STZ)-induced type 2 diabetic rats (Krishnaveni et al., 2010). In addition, diabetic rats fed with an ethanol extract of *PE* fruit showed a significant reduction in blood lipid levels

and an elevation in HDL cholesterol (Krishnaveni et al., 2010). In a study by Mehta et al., a maximum reduction of 27.3% in the blood glucose level was observed at the 6 h time point in fasting blood glucose studies in normal rats after the administration of 300 mg kg^{-1} BW of an aqueous extract of *PE* seeds. The same dose produced a 25.3% drop in normal rats during the glucose tolerance test (GTT) at 3 h after glucose administration and a maximum reduction of 34.1% and 41.6% compared to the control group in sub- and mildly diabetic animals, respectively (Mehta et al., 2009). Akhtar et al. (2011) studied the hypoglycaemic and lipid-lowering properties of *PE* fruit in normal and diabetic human volunteers (16 diabetic patients and 16 normal subjects of both sexes with an age range from 30 to 60). The results indicated a significant decrease ($P < 0.05$) in fasting and 2 h post-prandial blood glucose levels on day 21 in both normal and diabetic subjects receiving 1, 2 or 3 g *PE* powder per day compared with their baseline values (Akhtar et al., 2011).

An effective strategy for type 2 diabetes management is the strong inhibition of α-glucosidase and mild inhibition of pancreatic α-amylase to prevent the sudden rise in the blood glucose levels caused by consuming starch (Krentz et al., 2005). A study by Nampoothiri et al. (2011) revealed that an extract of *PE* fruit was able to inhibit both enzymes (IC_{50} value 1.0 and 94.3 μg ml^{-1} for α-amylase and α-glucosidase, respectively) significantly more efficiently than that of a reference compound, acarbose (IC_{50} value 45.20 and 175.35 μg ml^{-1} for α-amylase and α-glucosidase, respectively). These results may mechanistically explain some of the anti-diabetic effects of *PE*.

2.4.2.2. Effects on diabetic complications and metabolic syndrome

Diabetes can cause different types of complications in patients. These complications can be classified broadly as microvascular or macrovascular diseases. Microvascular complications include neuropathy (nerve damage), nephropathy (kidney disease) and vision disorders (e.g. retinopathy, glaucoma, cataract and corneal disease). Macrovascular complications include heart disease, stroke and peripheral vascular disease (which can lead to ulcers, gangrene and amputation). Studies have shown that *PE* and its tannins have beneficial effects on diabetic cataracts (Suryanarayana et al., 2004; Suryanarayana et al., 2007), diabetic neuropathy (Tiwari, et al., 2011) and diabetic uraemia (Chen et al., 2011b).

Diabetes is considered one of the major risk factors for cataracts. Prolonged exposure to uncontrolled chronic hyperglycaemia in diabetes may lead to various complications in the eye including cataract and retinopathy (Brownlee, 2001). Suryanarayana's group (2004; 2007) suggested that *PE* fruits and an enriched fraction of *PE* fruit tannins may be effective in delaying the development of diabetic cataracts in rats. Their results showed that an aqueous extract of *PE* fruit and the isolated tannins of *PE* fruits prevented aldose

reductase (AR) activation in rat lens organ culture and also inhibited sugar-induced osmotic changes (Suryanarayana et al., 2004). Regarding STZ-induced diabetic cataracts in rats, neither *PE* fruit nor its tannins showed any preventive effects on STZ-induced hyperglycaemia as assessed by blood glucose and insulin levels. However, slit lamp microscopic observations indicated that these supplements delayed cataract progression. *PE* fruit also prevented the aggregation and precipitation of lens proteins caused by hyperglycaemia (Suryanarayana et al., 2007).

Diabetic neuropathy is one of the most common microvascular complications of diabetes mellitus and affects more than 50% of diabetic patients. Tiwari et al. (2011) found that *PE* can correct functional, biochemical and molecular defects in experimental diabetic neuropathy by targeting the oxido-nitrosative stress mediated inflammatory cascade. Treatment with a *PE* aqueous extract (250–1000 mg kg^{-1} per day) significantly attenuated oxidative stress and levels of nitrite and cytokines (TNF-α, IL-1β and TGF-β1) both in the serum and in the sciatic nerve of diabetic rats in a dose-dependent manner (Tiwari, et al., 2011).

Chen et al. (2011) found that oral administration of a 1:1 mixture of epigallocatechin-3-gallate and *PE* extract for 3 months significantly improved antioxidant defences as well as diabetic and atherogenic indices in uremic patients with diabetes (Chen et al., 2011b).

Metabolic syndrome is characterised by insulin resistance, dyslipidaemia and hypertension and is associated with increased morbidity and mortality from several prevalent diseases, such as diabetes, cancer, myocardial infarction and stroke (Hwang, et al., 1987). Kim et al. (2010) found that the polyphenol-rich fraction of *PE* fruit could attenuate fructose-induced metabolic syndrome in a rat model. Their results showed that the ethyl acetate extract of *PE* ameliorated the high fructose diet-induced hypertriacylglycerolaemia and hypercholesterolaemia (Kim et al., 2010).

Table 4. Effects on diabetes and diabetic complications of *PE*.

Subject and method	Plant parts	Dose	Result	Reference
Alloxan-induced diabetic (type 1) rats, *in vivo*.	Methanolic (75%) extract of *Triphala* [a]	100 mg kg^{-1} BW	The extracts (100 mg kg^{-1} BW) reduced the blood sugar level in normal and in alloxan-induced diabetic rats significantly within 4 h of oral administration. Continuous, daily administration of the drug produced a sustained effect.	Sabu and Kuttan, 2002
Streptozotocin (STZ)-induced type 2 diabetes rats, *in vivo*.	Ethanolic extract of *PE* fruit	200 mg kg^{-1} BW for 45 days	Significant reduction in blood glucose and a significant increase in plasma insulin in diabetic rats.	Krishnaveni et al., 2010
Streptozotocin (STZ)-induced type 2 diabetes rats, *in vivo*.	Aqueous extract of *PE* seeds	100–400 mg kg^{-1} BW	Significant reduction in blood glucose.	Mehta et al., 2009
Normal and diabetic human volunteers, clinical trial.	*PE* fruit	1, 2 or 3 g *PE* fruit powder per day	Significant decrease (P<0.05) was found in fasting and 2h post-prandial blood glucose levels on the day 21 in both normal and diabetic subjects receiving 1, 2 or 3 g *PE* powder per day. Significant (P<0.05) decreases were observed in total cholesterol and triglycerides in both normal and diabetic volunteers on day 21 that were given either 2 or 3 g *PE* powder per day. The diabetic volunteers receiving 3 g *PE* powder per day exhibited a significant (P<0.05) decrease in total lipids on the day 21. Both normal and diabetic volunteers receiving 2 or 3 g *PE* powder showed significant (P<0.05) improvement in HDL cholesterol and LDL cholesterol levels	Akhtar et al., 2011
In vitro	Extract of *PE* fruit		Extract of *PE* fruit was able to inhibit α-amylase and α-glucosidase (IC_{50} value 1.0 and 94.3 μg ml^{-1}, respectively)	Nampoothiri et al., 2011
Fructose-induced metabolic syndrome in rat model	Ethyl acetate extract of *PE* fruit	10 or 20 mg kg^{-1} BW per day for 2 weeks	The ethyl acetate extract of *PE* fruit ameliorated the high fructose diet-induced hypertriacylglycerolaemia and hypercholesterolaemia.	Kim et al., 2010
Diabetic cataract, *in vitro*	*PE* fruit and an enriched fraction of *PE* fruit tannins		Aqueous extract of *PE* fruit inhibited aldose reductase (AR) of rat lens and recombinant human AR with IC_{50} values of 0.72 mg ml^{-1} and 0.88 mg ml^{-1}, respectively,	Suryanarayana et al., 2004
STZ-induced diabetic cataract in rats, *in vivo*.	*PE* fruit and an enriched fraction of *PE* fruit tannins	0.2 % diet	Supplements of *PE* fruit and *PE* fruit tannins delayed cataract progression	Suryanarayana et al., 2007
Diabetic neuropathy in rats, *in vivo*.	Aqueous extract of *PE*	250–1000 mg kg^{-1} per day	Insulin in combination with *PE* extract not only attenuated the diabetic condition but also reduced neuropathic pain through modulation of oxidative–nitrosative stress in diabetic rats	Tiwari, et al., 2011
Diabetic-uremic patients, clinical trial.	1:1 mixture of epigallocatechin-3-gallate and *PE* extract	100 mg EGCG and 100 mg *PE* extract 3 times per day	The mixture significantly improved antioxidant defence as well as diabetic and atherogenic indices in uremic patients with diabetes.	Chen et al., 2011b

[a] *Triphala*, *an* equal proportion of *Terminalia chebula*, *T. belerica*, *PE* extracts.

2.4.3. Effects on cardiovascular health

It has been shown that *PE* and *PE* extracts have beneficial effects on different cardiovascular diseases (**Table 5**). Oxygen-derived free radicals play an important role in the initiation and progression of all clinical manifestations of ischaemic heart disease. Myocardial cellular injury occurring during reperfusion of ischaemic cells, known as ischaemia-reperfusion injury, is primarily due to oxidative stress (McCord, 1985). Studies have shown that antioxidants exert a protective effect against cardiac ischaemia-reperfusion injury (IRI) (Harmaki et al., 1993; 1994).

Studies by Bhattcharya et al. (2002) and Rajak et al. (2004) have shown that *PE* fruit can ameliorate the oxidative stress induced by IRI. Oral administration of a *PE* extract enriched with emblicanin A and B (50 mg and 100 mg kg^{-1} BW twice per day for 14 days) significantly reversed the effects of IRI on SOD, CAT, GPx and LPO activities (Bhattcharya et al., 2002). Similar results were found in a study by Rajak et al. (2004). Fresh *PE* fruit homogenate (250–750 mg kg^{-1} per day) and saline were administered orally to Wistar albino rats for 30 days. There was a reduction in basal myocardial LPO, as evidenced by decreased TBARS levels, and an augmentation of myocardial endogenous antioxidants in the *PE*-treated rats compared to those in the saline group. The results indicate that chronic *PE* administration improves myocardial adaptation by augmenting endogenous antioxidants and protects the rat heart from oxidative stress associated with IRI (Rajak, et al., 2004).

Anila and Vijayalakshmi (2002) separated flavonoids from *PE* fruit by solvent extractions and silica gel column chromatography. The ethyl acetate:methanol (50:50) fraction provided the maximum yield of flavonoids (789.4 mg from 1.9 kg fresh fruit). The flavonoids were administrated orally (10 mg kg^{-1} BW per day) to male albino rats fed a high fat diet (coconut oil 15% and cholesterol 2%). After 90 days of treatment, significant decreases in lipid levels in serum and tissues were observed when compared with the control group (Anila and Vijayalakshmi, 2002). Duan et al. (2005) studied the antiatherogenic effects of two soluble tannins, corilagin and its analogue Dgg16 (1,6-di-*O*-galloyl-β-D-glucose) isolated from *PE* fruit. The results suggested that the two compounds are effective in inhibiting the progress of atherosclerosis by alleviating oxidation injury and by inhibiting oxidised LDL-induced vascular smooth muscle cell proliferation (Duan et al., 2005).

Hypercholesterolaemia is one of the risk factors for coronary artery disease. The results of Saravanan et al. have demonstrated the hypolipidaemic effects of *Triphala* (a polyherbal formulation containing an extract of *PE*) on experimentally-induced hypercholesterolaemia in rats (Saravanan et al., 2007).

Table 5. Effects on cardiovascular health of *PE*.

Subject and method	Plant parts	Dose	Result	Reference
Cardiac ischaemia-reperfusion injury (IRI) in rats	Emblicanin A and B enriched *PE* extract (prepared by eluting fresh *PE* juice though a Sephadex LH-20 column)	50 mg and 100 mg kg^{-1} BW twice per day for 14 days	The *PE* extract (50 mg and 100 mg kg^{-1} BW) significantly reversed the effects of IRI on SOD, CAT, GPx and LPO activities.	Bhattcharya et al., 2002
Cardiac ischaemia-reperfusion injury (IRI) in rats	Fresh *PE* fruit homogenate	250–750 mg kg^{-1} per day for 30 days	*PE* administration improves myocardial adaptation by augmenting endogenous antioxidants and protects rat hearts from oxidative stress associated with IRI.	Rajak, et al., 2004
Rats fed a high fat diet	Flavonoids separated from *PE* fruit	10 mg kg^{-1} BW per day for 90 days	Significant decrease in total cholesterol, triacylglycerol, phospholipids and free fatty acids levels in serum and tissues were observed when compared with the control group.	Anila and Vijayalakshmi, 2002
Human umbilical vein endothelial cells, ECV-304, incubated with ox-LDL (50 mg/l), *in vitro*	Tannins (corilagin/Dgg16) isolated from *PE* fruit	0.0001–0.1 mmol l^{-1}	Corilagin and Dgg16 are effective in inhibiting the progress of atherosclerosis by alleviating oxidation injury and by inhibiting ox-LDL-induced VSMC proliferation.	Duan et al., 2005
Hypercholesterolaemia in rats	*Triphala* [a]	1 g kg^{-1} BW per day for 48 days	*Triphala* has hypolipidaemic effects.	Saravanan et al., 2007
Deoxycorticosterone acetate/1% NaCl high salt (DOCA/HS)-induced hypertension model rat	Extract of *PE*	75–300 mg kg^{-1} BW per day for 5 weeks	Extract of *PE* reduces oxidative stress, prevents development and progression of hypertension as well as cardiac and renal hypertrophy in DOCA/HS-induced hypertension *via* activation of endothelial nitric oxide synthase (eNOS) and endogenous antioxidants as well as regulation of serum NO and electrolyte levels.	Bhatia et al., 2011

[a] *Triphala*, equal proportion of *Terminalia chebula*, *T. belerica*, *PE* extracts.

Bhatia et al. (2011) investigated the anti-hypertensive effect of *PE* in a deoxycorticosterone acetate/1% NaCl high salt (DOCA/HS)-induced hypertension model rat. Hypertension was induced in rats by the DOCAsalt (20 mg kg^{-1}, s.c.) and, at the same time, these rats received co-treatment with different doses of an extract of *PE* (75–300 mg kg^{-1} BW per day) for 5 weeks. The *PE* extract significantly decreased arterial blood pressure and heart rate as well as cardiac and renal hypertrophy in a dose-dependent fashion as compared to deoxycorticosterone acetate (DOCA) control rats. Increased TBARS and decreased endogenous antioxidants activity in serum, heart and kidney tissues of hypertensive rats were also normalised. These results demonstrate that an extract of *PE* can prevent the development and progression of hypertension as well as cardiac and renal hypertrophy in DOCA/HS-induced hypertension via the activation of endothelial nitric oxide synthase (eNOS) and endogenous antioxidants as well as regulation of serum NO and electrolyte levels (Bhatia et al., 2011).

2.4.4. Anti-inflammatory effects

A summary of the anti-inflammatory effects of *PE* is presented in **Table 6.**

Ihantola-Vormisto et al. (1997) and Asmawi et al. (1993) found that extracts of *PE* leaves have anti-inflammatory effects. In a study by Ihantola-Vormisto et al. (1997), the leaves of *PE* were extracted with different solvents. By studying the inhibitory activity of the extracts on human polymorphonuclear leukocyte (PMN) and platelet function, they found that the methanol, tetrahydrofuran and 1,4-dioxane extracts (50 μg ml^{-1}) inhibited leukotriene B_4 (LTB_4)-induced migration of human PMNs by 90% and *N*-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine (FMLP)-induced degranulation by 25-35%. The diethyl ether extract (50 μg ml^{-1}) inhibited calcium ionophore A23187-induced leukotriene B_4 (LTB_4) release from human PMNs by 40%, thromboxane B_2 (TXB_2) production in platelets during blood clotting by 40% and adrenaline-induced platelet aggregation by 36%. These results show that the leaves of *PE* have inhibitory activity on PMNs and platelets, which confirm their anti-inflammatory and antipyretic properties (Ihantola-Vormisto et al., 1997). Asmawi et al. (1993) examined the effects of *PE* leaf extracts using carrageenan- and dextran-induced rat hind paw models. Anti-inflammatory activity was found in the water fraction of methanol extract of the plant leaves.

The anti-inflammatory activities of *PE* fruit or *PE* fruit extracts were also studied in animal models (Sidhu et al., 2010; Muthuraman et al., 2010). Acute pancreatitis is a rapidly developing inflammation of the pancreas and causes high mortality. *PE* has been reported to have beneficial effects in the treatment of acute pancreatitis in rats (Sidhu et al., 2010). Serum levels of lipase and interleukin-10 were significantly lower in the *PE*-treated group than in the arginine and placebo-treated group. The nucleic acid

Table 6. Anti-inflammatory activities of *PE*.

Subject and method	Plant parts	Dose	Result	Reference
Human polymorphonuclear leukocyte (PMN) and platelet, *in vitro*	Ten different solvents (*n*-hexane, diethyl ether, methanol, tetrahydrofuran, acetic acid, dichloromethane, 1,4-dioxane, toluene, chloroform, and water) extracts of *PE* leaves		The leaves of *PE* have inhibitory activity on PMNs and platelets, which confirm the anti-inflammatory and antipyretic properties.	Ihantola-Vormisto et al., 1997
Carrageenan- and dextran-induced rat hind paw oedema	*PE* leaf extract		Anti-inflammatory activity was found in the water fraction of methanol extract of the plant leaves.	Asmawi et al., 1993
L-arginine induced acute pancreatitis in rats	*PE* fruit extract	100 mg kg^{-1} BW per day for 3, 7,14 and 28 days	*PE* treatment was found to be beneficial for treating acute pancreatitis.	Sidhu et al., 2010
Carrageenan and cotton pellet induced acute and chronic inflammatory in rat paw	Free and bound phenolic compounds from *PE* fruit (70 % ethanol extract)	20 and 40 mg kg^{-1} BW for 6 days	In both acute and chronic inflammation, both free and bound phenolics of *PE* reduced inflammation.	Muthuraman et al., 2010
IB3-1 CF bronchial epithelial cells exposed to the *P. aeruginosa* laboratory strain PAO1, *in vitro*	Ethanol extract of *PE*/ Pyrogallol	500 µg ml^{-1}/ 2, 20, 200 µM	Extracts from *PE* strongly inhibited the PAO1-dependent expression of the neutrophil chemokines IL-8, GRO-α, GRO-γ, of the adhesion molecule ICAM-1 and of the pro-inflammatory cytokine IL-6. Pyrogallol, one of the compounds isolated from extract of *PE*, inhibited the *P. aeruginosa*-dependent expression of these pro-inflammatory genes similarly to the whole extract from *PE*.	Nicolis et al., 2008

content, rate of DNA synthesis, pancreatic proteins and pancreatic amylase content were significantly improved (Sidhu et al., 2010). Muthuraman et al. (2010) studied the anti-inflammatory effects of free and bound phenolic compounds from *PE* in carrageenan- and cotton pellet-induced acute and chronic inflammatory animal models at dose levels of 20 and 40 mg kg^{-1}. In both acute and chronic inflammation, both the free and bound phenolics of *PE* reduced inflammation; at high doses, the effects of both fractions were comparable to treatment with diclofenac (Muthuraman et al., 2010).

Nicolis et al. (2008) suggested that pyrogallol, an active compound of *PE* can regulate the expression of pro-inflammatory genes in bronchial epithelial cells. The most relevant cause of morbidity and mortality in cystic fibrosis (CF) patients is chronic inflammation in the lung due to infection with *Pseudomonas aeruginosa*. Extracts from *PE* and pyrogallol, one of the compounds isolated from an extract of *PE*, inhibited the *P. aeruginosa*-dependent expression of these pro-inflammatory genes in a similar way. These results suggest that pyrogallol is the active compound responsible for the anti-inflammatory effect of extracts of *PE* (Nicolis et al., 2008).

2.4.5. Cytotoxic effects, protective effects against clastogenicity and anti-cancer activities

The anti-cancer effects of *PE* fruit were reviewed by Baliga and Dsouza (2011). They summarised that *PE* fruit and its extracts can be used 1) as antineoplastic agents, 2) as radioprotective agents and 3) as chemopreventive and chemomodulatory agents. The mechanism of the anti-cancer effects includes the following aspects: *PE* fruit or its extracts 1) are free radical scavengers; 2) can decrease the hepatic levels of phase I enzymes; 3) can increase levels of GST, a phase II enzyme; 4) can decrease levels of ornithine decarboxylase; 5) can increase levels of antioxidant enzymes; 6) can decrease LPO; 7) have antimutagenic effects; 8) possess immunomodulatory effects; 9) can modulate the levels of proteins important in cell cycle progression; 10) can cause apoptosis and cytotoxicity in neoplastic cells; 11) can prevent metastasis. The anti-cancer effects of *Triphala* were also reviewed by Baliga (2010); he concluded that *Triphala* was useful in the prevention of cancer. *Triphala* possesses antineoplastic, radioprotective and chemoprotective effects. **Table 7** presents the relevant studies on the anti-cancer effects of *PE*.

2.4.5.1. Cytotoxic effects

PE extracts have been shown to have cytotoxic effects on cancer cells *in vitro* and *in vivo*. Pinmal et al. (2008) studied the synergistic inhibitory effects of a *PE* extract with conventional cytotoxic agents (doxorubicin and cisplatin) against human hepatocellular carcinoma (HepG2) and lung cancer cells (A549). The *PE* extract demonstrated growth inhibitory activity, with a certain degree of selectivity between the two cancer cell lines

tested. Synergistic effects (CI < 1) between *PE* and doxorubicin as well as between *PE* and cisplatin were demonstrated on A549 and HepG2 cells at different dose levels (Pinmal et al., 2008). Pinmal et al. (2010) reported that an aqueous extract of *PE* exhibited cytotoxic activity on Vero cells with an IC_{50} value of 157.9 3 μg ml^{-1} and with a selectivity index (SI) of 11. They also reported the *in vivo* antiplasmodial activity of an aqueous extract of *PE* with good suppression activity by 69.46% using a standard 4-day suppressive test on *P. berghei*-infected mice (Pinmal et al., 2010).

Sumantran et al. (2007) investigated the short- and long-term growth inhibitory effects of an aqueous extract *PE* fruit on Chinese hamster ovary (CHO) cells. A standard 96-well plate assay was used to measure short-term growth. An aqueous extract of *PE* fruit (50 μg ml^{-1}) caused 42% growth inhibition in CHO cells. For an assessment of long-term growth, the colony formation assay (CFA) was used, which measures clonogenic potential. The aqueous extract *PE* fruit did not significantly alter the colony forming efficiency of CHO cells (Sumantran et al., 2007).

Sandhya et al. (2006) investigated the cytotoxic effects of an aqueous extract of *Triphala* on a human breast cancer cell line (MCF-7) and a transplantable mouse thymic lymphoma (barcl-95) *in vitro* and *in vivo*. These results suggest that *Triphala* possesses cytotoxicity in tumour cells without a clear influence on normal cells (Sandhya et al., 2006).

2.4.5.2. Protective effects against metal-induced clastogenicity

The protective effects of *PE* against chromosome aberrations (CA) induced by metal salts were also reported. These metal salts included caesium chloride (CsCl) (Ghosh et al., 1992), nickel chloride (Dhir et al., 1990), lead nitrate (Dhir et al., 1991) and aluminium sulphate (Roy et al., 1992). Ghosh et al. (1992) reported that the oral administration of an aqueous extract of *PE* fruit (685 mg kg^{-1} BW) for 7 days significantly reduced the frequency of CA on bone marrow cells induced by CsCl (125, 250 and 500 mg kg^{-1} BW) in Swiss albino mice (Ghosh et al., 1992). The counteracting effects of *PE* against nickel, lead and aluminium-induced clastogenicity in mice were also reported (Dhir et al., 1990; Dhir et al., 1991; Roy et al., 1992). An aqueous extract of edible parts of dried *PE* fruit was fed to *Mus musculus* for seven consecutive days prior to treatment with nickel chloride (10–40 mg kg^{-1} BW), lead nitrate (10–40 mg kg^{-1} BW) or aluminium sulphate (250–1000 mg kg^{-1} BW). The fruit extract significantly reduced the frequency of CA/cell, the percentage of aberrant cells and the frequency of micronuclei induced by all metal salts at all doses in the bone marrow cells of treated mice (Dhir et al., 1990; Dhir et al., 1991; Roy et al., 1992).

2.4.5.3. Radioprotective effects

The radioprotective effects of *PE* have been investigated in animal models. Singh et al. (2005) studied the radioprotective properties of an aqueous extract of *PE* fruit against

sublethal gamma radiation (9 Gy) in Swiss albino mice. The dose of the fruit pulp extract found to be most effective against radiation was 100 mg kg^{-1} BW with 87.5% survival after 30 days (Singh et al., 2005). Hari Kumar et al. (2004) found that the fruit pulp of *PE* significantly reduced the effects of radiation on Swiss albino mice, and they suggested that *PE* extract may be useful in reducing the side effects produced during radiation therapy. (Hari Kumar et al., 2004).

Jagetia et al. (2002) demonstrated that *Triphala* is a good radioprotective agent in mice exposed to γ-radiation.

2.4.5.4. Protective effects against chemical-induced carcinogenesis

Jeena et al. (1999) reported that an extract of *PE* fruit significantly inhibited hepatocarcinogenesis induced by *N*-nitrosodiethylamine (NDEA) in a dose-dependent manner. The anticarcinogenic activity of the extract was evaluated by its effects on tumour incidence, levels of carcinogen metabolising enzymes, levels of cancer markers and injury markers in the liver. Animals treated with NDEA alone showed 100% tumour incidence and significantly elevated tissue levels of drug metabolising enzymes such as GST and aniline hydroxylase (AH). Treatment with the extract significantly reduced the levels of these indicators. Levels of γ-glutamyl transpeptidase (GGT) were also found to be elevated both in the serum and tissues of tumour-bearing animals. A similar reduction was seen in the tissue levels of GSH. Serum levels of LPO, alkaline phosphatase (ALP) and glutamate pyruvate transaminase (GPT), which are markers of liver injury, were also elevated. The morphology of liver tissue and levels of marker enzymes indicated that the *PE* extract offered protection against chemical carcinogenesis (Jeena et al., 1999).

Veena et al. (2006a; 2006b; 2007) studied the potency of *Kalpaamruthaa* [a modified Siddha preparation, which contains *Semecarpus anacardium* Linn., *PE* and honey] against breast cancer induced by 7,12-dimethylbenz(a)anthracene (DMBA) in rats. Changes in the levels of glycoprotein components, marker enzymes [lactate dehydrogenase (LDH) and 5′ nucleotidase (5′ ND)], lysosomal enzymes, plasma lipids, lipid-metabolising enzymes, lipid peroxides and antioxidants in the blood and vital organs (liver, kidney and breast tissue) were investigated in mammary carcinoma-bearing rats. Changes in body weight and the volume of cancer were also determined. These results provide evidence for the therapeutic effects of *Kalpaamruthaa* against mammary carcinoma (Veena et al., 2007).

2.4.5.5. Protective effects against the toxicity of anti-cancer medicine

Cyclophosphamide (CP) is one of the most commonly used alkylating anticancer drugs, but has toxic side effects including immunotoxicity, hematotoxicity and mutagenicity. Haque et al. (2001) found that oral administration of an extract of *PE*

Table 7. Cytotoxic effects, protective effects against clastogenicity and anti-cancer activities of *PE*.

Subject and method	Plant parts	Dose	Result	Reference
Human hepatocellular carcinoma (HepG2) and lung cancer cells (A549), *in vitro*	Aqueous extract of *PE* fruit	4-48 µg ml^{-1} for A549/ 25-200 µg ml^{-1} for HepG2	*PE* extract demonstrated growth inhibitory activity, with a certain degree of selectivity between the two cancer cell lines tested. Synergistic effects (CI < 1) between *PE* and doxorubicin as well as between *PE* and cisplatin were demonstrated on A549 and HepG2 cells at different dose levels	Pinmal et al., 2008
Vero cell line, *in vitro*; *Plasmodium falciparum* infected mice, *in vivo*.	Aqueous extract of *PE*	250 mg kg^{-1} BW per day (*in vivo*)	Extract of *PE* exhibited interesting *in vitro* and *in vivo* antiplasmodial activity with good selectivity	Pinmal et al., 2010
Chinese Hamster ovary (CHO) cell line	Aqueous extract of *PE* fruit	50 µg ml^{-1}	The aqueous extract of *PE* fruit caused 42% growth inhibition on CHO cells. The aqueous extract of *PE* fruit did not significantly alter the colony forming efficiency of CHO cells	Sumantran et al., 2007
Human breast cancer cell line (MCF-7), *in vitro*; Transplantable mouse thymic lymphoma (barcl-95), *in vivo*.	Aqueous extract of *Triphala*	0.025-0.5 mg ml^{-1} (*in vitro*); 40 mg kg^{-1} BW (*in vivo*)	Aqueous extract of *Triphala* has cytotoxic effects of on human breast cancer cell line (MCF-7) and a transplantable mouse thymic lymphoma (barcl-95) *in vitro* and *in vivo*.	Sandhya et al., 2006
Chromosome aberrations (CA) on bone marrow cells induced by CsCl in mice	Aqueous extract of *PE* fruit	685 mg kg^{-1} BW per day for 7 days	Significantly reduced the frequency of CA on bone marrow cells induced by CsCl (125, 250 and 500 mg kg^{-1} BW) in Swiss albino mice.	Ghosh et al., 1992
CA on bone marrow cells induced by nickel chloride in mice	Aqueous extract of *PE* fruit	685 mg kg^{-1} BW per day for 7 days	Significantly reduced the frequency of CA on bone marrow cells induced by nickel chloride in mice	Dhir et al., 1990
CA on bone marrow cells induced by lead nitrate in mice	Aqueous extract of *PE* fruit	685 mg kg^{-1} BW per day for 7 days	Significantly reduced the frequency of CA on bone marrow cells induced by lead nitrate in mice	Dhir et al., 1991
CA on bone marrow cells induced by aluminium sulphate in mice	Aqueous extract of *PE* fruit	685 mg kg^{-1} BW per day for 7 days	Significantly reduced the frequency of CA on bone marrow cells induced by aluminium sulphate in mice	Roy et al., 1992
Mice exposed to 9 Gy of γ-radiation	Aqueous extract of *PE* fruit	50-800 mg kg^{-1} BW per day for 7 to 30 days.	The extract had radioprotective effects. The optimum dose was 100 mg kg^{-1} BW.	Singh et al., 2005

Subject and method	Plant parts	Dose	Result	Reference
Mice exposed to 7 Gy of γ-radiation	Fruit pulp of *PE*	2.5 g kg^{-1} BW for 10 days	Administration of *PE* significantly increased the total leukocyte count, bone marrow viability and the level of haemoglobin. Administration of *PE* significantly enhanced the activity of the various antioxidant enzymes and GST as well as glutathione system in the blood and treatment with *PE* suppressed the elevation in lipid peroxides in the serum.	Hari Kumar et al., 2004
Mice exposed to 10 Gy of γ-radiation	Aqueous extract of *Triphala* [a]	0–80 mg kg^{-1} BW	Treatment of mice with *Triphala* consecutively for five days before irradiation delayed the onset of mortality and reduced the symptoms of radiation sickness when compared with the non-drug treated irradiated controls without drug treatment. The highest protection against gastrointestinal (GI) death was observed in treatment with *Triphala* at 12.5 mg kg^{-1} BW.	Jagetia et al., 2002
Hepatocarcinogenesis induced by NDEA in rats	Aqueous extract of *PE* fruit	50–250 mg kg^{-1} BW per day for 20 weeks	Extract of *PE* significantly inhibited hepatocarcinogenesis induced by NDEA in a dose dependent manner.	Jeena et al., 1999
Breast cancer induced by 7,12-dimethylbenz(a) anthracene (DMBA) in rats	*Kalpaamruthaa* [b]	100–500 mg kg^{-1} BW in olive oil per day for 14 days	*Kalpaamruthaa* possesses therapeutic effects against mammary carcinoma. *Kalpaamruthaa* is effective at the dosage level of 300 mg kg^{-1} BW.	Veena et al. (2006a; 2006b; 2007)
Cyclophosphamide (CP)-treated mice, *in vivo*.	Aqueous extract of *PE* fruit	100 mg kg^{-1} BW per day for 10 days	Extract of *PE* in particular was very effective in reducing CP-induced suppression of humoral immunity. Pretreatment with extract of *PE* also preserved antioxidants in kidney of CP-treated rats.	Haque et al., 2001
Cyclophosphamide (CP)-treated mice, *in vivo*.	Immu-21[c]	100 mg kg^{-1} BW per day for 7 days/ 30 mg kg^{-1} BW per day for 14 days	Treatment with Immu-21 prevented CP-induced genotoxicity in mice.	Jena et al., 2003

[a] *Triphala,* equal proportion of *Terminalia chebula*, *T. belerica*, *PE* extracts.

[b] *Kalpaamruthaa*, a modified Siddha preparation, which contains *Semecarpus anacardium* Linn., *PE* and honey.

[c] Immu-21, a polyherbal formulation containing extracts of *Ocimum sanctum*, *Withania somnifera*, *PE* and *Tinospora cordiafolia.*

to rats at a dose of 100 mg kg^{-1} BW per day for 10 days resulted in the modulation of immunological parameters and antioxidants in the kidney and liver in normal as well as cyclophosphamide (50 mg kg^{-1})-treated animals. The *PE* extract, in particular, was very effective in reducing the cyclophosphamide-induced suppression of humoral immunity. Pretreatment with an extract of *PE* also preserved antioxidant levels in the kidneys of cyclophosphamide-treated rats. GSH levels were significantly ($P < 0.001$) increased and antioxidant enzymes were restored by the *PE* extract compared with cyclophosphamide treatment alone (Haque et al., 2001). The preventive effects of Immu-21 (a polyherbal formulation containing extracts of *Ocimum sanctum*, *Withania somnifera*, *PE* and *Tinospora cordiafolia*) against genotoxicity induced by cyclophosphamide were also found in mice (Jena et al., 2003).

2.4.6. Effects on the nervous system

PE is traditionally used to treat disorders in the central nervous system (CNS) (Golechha et al., 2011). The effects of a standardised hydroalcoholic extract of *PE* fruits against kainic acid-induced seizures, cognitive deficits and on markers of oxidative stress in rats were studied by Golechha et al. (2011). The results showed that pretreatment with an extract of *PE* fruit (500 and 700 mg kg^{-1} i.p.) significantly ($P < 0.001$) increased the latency of seizures compared with the vehicle-treated kainic acid group. The *PE* fruit extract significantly prevented the increase in TBARS levels and ameliorated the fall in GSH. Furthermore, the *PE* fruit extract dose-dependently attenuated the kainic acid-induced increase in TNF-α levels in the brain. The *PE* fruit extract also significantly improved the cognitive deficits induced by kainic acid. The authors suggested that the neuroprotective effects may be due to the antioxidant and anti-inflammatory effects of *PE* (Golechha et al., 2011).

Vasudevan and Parle (2007) investigated the memory-enhancing activity of *PE*. The elevated plus maze and passive avoidance apparatus served as the exteroceptive behavioural models for testing memory. Diazepam-, scopolamine- and aging-induced amnesia served as the interoceptive behavioural models. *PE* produced a dose-dependent improvement in memory scores in young and aged mice. Furthermore, it reversed the amnesia induced by scopolamine (0.4 mg kg^{-1} BW) and diazepam (1 mg kg^{-1} BW). Brain cholinesterase activity and total cholesterol levels were also reduced by *PE* administered orally. The authors suggested that *PE* may be a useful remedy for the management of Alzheimer's disease on account of its multiple beneficial effects such as its memory improving, cholesterol lowering and anticholinesterase activities (Vasudevan and Parle, 2007).

2.4.7. Immunomodulating effects

Immune activation is an effective as well as protective approach against emerging infectious diseases. Studies have shown that *PE* and *PE* extracts have immunomodulating effects (**Table 8**). Srikumar et al. (2005; 2006) studied the immunomodulatory activities of *Triphala* by testing various functions of neutrophils such as adherence, the phagocytic index (P.I.) and the avidity index (A.I.), as well as nitro blue tetrazolium (NBT) reduction on noise-induced stress in albino rats. They found that supplementation with *Triphala* prevented the noise-stress induced changes in the antioxidant as well as cell-mediated immune response in rats (Srikumar et al., 2006).

Sai Ram et al. (2002) investigated the cytoprotective and immunomodulating properties of a 90% ethanol extract of dry *PE* fruit on lymphocytes using an *in vitro* method. Chromium (VI) was used as an immunosuppressive agent. The *PE* extract significantly inhibited Cr-induced free radical production and restored the anti-oxidant status back to the control level. The *PE* extract also inhibited apoptosis and DNA fragmentation induced by Cr, relieved the immunosuppressive effects of Cr on lymphocyte proliferation, and returned IL-2 and γ-interferon (γ-IFN) production to control levels. The results showed that the fruit extract of *PE* had cytoprotective and immunomodulatory properties (Sai Ram et al., 2002). The cytoprotective and immunomodulating properties of a *PE* extract against chromium (VI)-induced oxidative injury in murine macrophages were reported by Sai Ram et al. (2003). Chromium (VI) treatment at 1 μg ml^{-1} increased free radical production and decreased GSH levels and GPx activity in macrophages. The presences of the *PE* extract enhanced cell survival, decreased free radical production and maintained antioxidant levels close to those of the control cells. Further, chromium (VI) treatment resulted in decreased phagocytosis and γ-IFN production which were restored by the *PE* extract (Sai Ram et al., 2003).

Suresh and Vasudevan (1994) found that *PE* could enhance natural killer (NK) cell activity and antibody-dependent cellular cytotoxicity (ADCC) in syngeneic BALB/c mice bearing Dalton's lymphoma ascites (DLA) tumours (Suresh and Vasudevan, 1994).

The immunomodulatory effects of *PE* were evaluated in an adjuvant-induced arthritic (AIA) rat model; the results showed *PE* extract can cause immunosuppression in AIA rats (Ganju et al., 2003).

Table 8. Immunomodulating effects of *PE*.

Subject and method	Plant parts	Dose	Result	Reference
Noise-induced stress in rats	*Triphala* [a]	1 g kg^{-1} per day for 48 days	Oral administration of *Triphala* stimulated the neutrophil functions in the immunised rats and prevented stress-induced suppression of neutrophil functions. Supplementation with *Triphala* prevented noise-stress induced changes in antioxidant levels as well as the cell-mediated immune response in rats.	Srikumar et al., 2005
Lymphocytes, *in vitro*	90% ethanol extract of *PE* dry fruit	10 µg–1 mg ml^{-1}	*PE* extract also inhibited apoptosis and DNA fragmentation induced by Cr, relieved the immunosuppressive effects of Cr on lymphocyte proliferation, and restored the IL-2 and γ-IFN production considerably. The results showed the fruit extract of *PE* had cytoprotective and immunomodulatory properties.	Sai Ram et al., 2002
Chromium (VI) induced oxidative injury in murine macrophages, *in vitro*	70% ethanol extract of *PE* dry fruit	250 µg ml^{-1}	The presences of the PE extract enhanced cell survival, decreased free radical production and maintained antioxidant levels close to that of the control cells. Further, chromium (VI) treatment resulted in decreased phagocytosis and γ-IFN production which were restored by the *PE* extract.	Sai Ram et al., 2003
Mice bearing Dalton's lymphoma ascites (DLA) tumour	*PE* fruit powder	20 mg kg^{-1} BW per day	*PE* enhanced natural killer (NK) cell activity and antibody-dependent cellular cytotoxicity (ADCC) in syngeneic BALB/c mice bearing Dalton's lymphoma ascites (DLA) tumours.	Suresh and Vasudevan, 1994
Adjuvant induced arthritic (AIA) rat model	Crude extract of *PE*	6.25–100 mg kg^{-1} BW	*PE* extract can cause immunosuppression in AIA rats.	Ganju et al., 2003

[a] *Triphala,* equal proportion of *Terminalia chebula*, *T. belerica*, *PE* extracts.

2.4.8. Anti-microbial and cytotoxic activity

The anti-microbial and cytotoxic activities of *PE* were studied by Rahman et al. (2009), Saini et al. (2008) Srikumar et al. (2007) and Saeed and Tariq (2007). Rahman et al. (2009) found that the alkaloids of *PE* had antimicrobial and cytotoxic activities. The active components were in the chloroform fraction separated from the methanolic extract of the fresh ripe fruit of *PE*. This fraction showed the strongest inhibitory effect against *Bacillus subtilis* and moderate inhibitory activity against *Salmonella typhi*, *Bacillus cereus*, *Pseudomonas aeruginosa,*, *Shigella boydii*, *Shigella dysenteriai*, *Staphylococcus aureus*, *Sternbergia lutea*, *Escherichia coli*, *Salmonella paratyphi*, *Vibrio parahaemolyticus* and *Vibrio mimicus* (Rahman et al., 2009). Srikumar et al. (2007) showed that aqueous and ethanol extracts of *Triphala* and its individual herbal components had antibacterial activity against several bacterial isolates (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Shigella sonnei*, *S. flexneri*, *Staphylococcus aureus*, *Vibrio cholerae*, *Salmonella paratyphi-B*, *Escherichia coli*, *Enterococcus faecalis* and *Salmonella typhi*) obtained from HIV-infected patients using the Kirby-Bauer disk diffusion and minimum inhibitory concentration (MIC) methods. The antibacterial activity of *PE* against 345 bacterial isolates belonging to six different genera of Gram-negative bacterial populations isolated from urine specimens were evaluated by Saeed and Tariq (2007). Aqueous infusion and decoction of *PE* exhibited potent antibacterial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *K. ozaenae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *S. paratyphi* A, *S. paratyphi* B and *Serratia marcescens*, but did not show any antibacterial activity against Gram-negative urinary pathogens (Saeed and Tariq, 2007).

Saini et al. (2008) studied the protective efficacy of *PE* against *Klebsiella pneumoniae*-induced pneumonia in mice. The effect of short- (15 days) and long-term (30 days) feeding of *PE* in mice over the course of *K. pneumoniae* ATCC43816 infection in the lungs was studied in terms of bacterial colonisation, macrophage activity, MDA and nitrite production in the bronchoalveolar lavage fluid. TNF-α levels in serum were also assessed. The results in the long-term feeding experimental model suggested that supplementation with *PE* might reduce bacterial colonisation in the lung (Saini et al., 2008).

2.4.9. Antidiarrhoeal effects

Perianayagam et al. (2005) found that the methanol extract of *PE* fruit showed a significant inhibitory effect on diarrhoea in Wistar albino rats induced by castor oil and magnesium sulphate. Oral administration of the extract (50–150 mg kg^{-1} BW) produced a significant dose-related reduction in gastrointestinal motility in charcoal meal tests in rats. It also significantly inhibited the production of prostaglandin E_2 (PGE_2)-induced enteropooling

as compared to control animals (Perianayagam et al., 2005). Mehmood et al. (2011) studied the possible medicinal use of *PE* in diarrhoea *in vivo* and *in vitro*. The *in vivo* studies were conducted in mice, while isolated rabbit jejunum and guinea pig ileum were used for the *in vitro* experiments. The results showed that the crude extract of *PE* caused an inhibition in castor oil-induced diarrhoea and intestinal fluid accumulation in mice at 500–700 mg kg^{-1} BW. The results of the *in vitro* studies indicated that the *PE* fruit extract possesses antidiarrhoeal and spasmolytic activities, possibly mediated through dual blockade of muscarinic receptors and Ca^{2+} channels (Mehmood et al., 2011).

2.4.10. Hepatoprotective effects

PE fruit and its extract were found to have beneficial effects on hepatic injury induced by chemical agents (Chen et al., 2011a; Verma and Chakraborty, 2008; Pramyothin et al., 2006; Tasduq et al., 2005b). Moreover, it was found that the fruit of *PE* could reverse fibrosis in the liver (Tasduq et al., 2005a; Mir et al., 2007).

Chen et al. (2011a) elucidated the effects of *PE* fruit supplementation (100 mg ml^{-1} BW) on NDEA-induced injury in rats by evaluating ROS responses in the liver and bile. They found that *PE* fruit significantly preserved the expression of MnSOD and CAT and decreased the expression of iNOS and cytochrome P450 2E1 (CYP2E1) protein in the livers of NDEA-treated rats. *PE* fruit also decreased NDEA-enhanced hepatic apoptosis and autophagy *via* downregulation of the bax/bcl-2 ratio and beclin-1 expression (Chen et al., 2011a). In study by Verma and Chakraborty (2008), administration of a *PE* fruit aqueous extract (2 mg/animal/day) for 45 days along with ochratoxin caused significant amelioration in the ochratoxin-induced reduction in DNA, RNA and protein contents in the livers and kidneys of mice (Verma and Chakraborty, 2008). Pramyothin et al. (2006) found that pretreatment of rats with a *PE* extract with a single oral dose of 25, 50 or 75 mg kg^{-1}, 4 h before ethanol treatment, lowered the ethanol-induced levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and IL-1β in rats. The 75 mg kg^{-1} *PE* dose gave the best result. Treatment of rats with *PE* extract (75 mg kg^{-1} per day) also enhanced liver cell recovery by bringing the levels of AST, ALT and IL-1β back to normal (Pramyothin et al., 2006).

Tasduq et al. (2005a) found that a hydroalcoholic (50%) extract of *PE* fruit could reverse fibrogenic events in the liver, and suggested that the effect was due to the antioxidant activity. A similar study carried out by Mir et al. (2007) supported this conclusion. The protective effect of the same extract used by Tasduq et al. (2005a) against anti-tuberculosis (anti-TB) drug-induced liver toxicity was studied by the same group. *In vitro* studies were done on suspension cultures of rat hepatocytes while sub-acute studies were carried out in rats. The *PE* extract was found to be hepatoprotective and the authors

suggested that these effects were due to its membrane stabilising, antioxidant and CYP 2E1 inhibitory effects (Tasduq et al., 2005b).

2.4.11.Effects on gastric ulceration

The healing properties of *PE* fruit and its extracts against gastric ulceration have been studied. Most of these studies were carried out in animal models (Bhattacharya et al., 2007; Sairam et al., 2002; Bandyopadhyay et al., 2000). Bandyopadhyay et al. suggested that the antioxidant property appeared to be primarily responsible for the healing effects of the *PE* extract (Bandyopadhyay et al., 2000). An extract of *PE* also showed significant antioxidant effects in stressed animals (Sairam et al., 2002).

Bhattacharya et al. (2007) suggested that a 95% ethanol extract of sun-dried *PE* fruit (100 mg kg^{-1} per day) accelerated the healing process of ulcers. The results of Sairam et al. (2002) showed that a methanolic extract of *PE* had significant gastroprotective and ulcer-healing effects, which might be due to its effects both on offensive and defensive mucosal factors. Bandyopadhyay et al. (2000) found that pretreatment with the butanol fraction of the aqueous extract of *PE* fruit at a dose of 100 mg kg^{-1} BW per day, orally administered to rats for 10 consecutive days, enhanced the secretion of gastric mucus and hexosamine ($P< 0.001$) in the context of indomethacin-induced ulceration in rats. The morphological observations also supported a protective effect of the extract on the stomach wall. Indomethacin treatment of animals pretreated with the *PE* extract hardly affected either the MDA or SOD levels in gastric tissue, while treatment with the ulcerative agent alone significantly enhanced both the levels of SOD and MDA (Bandyopadhyay et al., 2000). Bafna and Balaraman (2005) suggested that *Pepticare* (a herbomineral formulation, consisting of *Glycyrrhiza glabra*, *PE* and *Tinospora cordifolia*) could ameliorate gastric ulcers in rats.

Chatterjee et al. (2011) suggested that the ethanolic extract of *PE* showed biphasic activity in non-steroidal anti-inflammatory drug (NSAID)-induced ulcers in mice, with the healing effect observed at 60 mg kg^{-1} and an adverse effect at 120 mg kg^{-1}.

2.4.12. Other functions

Beyond the health effects mentioned above, some studies also suggest that extracts of *PE* may possess anti-pyretic and analgesic activity, skin protective effects and wound-healing effects (Perianayagam et al., 2004; Adil et al., 2010; Kumar et al., 2008; Sumitra et al., 2009).

Perianayagam et al. (2006) found that a single oral dose of the ethanol and aqueous extracts of *PE* fruit (500 mg kg^{-1} BW, i.p.) led to a significant reduction in brewer's yeast-induced hyperthermia in rats. Ethanol and aqueous extracts of *PE* fruit also elicited

pronounced inhibitory effects on the acetic acid-induced writhing response in mice in a test for analgesic activity (Perianayagam et al., 2004).

Effect of *PE* fruit against UVB-induced photo-aging in human skin fibroblasts was studied by Adil et al. (2010) *in vitro*. The results suggested that *PE* fruit effectively inhibits UVB-induced photo-aging in human skin fibroblasts *via* its strong ROS scavenging ability (Adil et al., 2010).

Kumar et al. (2008) found that an alcoholic extract of *Triphala* promoted the healing of infected full-thickness dermal wounds. Sumitra et al. (2009) proved that the topical application of a 90% ethanol extract of dry *PE* fruit powder exerted wound healing action through the upregulation of collagen expression and extracellular signal-regulated kinase (ERK1/2) signalling.

3. SUMMARY OF THE PRESENT STUDY

3.1. Objectives of the study

The objectives of the present investigation were to:

1. Investigate the acids, sugars and sugar alcohols in Chinese hawthorn (*Crataegus* sp.) fruits and to compare the differences in these compounds among different species/cultivars.

2. Characterise the phenolic compounds of fruits of the *Crataegus pinnatifida* var. *major*.

3. Compare the differences in compositional profile of the phenolics of fruits of different species and cultivars of Chinese hawthorn.

4. Characterise the phenolic compounds of fruits and leaves of *Crataegus grayana* and follow the changes in the contents of phenolics of fruits and leaves of *C. grayana* during fruit ripening and harvesting.

5. Characterise the phenolic compounds of fruits of *Pyllanthus emblica*.

3.2. Materials and methods

3.2.1. Plant samples

Fruits of *C. pinnatifida.* var. *major* were collected from Qingzhou, Shandong Province, China, 2006. After harvesting, the fruits were dried, and the seeds were removed (**II**).

Hawthorn fruits of 22 cultivars and origins were collected from different sources in China (**I, III**) (**Table 9**). After harvesting, the fruits were sliced and dried in the shade. Among these, a sample of *C. scabrifolia* was collected in Kunming, Yun'nan Province, China. The other samples were collected in the Chinese National Fruit Germplasm Repository, Shenyang Hawthorn Garden (Shenyang, Liaoning Province, China)

Fruits and leaves of *C. grayana* (**IV**) were picked in Turku, Finland, between 11 August and 5 October, 2009 at roughly one-week intervals. Three fruit samples and three leaf samples were collected at each harvesting date and each sample was collected from three trees. Immediately after harvesting, the fruits and leaves were frozen and stored at -18°C until analysis.

Four emblic leafflower fruit (*P. emblica*) samples were collected from Guangxi and Fujian provinces in China, 2006. The fruits of the variety Ping Dan No 1 (EMB 1) and Fruity (EMB 2) were collected from a cultivation site in the town of Danzhu, Pingnan County, Guangxi Province, China. Fruit samples of wild Tian Chuan emblic leafflower were collected from two different growth sites in Lantian County (EMB3) and Weishan County (EMB4), Fujian Province, China (EMB3 and EMB4). After harvesting, the fruits were sliced and air-dried for one day, followed by hot air-drying at 60°C for 8 hours.

Table 9. Hawthorn fruits of 22 cultivars and origins were collected in China.

Species	Cultivar/origin	Harvesting year
C. pinnatifida var. *major*	947	2007
	8321	2007
	Dajinxing	2007
	Huixiandahong	2007
	Jiangou 2	2007
	Mopan	2007
	Qiujinxing	2007
	Shandongdajinxing	2007
	Shen78201	2007
	Zizhenzhu	2007
C. brettschneideri	Caihong	2008
	Hongroushanlihong	2008
	Hongroushanzha	2008
	Jifu 1	2008
	Jifu 3	2008
	Xinghong 2	2008
	Zuofu 1	2008
	Zuofu 2	2008
C. pinnatifida	Shanzha 1	2008
	Shanzha 2	2008
	Shanzha 3	2008
C. scabrifolia	Yun'nan shanzha	2007

3.2.2. Analysis of acids, sugars and sugar alcohols in Chinese hawthorn fruits (Study I)

3.2.2.1. Sample preparation

The dried, seedless hawthorn fruits were milled into a fine powder in a mortar with the aid of liquid nitrogen and extracted with MilliQ water in a sonication bath. The extracts were evaporated to dryness under nitrogen flow at 40°C and dried further in a desiccator above P_2O_5 overnight. Trimethylsilyl (TMS) derivatives of acids, sugars and sugar alcohols of the samples were prepared by adding the Tri-Sil HTP reagent to the extracts, followed by incubation at 60°C for 30 min.

3.2.2.2. Gas chromatography

TMS derivatives of the samples were first analysed with a Hewlett Packard 5890 Series II gas chromatograph (GC, Hewlett-Packard Co., Palo Alto, CA) equipped with a flame ionisation detector (FID) and a Hewlett Packard 7673 auto-sampler. The analyses were carried out with a Supelco Simplicity-1 fused silica column (30 m L × 0.25 mm i.d. × 0.25 μm d_f) (Bellefonte, PA). A sample of 1 μL was injected into a split/splitless injector (in splitless mode). The average flow rate of the helium carrier gas was 1.4 ml/min. The temperature of the injector was 210°C and that of the detector 290°C. The column temperature was programmed as 2 min at 150°C, raised to 210°C at a rate of 6°C/min, before reaching the final temperature of 275°C at a rate of 40°C/min, and held at 275°C for 10 min.

TMS derivatives of the samples and the reference compounds were also analysed using a Shimadzu QP 5000 MSD GC-MS (Kyoto, Japan). The column used was DB-1MS (30 m L × 0.25 mm i.d. × 0.25 μm d_f) (J & W Scientific, Agilent, Folsom, CA). A sample of 0.5 μL was injected manually into a split (1:24) injector. The flow rate of the helium carrier gas was 1.3 mL/min. The temperature of the injector and the column temperature program were the same as in the corresponding GC-FID analysis. The temperature of the interface was 290°C.

3.2.2.3. Qualitative and quantitative analysis

Acids, sugars, and sugar alcohols were identified by comparing the retention times and mass spectra of the analytes with those of the reference compounds and by co-analyses of the sample and reference compounds. Quantitative analysis of the acids, sugars and sugar alcohols in the fruits were carried out by co-analyses of the sample with internal standard solutions (tartaric acid for acids, xylose for sugars and mannitol for sugar alcohols).

3.2.3. Analysis of phenolic compounds in hawthorn fruits and leaves (Studies II, III and IV)

3.2.3.1. Sample preparation for qualitative analysis of phenolic compounds in fruits of *C. pinnatifida* var. *major*

Dried, seedless hawthorn fruits were milled into a fine powder with the aid of liquid nitrogen using a Retsch electric mill (Haan, Germany) and kept in a desiccator overnight before extraction.

The crude ethanol extract samples were prepared by extracting hawthorn fruit powder with 80% aqueous ethanol in an ultrasonicator bath. After the removal of ethanol with a vacuum rotary evaporator, the extract was dissolved in methanol, followed by filtration

through a 0.45 µm filter. After this, the sample was analysed by HPLC-DAD and HPLC-ESI-MS.

The crude ethanol extract was fractionated in nineteen fractions on a 20 mm i.d. × 370 mm glass column packed with 25 g Polyamide 6 (bulk density: 0.25 g/ml, particle size 50–160 µm, Sigma-Aldrich, Buchs, Germany). After application of the sample, the column was sequentially eluted with 400 mL MilliQ H_2O (water fraction), 10 × 100 mL methanol (Fractions I–X) and 8 × 100 mL aqueous acetone (70%, fractions XI–XVIII). After the removal of solvents (vacuum rotary evaporator at 35°C), each fraction was redissolved in methanol, filtered through a 0.45 µm filter and analysed by HPLC-DAD and HPLC-ESI(+)-MS.

3.2.3.2. Sample preparation for quantitative analysis of phenolic compounds in fruits of Chinese hawthorn (Study III)

Dried, seedless hawthorn fruits were milled into a fine powder with the aid of liquid nitrogen in a mortar and kept in a desiccator overnight before extraction. The fruit powder was extracted with 80% aqueous ethanol and a 1.0 mL sample was taken and filtered through a 0.45 µm filter and analysed by HPLC-DAD-ESI(+)-MS.

3.2.3.3. Sample preparation for quantitative analysis of fruits and leaves of *C. grayana* (Study IV)

The frozen hawthorn fruits and leaves were manually cut into small pieces and the seeds of the fruits were removed. The samples were extracted with methanol and analysed by HPLC-DAD and HPLC-ESI(+)-MS.

3.2.3.4. HPLC-DAD and HPLC-ESI-MS (Studies II, III and IV)

The HPLC-DAD system consisted of a GT-154 vacuum degasser, two LC-10AT pumps, a SIL-10A automatic injector, a CTO-10A column oven, an SPD-M10A VP photo diode array detector (DAD) and an SCL-10A VP system controller (Shimadzu, Kyoto, Japan). The system was operated using Class-VP 6.1 Workstation software. A Phenomenex Prodigy RP-18 ODS (3) column (5 µm, 250 × 4.60 mm, Torrance, CA) combined with a Phenomenex Prodigy guard column (5 µm, 30 × 4.60 mm, Torrance, CA) was used. A binary solvent system was employed consisting of formic acid/water (0.5:99.5, v/v) as solvent A and acetonitrile /methanol (80:20, v/v) as solvent B. The gradient program was 0–5 min with 10% solvent B, 5–15 min with 10–18% B, 15–25 min with 18% B, 25–30 min with 18–25% B, 30–35 min with 25% B, 35–40 min with 25–35% B, 40–45 min with 35–60% B, 45–50 min with 60–10% B and 50–55 min with 10% B. The flow rate of the mobile phase was 1 mL/min, and the injection volume was 10 µL. The peaks were monitored at three different wavelengths: 280, 360 and 520 nm.

HPLC-ESI(+)-MS analysis was performed using a Waters Acquity Ultra Performance LC system in combination with a Waters Quattro Premier mass spectrometer (Waters Corp., Milford, MA) equipped with an ion-spray interface. The HPLC columns, injection volume, flow rate and gradient programme were the same as in the HPLC-DAD analysis. The capillary voltage was set to 4.0 kV, the cone voltage 22 V and the extractor voltage 3 V. The source temperature was 150°C and the desolvation temperature was 300°C. The HPLC-ESI(+)-MS system was operated using MassLynx 4.1 software.

The full scan method was used in study II and both full scan and selected ion recording (SIR) method were used in study III and IV.

3.2.4. Statistical analysis (Studies I and III)

In study I and III, statistical analyses were performed using SPSS 16.0.1 (SPSS Inc., Chicago, IL) and Unscrambler 9.8 (Camo Process AS, Oslo, Norway). Differences in chemical composition among the species were analysed using one-way analysis of variance (ANOVA) and with the Games–Howell and Student–Newman–Keuls (SNK) tests. Differences reaching a confidence level of 95% were considered significant. Pearson's correlation coefficient analysis was carried out to investigate the correlation between the contents of different phenolic compounds in hawthorn samples. Principal component analysis (PCA) was used to interpret differences between hawthorn samples based on the contents of phenolics analysed in this study.

3.2.5. Analysis of phenolic compounds in emblic leafflower fruits

3.2.5.1. Sample preparation

Dried, seedless emblic leafflower fruits were milled into a fine powder using a Retsch electric mill (Haan, Germany) with a 0.5 mm sieve and stored in a desiccator until extraction.

Preparation of the crude methanolic extract was carried out by extracting dry emblic leafflower fruit powder with methanol in an ultrasonicator bath.

Preparation of Sephadex LH-20 column chromatographic fractions was carried out by applying the crude extract to a 22 mm i.d. × 470 mm L. glass column (Wright Scientific Ltd., England) packed with 20 g Sephadex LH-20 medium (dry particle size 18-111 μm) and by sequentially eluting with solvents as listed in **Table 10** to yield ten fractions. After the organic solvents were removed by nitrogen flow, the fractions were freeze-dried and redissolved in methanol and filtered through a 0.45 μm filter. The fractions were analysed with HPLC-ESI(–)-MS.

Table 10. Eluotropic series for Sephadex LH-20 column chromatography.

Fraction	H_2O (%)	MeOH (%)	Acetone (%)
F1	100	0	0
F2	70	30	0
F3	50	50	0
F4	50	40	10
F5	50	30	20
F6	50	20	30
F7	50	10	40
F8	50	0	50
F9	30	0	70
F10	20	0	80

3.2.5.2. HPLC analysis (Study V)

In study V, HPLC-DAD, HPLC-ESI(-)-MS and LC-DAD-ESI(–)-TOF-MS analyses were employed to identify the components of samples.

The HPLC-DAD and HPLC-ESI(-)-MS system were the same as described in 3.2.3.4. A binary solvent system was employed consisting of 0.1% formic acid in MilliQ H_2O as solvent A and acetonitrile/methanol (4:1, v/v) as solvent B. The gradient program was 0–5 min with 0% solvent B, 5–15 min with 0–5 % B, 15–20 min with 5–10 % B, 20–25 min with 10–15 % B, 25–30 min with 15–20 % B, 30–35 min with 20–25 % B, 35–40 min with 25 % B, 40–55 min with 25–60 % B, 55–60 min with 60–0 % B and 60–65 min 0% with B. The flow rate of the mobile phase was 1 mL/min, and the injection volume was 10 µL.

In the HPLC-ESI(-)-MS system, the capillary voltage was set to 3.0 kV, the cone voltage 25 V and the extractor voltage 8 V. The source temperature was 120°C and the desolvation temperature was 300°C. The mass spectra were obtained by scanning ions between *m/z* 100 and 1300. The HPLC-ESI(–)-MS system was operated using MassLynx 4.1 software (Waters Corp., Milford, MA).

The crude extract and the fractions of sample EMB1 were also analysed with a HPLC-DAD-ESI(–)-TOF-MS system, which consisted of an Agilent HPLC 1200 Series equipped with diode array detector (Agilent Technologies, Waldbronn, Germany) and micrOTOF$_Q$ ESI-mass spectrometer (Bruker Daltonics, Bremen, Germany). Chromatographic separations were performed using an XBridge™ column (2.1×100 mm, C-18, 3.5 µm, Waters, Dublin, Ireland). The binary mobile phase consisted of acetonitrile (A) and water and formic acid (99.6:0.4, v/v) (B). The elution started with 0-2 min 0% A and a linear gradient elution was performed to obtain 30% A at 33 min and 70% A at 35 min

staying constant until 43 min. The flow rate was 0.3 mL/min and the injection volume was 5 µL. Chromatograms were recorded at 280 nm. The HPLC system was controlled by Hystar software (version 3.2., Bruker BioSpin, Rheinstetten, Germany). The mass spectrometer was controlled by Bruker Compass micrOTOF control software (Bruker Daltonics, Bremen, Germany) and operated in negative ion mode. The capillary voltage was maintained at +4000 V with the end plate offset at -500 V. The pressure for the nebuliser gas (N_2) was set at 1.6 bar, the drying gas (N_2) flow was 8.0 L/min and the drying gas temperature was 200°C. The full scan mass ranged from *m/z* 100 up to *m/z* 2000. Calibration with 5 mM sodium formate injected via a six-port-valve was used at the end of the LC-MS experiment in order to provide high-accuracy mass measurements. The data were handled by Bruker Compass DataAnalysis (version 4.0; Bruker Daltonics, Bremen, Germany).

3.3. Results and discussion

3.3.1. Acids, sugars and sugar alcohols in Chinese hawthorn fruits (Study I)

The identification of acids, sugar and sugar alcohols in fruits of Chinese hawthorn was carried out in study I using GC-FID and GC-MS. Malic acid, citric acid and quinic acid were the major organic acids in all the samples analysed. In some samples, ascorbic acid was detected in trace amounts. Fructose, glucose, sucrose, sorbitol and *myo*-inositol were the major sugars and sugar alcohols found in these fruits. Sucrose was detected in four samples only, of which three belonged to *C. pinnatifida* var. *major* and one to *C. scabrifolia.*

The total acid content of the fruits varied from 31 to 118 g kg^{-1} DM. High acid contents were found in cultivars 947 (118 g kg^{-1} DM) and 8321 (115 g kg^{-1} DM) of *C. pinnatifida* var. *major*. The samples with low acid contents were Jiangou 2 (31 g kg^{-1} DM) and Shen78201 (38 g kg^{-1} DM) of *C. pinnatifida* var. *major*. A wide variation was seen (31 to 118 g kg^{-1} DM) in the acid content among the cultivars of this variety ($P < 0.05$). The total acid content of eight cultivars of *C. brettschneideri* ranged from 51 to 74 g kg^{-1} DM with clearly smaller variation within species than within *C. pinnatifida* var. *major*.

The total sugar content, including sugar alcohols, ranged from 185 to 445 g kg^{-1} DM. All three natural origins of *C. pinnatifida* had lower total sugar contents (185–271 g kg^{-1} DM) than *C. scabrifolia* and cultivars of *C. brettschneideri* and *C. pinnatifida* var. *major* (282–445 g kg^{-1} DM, $P < 0.01$).

The sugar/acid (S/A) ratio varied widely from 2.4 to 13.2 among the cultivars of *C. pinnatifida* var. *major*. Less variation (4.1–8.8) was seen within the species *C.*

brettschneideri. The S/A ratios in the fruits of *C. scabrifolia* (3.7) and the natural origins of *C. pinnatifida* (2.5–3.8) were low, mainly because of the low sugar content.

Sucrose was detected in only three cultivars (8321, Huixiandahong and Shangdongdajinxing) of *C. pinnatifida* var. *major* and of *C. scabrifolia* origin. The clear on/off situation of sucrose in the fruits of cultivars within *C. pinnatifida* var. *major* indicates significant differences in sucrose metabolism, probably because of the introduction of a different genotype to some of the cultivars during breeding.

Principle component analysis (PCA) revealed that *C. pinnatifida* var. *major* fell into two groups, one rich in fruit acids, the other rich in sugars. In contrast, the cultivars of *C. brettschneideri* were more homogenous, belonging mostly to the sugar-rich group.

3.3.2. Purification and enrichment of phenolic compounds with polyamide column chromatography (Study II)

Polyamide column chromatography has been widely used for the purification of flavonoids. A number of studies have indicated the good capacity of polyamide column chromatography in the fractionation of procyanidins and phenolic compounds (Svedström et al., 2002a; Svedström et al., 2002b; Svedström et al., 2006).

HPLC-DAD chromatography of the crude aqueous ethanolic extract showed the complexity of the phenolic components in hawthorn fruit. To obtain a more detailed characterisation of these components, polyamide column chromatography was employed to fractionate the ethanolic extract into 19 fractions (a water fraction, fractions I–X eluted with methanol and XI–XVIII with 70% aqueous acetone).

Mainly sugars and fruit acids were eluted with water. No phenolic compounds were detected in the water fraction. Only traces of phenolic compounds were found in fractions I, XVII and XVIII, and the quantities were too small for further identification by UV and MS. The phenolics were mainly eluted in fractions II–XVI. **Figure 16** shows the HPLC-DAD chromatograms of fractions II–XVI. Fraction II is presented at 280, 360 and 520 nm. Fractions III–XVI contained mostly procyanidins, and therefore HPLC-DAD chromatograms of these fractions are presented at 280 nm. Peaks with identical retention times, UV spectra and mass spectra appearing in successive fractions were considered as the same compounds.

Pre-separation of the aqueous ethanol extract with polyamide column chromatography resulted in much improved separation in the subsequent HPLC-DAD and HPLC-MS analyses. Procyanidins were eluted from the polyamide column in a sequence related to the degree of polymerisation, such that the glycosides eluted earlier than the corresponding aglycons. Procyanidins with a polymerisation index (PI) higher than 2

were mostly eluted with aqueous acetone. Flavonol glycosides were found primarily in fractions II–IV.

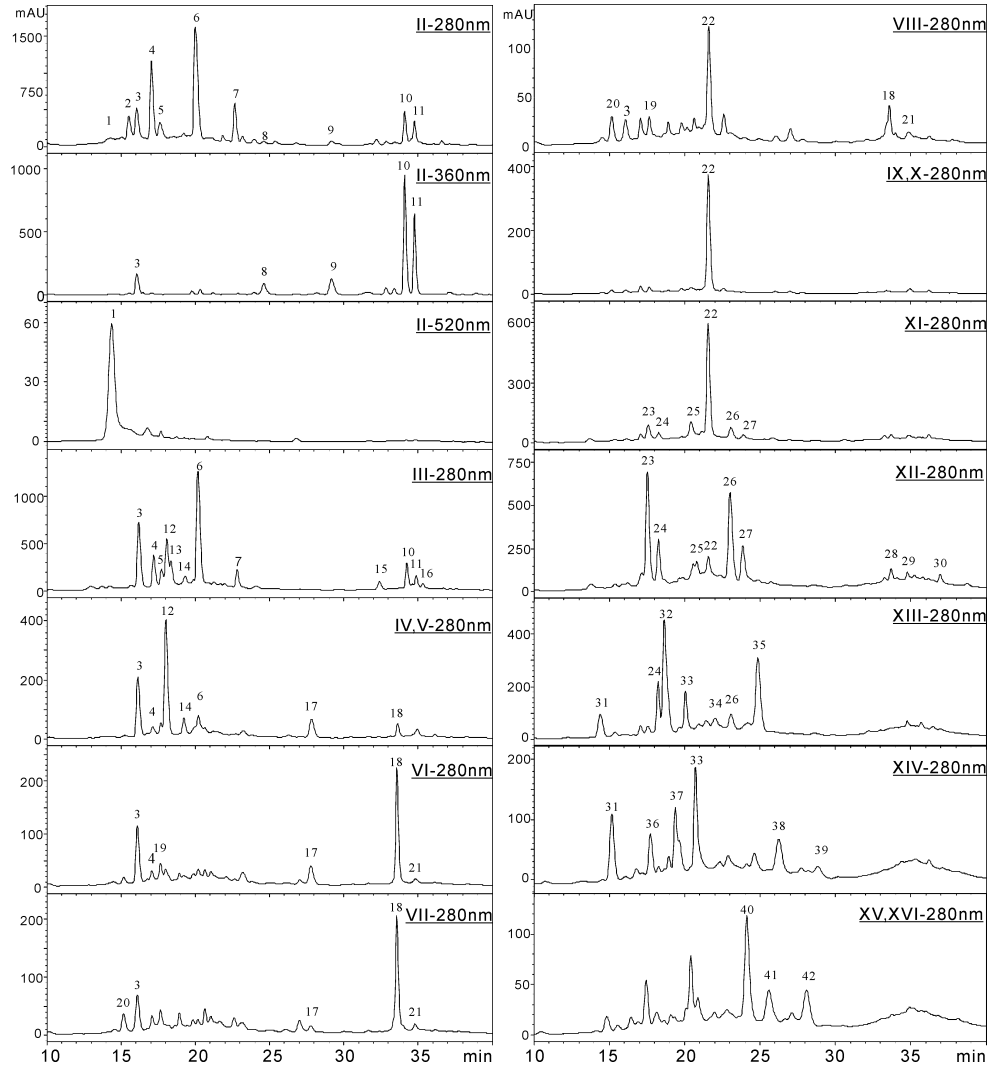


Figure 16. HPLC-DAD chromatograms of fractions II–XVI of ethanolic extract of hawthorn fruit. (A) fraction II at 280, 360 and 520 nm; (B) fractions III–IX at 280 nm; (C) fractions X–XVI at 280 nm.

3.3.3. Identification of phenolic compounds in Chinese hawthorn fruits (Study II)

In study II, forty-two phenolic compounds were identified/tentatively identified in the ethanol extracts of fruits of *C. pinnatifida* var. *major* by using HPLC-DAD, HPLC-ESI(+)-MS combined with polyamide column chromatography. Ideain (cyanidin-3-*O*-

galactoside), chlorogenic acid, procyanidin B2 [(-)-epicatechin-(4β→8)-(-)-epicatechin], (-)-epicatechin, hyperoside (quercetin-3-*O*-galactoside) and isoquercitrin (quercetin-3-*O*-glucoside) were identified using UV spectra, mass spectra and reference compounds. In addition, 35 compounds were tentatively identified based on the UV and mass spectra. These compounds were mostly B-type procyanidins (PC) and their glycosides, including aglycons of three dimers, three trimers, eight tetramers, four pentamers, two hexamers and two hexosides of PC monomers, seven hexosides of PC dimers, a hexoside of a PC trimer, two hexosides of PC tetramers, a hexoside of a PC pentamer and two glycosides of quercetin. A PC derivation with a MW of 403 was found.

3.3.4. Quantitative analysis of phenolic compounds in hawthorn using HPLC-ESI-MS-SIR (Study III and IV)

Quantitative analysis of phenolic compounds, especially of PCs in plant materials, is a challenging task. This is largely because of the lack of commercial reference compounds and deficient separation between PCs using high performance liquid chromatography (HPLC). To simplify the analysis and to improve the accuracy of quantification, single ion recording function of HPLC-electrospray ionisation mass spectrometry (HPLC-ESI-MS-SIR) was applied to quantify the phenolic compounds in extracts of hawthorn fruits and leaves without the prefractionation steps used in study III and V.

Figure 17 presents the chromatograms of the 80% ethanolic extract of the fruits of the hawthorn cultivar Mopan (*C. pinnatifida. var. major*), obtained by HPLC-DAD analysis at 280 nm and HPLC-ESI(+)-MS-SIR analysis at different *m/z* channels. The peaks were identified based on retention times and mass spectra. The humps under the peaks and the obvious overlapping in the HPLC-DAD chromatogram were because of insufficient separation. Comparing with the resolution of peaks in the HPLC-UV chromatogram at 280 nm and in the SIR chromatograms of HPLC-ESI(+)-MS, the peaks of the phenolic compounds in the 80% ethanolic extracts of the hawthorn fruit were clearly better for integration in the SIR chromatograms of HPLC-ESI(+)-MS, resulting in an increased accuracy of quantification.

In the concentration range of our study, when linear regressions were used for the calculations, the R^2 values of the calibration lines of all the reference compounds were between 0.986–0.999 and the linearity was high enough for quantification.

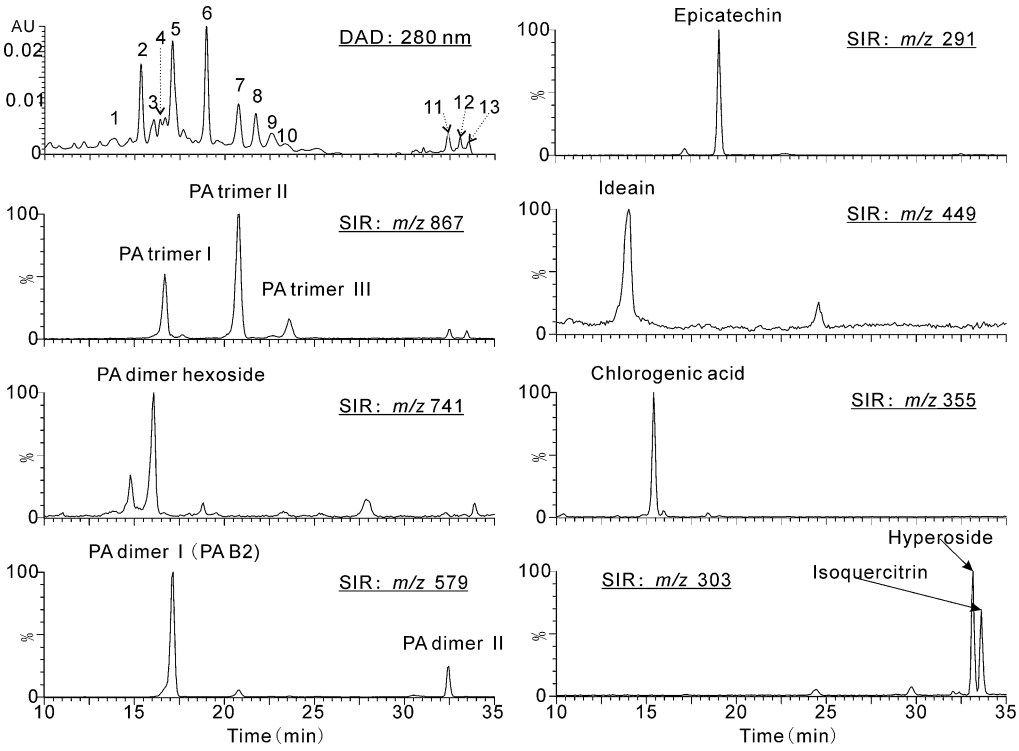


Figure 17. HPLC-DAD and HPLC-ESI(+)-MS chromatograms of the 80% ethanolic extracts of hawthorn fruits of the cultivar Mopan (*C. pinnatifida* var. *major*). Peaks in HPLC-DAD: 1, ideain (14.01 min); 2, chlorogenic acid (15.33 min); 3, PC dimer-hexoside (16.03 min); 4, PC trimer I (16.43 min); 5, PC dimer I (PC B2) (17.08 min); 6, (-)-epicatechin (18.98 min); 7, PC trimer II (20.75 min); 8, unknown PC derivative (21.72 min); 9, PC tetramer (22.60 min); 10, PC trimer III (23.40 min); 11, PC dimer II (32.40 min); 12, hyperoside (33.07 min); 13, isoquercitrin (33.61 min).

3.3.4.1. Quantitative analysis of phenolic compounds in Chinese hawthorn fruits (Study III)

The contents of hyperoside, isoquercitrin, chlorogenic acid, ideain, (-)-epicatechin, PC dimer I (PC B2), PC dimer II (PC B5), PC trimers (I, II, III) and PC dimer-hexoside in 22 Chinese hawthorn samples were determined by the HPLC-ESI-MS-SIR method used in study III.

Hyperoside and isoquercitrin were found to be the most abundant flavonol glycosides in the extracts of hawthorn fruits in all the 22 samples. In addition, other minor flavonol glycosides were detected, but the peaks were too small to be integrated.

The total contents of flavonol glycosides in the fruits varied from 0.2 to 1.1 g kg^{-1} DM. *C. brettschneideri* had the highest content of flavonol glycosides (0.7 g kg^{-1} DM as average content of eight cultivars in the species) among the species studied ($P < 0.05$). The fruits

of *C. pinnatifida* var. *major* contained less flavonol glycosides (0.4 g kg^{-1} DM as average content of 10 cultivars in the species) than those of the other species ($P < 0.05$). The content of hyperoside (0.1–0.8 g kg^{-1} DM) was higher than that of isoquercitrin (0.1–0.3 g kg^{-1} DM) in almost all the samples except the cultivar Huixiandahong of *C. pinnatifida* var. *major*. The content of chlorogenic acid varied from 0.3 to 1.6 g kg^{-1} DM. The fruits of *C. pinnatifida* var. *major* contained the highest level of chlorogenic acid (1.1 g kg^{-1} DM) and *C. scabrifolia* the lowest (0.3 g kg^{-1} DM) among the hawthorn species. Ideain was not detected in the fruits of *C. scabrifolia*. *C. brettschneideri* contained more ideain (0.4 g kg^{-1} DM) than the other species ($P < 0.05$).

The content of (-)-epicatechin in the fruits analysed in study III varied from 0.9 to 11.7 g kg^{-1} DM. Most samples contained (-)-epicatechin between 2–6 g kg^{-1} DM, but several samples had extremely high levels of the compound, e.g. Shandongdajinxing of *C. pinnatifida* var. *major* (11.7 g kg^{-1} DM). The levels of PC B2 ranged from 0.7 to 12.4 g kg^{-1} DM and PC dimer II from 0.1 to 1.5 g kg^{-1} DM. The contents of PC B2 and (-)-epicatechin were always close to each other. Three major PC trimers were detected in hawthorn fruit samples analysed in study III. PC trimer II was the most abundant (0.7 to 6.9 g kg^{-1} DM) followed by PC trimer I (0.1 to 2.7 g kg^{-1} DM). The content of PC trimer III (0.01 to 1.2 g kg^{-1} DM) was always the lowest among the PC trimers. The highest level (1.1 g kg^{-1} DM) of the PC dimer-hexoside was found in *C. scabrifolia* fruits. In addition, the dimer-hexoside was a typical compound in the cultivars of *C. pinnatifida* var. *major*, with varieties 947 and Mopan containing the highest amounts of the compound. The total contents of the eight PCs quantified in the 22 samples varied widely from 2.5 to 36.7 mg/g DM. The fruits of *C. pinnatifida* var. *major* and *C. scabrifolia* had higher PC contents than those of the two other species ($P < 0.05$).

The PCA results show that all the samples fell into two groups, one rich in procyanidins and the other rich in flavonols.

3.3.4.2. Correlation between the contents of different phenolic compounds in Chinese hawthorn fruits (Study III)

Pearson's correlation coefficient analyses were performed to investigate the correlation between the contents of phenolics in Chinese hawthorn fruits in study III. There was a positive correlation between the contents of hyperoside and isoquercitrin ($R^2 = 0.49$, $P < 0.01$). Significant positive correlations ($R^2 = 0.85–0.97$, $P < 0.01$) were found between the content of (-)-epicatechin and the levels of practically all PCs of different DP values except a PC dimer-hexoside. A positive correlation also existed between the contents of PC B2 and PC trimer II. This might provide useful compositional information with a simple analysis, especially if there is a lack of sophisticated chromatographic-mass spectrometric equipment and reference compounds available.

3.3.4.3. Phenolic compounds in fruits and leaves of *C. grayana* and changes during fruit ripening (Study IV)

Twenty-two phenolic compounds were found in fruits and leaves of *C. garayana*. Ideain, chlorogenic acid, PC B2, (-)-epicatechin and hyperoside were identified using UV spectra, mass spectra and reference compounds. In addition, seventeen compounds were tentatively identified based on the UV and mass spectra. These compounds belong to the B-type procyanidins, flavonol glycosides, *C*-glycosyl flavones and hydroxycinnamic acids.

Thirteen major phenolic compounds in fruits and fifteen in leaves of *C. grayana* were analysed quantitatively by the HPLC-MS-ESI(+)-SIR method used in study IV. The changing trends during fruits ripening and harvesting were also followed.

3.3.4.3.1. Fruits

Procyanidins were the major phenolic compounds in the fruits of *C. grayana*. The content of total procyanidins varied from 6 to 17 g kg^{-1} DM during fruit ripening. (-)-Epicatechin (1–7 g kg^{-1} DM), PC B2 (2–4 g kg^{-1} DM) and PC trimer II (tentatively identified as PC C1, 2–4 g kg^{-1} DM) were the most abundant procyanidins. All the procyanidins showed quite similar changing trends in the fruit ripening period. The highest contents were typically found in the fruits collected on 19 August. From mid-August to early September, the content of procyanidins was at a high level (15–17 g kg^{-1} DM in total). From the beginning to the end of September, the levels of procyanidins decreased dramatically (from 15 to 6 g kg^{-1} DM in total). By the beginning of October, the total content of procyanidins was up to 10 g kg^{-1} DM again.

Compared with the content of procyanidins, those of flavonol glycosides were quite low (1–1.5 g kg^{-1} DM). Hyperoside (0.5–1 g kg^{-1} DM) and quercetin-pentoside II (0.3–0.5 g kg^{-1} DM) were the major flavonol glycosides. The contents of flavonols increased slowly from 11-27 August to and then decreased for the rest of the period. The highest contents were detected in fruits collected on 27 August, with a total content of quercetin derivatives being close to 2 g kg^{-1} DM.

Chlorogenic acid increased from 0.5 g kg^{-1} DM in early August to 0.8 g kg^{-1} DM in early September and then slightly decreased during the following month. In the end, it reached a level above 1 g kg^{-1} DM by 5 October. The content of its isomer (5-*O*-caffeoylquinic acid) decreased over the entire period, from 0.4 g kg^{-1} DM to 0.3 g kg^{-1} DM.

The level of ideain in fruits increased from 0.2 to 3 g kg^{-1} DM during this period. The contents of *C*-glycosyl flavones in the fruits were too low to be quantified.

The content of ideain in the fruits increased continuously from 0.1 g kg^{-1} DM to 3 g kg^{-1} DM d over the entire period.

3.3.4.3.2. Leaves

The total procyanidin content in the leaves ranged from 5.4 to 30 g kg^{-1} DM. As in fruits, (-)-epicatechin (1–10 g kg^{-1} DM), PC B2 (1–8 g kg^{-1} DM) and PC trimer II (2–8 g kg^{-1} DM) were the major procyanidins. The contents of procyanidins remained stable in August and increased progressively in September until reaching the highest levels by the end of the month (30 g kg^{-1} DM). The content of procyanidins seemed to decrease after September. The changing trend in the content of chlorogenic acid in leaves was close to that of procyanidins. The content of chlorogenic acid in the leaves varied from 3 to 11 g kg^{-1} DM.

The content of total flavonol glycosides varied in the range of 7–21 g kg^{-1} DM during autumn. Hyperoside was the most abundant flavonol glycoside at levels of 3–11 g kg^{-1} DM, accounting for almost half of the total content of flavonol glycosides. The total *C*-glycosyl flavone content varied from 2 to 5 g kg^{-1} DM. The content of chlorogenic acid in leaves varied from 3 to 11 g kg^{-1} DM. The changing trend of flavonol and *C*-glycosyl flavone were quite similar, keeping constant in August and increasing sharply by two- to three-fold in early September and then increasing slowly thereafter.

3.3.5. Composition of phenolic compounds in emblic leafflower fruits (Study V)

Hydrolysable tannins were found to be the major phenolic compounds in emblic leafflower fruits. A mucic acid gallate, three mucic acid lactone gallate isomers, a galloylglucose, gallic acid, a digalloylglucose, putranjivain A, a galloyl-HHDP-glucose, elaeocarpusin and chebulagic acid were the most abundant compounds in crude methanol extracts of the fruits. In addition, 130 peaks were detected, of which sixty-three were identified/tentatively as identified simple gallic acid derivatives, derivatives of chebulic acid and chebuloylglucose, as well as ellagitannins and flavonoids. Neochebulagic acid, isomers of neochebuloyl galloylglucose (phyllanemblinins D, E and F), punicalagin, geraniinic acid, ellagic acid glycosides and quercetin glycosides were reported in emblic leafflower fruits for the first time. Furthermore, several isomers existed for compounds previously reported in the plant.

The fruits of two varieties (Ping Dan No 1 and Fruity) from Guangxi Province differed significantly from those of wild Tian Chuan emblic leafflower from Fujian Province in the content and profile of phenolic compounds. The contents of phenolic compounds were higher in the extracts of emblic leafflower fruits from Guangxi Province than in the extracts of those from Fujian Province, reflected as a greater (roughly two-fold) total peak area in the chromatograms of the crude extracts. Gallic acid and simple gallate esters represented the most abundant phenolic compounds in all the samples analysed The two varieties from Dayu County, Guangxi Province also contained high levels of ellagitannins and derivatives of chebulic acid and chebuloylglucose. In contrast, these groups of compounds were present at a much lower proportion in the two samples of Tian Chuan emblic leafflower.

3.4. Conclusion

Malic acid, citric acid and quinic acid were the major organic acids and fructose, glucose, sucrose, sorbitol and *myo*-inositol were the major sugars and sugar alcohols in hawthorn fruits. Sucrose was detected only in *C. pinnatifida* var. *major* and *C. scabrifolia.* Significant differences were found in the contents and composition of acids and sugars between different hawthorn species. The hawthorn samples analysed fell into two groups, those rich in sugars and those rich in acids.

Hawthorn fruits and leaves of different origins and fruits were rich in phenolic compounds. Procyanidins and flavonoids were the major phenolics in hawthorn.

A simple and reliable HPLC-ESI-SIR method was optimised and applied for the quantitative analysis of phenolic compounds in Chinese hawthorn fruits. The samples of *C. pinnatifida* var. *major* differed from *C. pinnatifida* and *C. brettschneideri* with higher contents of PCs ($P<0.05$). The content of ideain in *C. brettschneideri* was typically higher compared with levels in *C. pinnatifida* var. *major*, *C. scabrifolia* and *C. pinnatifida*. *C. scabrifolia* contained a higher level of PC dimer-hexoside than the rest of the samples studied, but contained no ideain. Among all the species, *C. pinnatifida* var. *major* contained the highest level of chlorogenic acid. Based on PCA analysis, the hawthorn samples analysed fell into two groups: those rich in PAs and those rich in flavonols.

The leaves and fruits of *C. grayana* differed in their contents and composition of phenolic compounds. The leaves of *C. garayana* contained a higher level of total phenolics, total flavonols and *C*-glycosyl flavones. Differences were also found in the compositional profiles of phenolics between the fruits of *C. grayana* and Chinese hawthorn.

Harvesting time affects the phenolic content in hawthorn fruits and leaves. Unripe fruits contain more phenolic compounds than ripe fruits.

Hydrolysable tannins were found to be the major phenolic compounds in emblic leafflower fruits. A mucic acid gallate, three mucic acid lactone gallate isomers, a galloylglucose, gallic acid, a digalloylglucose, putranjivain A, a galloyl-HHDP-glucose, elaeocarpusin and chebulagic acid were the most abundant compounds in the crude methanol extracts of the fruits. The fruits of two varieties (Ping Dan No 1 and Fruity) from Guangxi Province differed significantly from those of wild Tian Chuan emblic leafflower from Fujian Province in the content and profile of phenolic compounds. The contents of phenolic compounds were higher in the extracts of emblic leafflower fruits from Guangxi Province than in the extracts of fruits from Fujian Province.

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Turku, April, 2012

Pengzhan Liu

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