CHAPTER 2

LITERATURE REVIEW

2.1 Free radicals and skin aging

The idea that free radicals can cause aging was offered by Denham Herman in 1956. He proposed that aging and age-related disease might be due to the long term effect of oxidative damage by free radicals which, in turn, are modified by genetic and environmental factors (Wickens, 2001).

According to the free radical theory of aging, cellular senescence is a cumulative oxidative damage by free radicals, a causative factor in aging. Skin is constantly exposed to various environmental insults such as exposure to UV (Figure 2.1) and ionizing radiation, oxygen, ozone and pollutants that may deleteriously augment the normally occurring intracellular oxidative stress. Skin is also a major candidate and target of oxidative damage by free radicals. Lipid, proteins and DNA are biological sites of the skin aging are commonly associated with increased wrinkling, sagging and increased laxity (Jenkins, 2002).

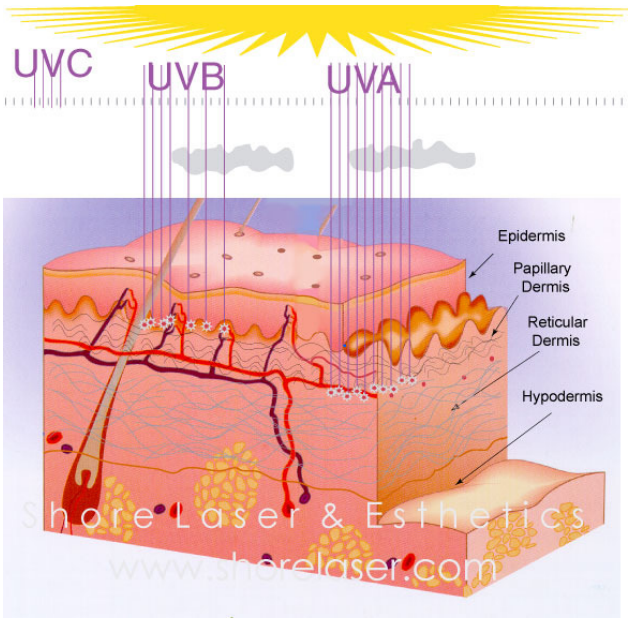


Figure 2.1 Skin exposed to ultraviolet (UV) light

(www. goremedical.com)

Free radicals are normal biochemical intermediates of any metabolic reactions. They consist of any chemical species (atom, ions or molecules) that contain one or more unpaired electrons in their outer atomic or molecular orbital. This makes them highly unstable and violently reactive (Benedetto, 1998 and Wickens, 2001). The most important free radicals found are reactive oxygen species (ROS) which include oxygen free radicals or oxygen-centered free radicals and non radical species as illustrated in Table 2.1.

Table 2.1 Reactive oxygen species (ROS)

Oxygen radical		**Non-radical oxygen**	
Superoxide anion	$O_2^{\bullet -}$	Hydrogen peroxide	H_2O_2
Hydroxyl	$^{\bullet}OH$	Hypochlorous acid	HOCL
Peroxyl	$ROO^{\bullet}$	Ozone	O_3
Alkoxyl	$RO^{\bullet}$	Singlet oxygen	
Hydroperoxyl	$HOO^{\bullet}$		

ROS are one of the major and important contributions to skin aging (Figure 2.2), skin disorders and skin diseases (Benedetto, 1998 and Kohen, 1999). They are mostly formed in mitochondria where oxygen is reduced in four sequential steps to produce water. This chain reaction produces a number of short-lived intermediates including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) (Ames *et al*., 1993). In addition, the intracellular formation of these free radicals can be stimulated by the environmental sources, especially UVA and UVB radiation (Halliwell and Gutteridge, 1989). H_2O_2 may initiate a peroxidation process in either lipids or proteins. This lipid peroxidation process may lead to a change in the fluidity of the plasma membranes resulting in a molecule leakage and a subsequent dysfunction in its essential roles. In addition, ROS may also directly inactivate enzymes and cause protein and DNA degradation. Damage to DNA may result in deleterious process, aging, as well as onset of cancer and other pathological disorders (Kohen, 1999 and Vendemiale, *et al*., 1999).

Figure 2.2 Skin aging

(www.wikipedia.org)

2.2 Defense mechanism of skin against oxidative damage

The epidermis of the skin possesses an extremely efficient antioxidant activity that is superior to most tissues (Jenkins, 2002). There are two types of antioxidants, the enzymatic and non enzymatic antioxidants (Benedetto, 1998).

The enzymatic antioxidants including superoxide dismutases (SOD), catalase, glutathione peroxidase (GSHP) and glucose-6-phosphaste dehydrogenase (G-6-PD), protect cells by hastening biochemical reaction. Moreover, thioredoxin reductase, catalase and GSHP/reductase are the main antioxidant enzymes which are involved in the protection of the epidermis against UV-radiation-generated ROS (Benedetto, 1998).

The nonenzymatic antioxidants, including ascorbic acid (vitamin C), α-tocopherol (vitamin E), retinol (vitamin A), β-carotene and glutathione, help dissipate intracellular oxidants or ROS by acting as free radical scavengers, thereby maintaining intracellular redox homeostasis and reducing the potential for cellular oxidative damage (Benedetto, 1998). However, UV radiation exposure (both acute and chronic) causes a decrease of these nonenzymatic antioxidants in cell diminishing their role as oxidant quenchers and free radical scavengers. Hence, the rationale for the suggestion of vitamins A, C, E and their

derivative might be effective in the prevention of skin damage caused by ROS that are induced by UV radiation (Benedetto, 1998).

2.3 Pomegranate

Scientific name: *Punica granatum* Linn.

Synonym: *Punica nana* Linn.

Common name: Pomegranate, Tubtim, Siae lin (Chinese), Phi laa (Nong khai), Philaa khaao (Nan), Ma koh (Northern), Maak-chang (Shan-Mae Hong Son) (นันทวัน บุณยะ-ประภัศร, 2541).

Family name: Punicaceae

Botanical description: The plant is an erect shrub up to 3 m high, much branched from the base, having branchlets slender, often ending in a spine. Leaves are simple, oblong-lanceolate, 1-9 by 0.5-2.5 cm, and consisting of obtuse or emarginated apex, acute base, shiny and glabrous texture. Flowers are showy, orange red, about 3 cm in diameter, 1-5 borned at branch tips, the others solitary in highest leaf-axils, sessile or subsessile; consisting of calyx 2-3 cm long, tubular, lobes erect or recurved, thick, coriaceous; petals the same numbers as the calyx lobes, rounded or very obtuse, from edge of hypanthium, caduceus; stamen numerous within upper half of hypanthium, filament free; inferior ovary, ovules numerous, style1, stigma capitate. Fruit is globose berry, crowded by persistent calyx-lobes, having pericarp leathery filled with numerous seeds, which are surrounded by pink and red, transparent, juicy, acid, pleasant-tasting pulp (Figure 2.3) (Farnsworth and Bunyapraphatsara, 1992). In each sac there is one angular, soft or hard seed.

Figure 2.3 Pomegranate fruit

Growth Habits: The pomegranate is a neat, rounded shrub or small tree that can grow to 20 or 30 feet, but more typically to 12 to 16 feet in height. Dwarf varieties are also known. It is usually deciduous, but in certain areas the leaves will persist on the tree. The trunk is covered by a red-brown bark which later becomes gray. The branches are stiff, angular and often spiny. There is a strong tendency to sucker from the base. Pomegranates are also long-lived. There are specimens in Europe that are known to be over 200 years of age. The vigor of a pomegranate declines after about 15 years, however. High temperatures are essential during the fruiting period to get the best flavor. The pomegranate may begin to bear in 1 year after planting out, but 2 ½ to 3 years is more common. Under suitable conditions the fruit should mature some 5 to 7 months after bloom.

Many researches have focused on antioxidant actions *in vitro*, *ex vivo* and *in vivo*, while other work has elaborated on the ability of pomegranate juice, seed oil, peel or flower extracts, and their derivatives to kill bacteria and viruses, or to fight vascular disease, diabetes and cancer. In everything from improving erectile insufficiency in rabbits to healing ethanol induced stomach ulcers in rats, antioxidant action is given as the leitmotif and root of the observed

beneficial effects. Driven by such studies, sales of pomegranate juice are soaring worldwide, with even pomegranate seed oil beginning to appear in the marketplace.

2.3.1 Chemistry

While detailed knowledge of relationships of the chemical content of pomegranates and their desirable pharmacologic endpoints has yet to be obtained, significant progress has been made over the past 8 years toward a much more comprehensive understanding of some of the important pharmacologic components of pomegranate. Chemical studies of pomegranate have reported on many compounds isolated from different parts of the plant. List of the compounds found in pomegranate is shown in table 2.2. In addition to the more common anthocyanins shown in the table, pentose glycosides of malvidine and pentunidin have been described in the pericarp and juice (Sharma and Seshadri, 1955). Although some limited knowledge of the abundance of selected compounds does exist, e.g., vitamin C in the juice at 0.47 mg/100 g (Veres, 1976), in general such quantitative knowledge is lacking, and hence has been left out.

Table 2.2 Chemical constituents of pomegranate

Chemical class	**Compound name**	**Plant part**	**References**
Hydroxybenzoic acids	Gallic acid	Juice, Peel, Fruit	Amakura *et al*., 2000b Huang *et al*., 2005b
Hydroxybenzoic acids	Ellagic acid	Juice, Peel, Seed	Amakura *et al*., 2000b Wang *et al*., 2004
Hydroxycinnamic acids (phenylpropanoids)	Caffeic acid	Juice, Peel	Artik, 1998, Amakura *et al*., 2000a
Hydroxycinnamic acids (phenylpropanoids)	Chlorogenic acid	Juice, Peel	Artik, 1998, Amakura *et al*., 2000a

Table 2.2 Chemical constituents of pomegranate (*Continued*)

Chemical class	Compound name	Plant part	References
Hydroxycinnamic acids (phenylpropanoids)	ρ-Coumaric acid	Juice, Peel	Artik, 1998, Amakura *et al*., 2000a
Cyclitol carboxylic acids And their salts	Quinic acid	Juice, Peel	Artik, 1998, Amakura *et al*., 2000a
Flavan-3-ols	Flavan-3-ol	Juice, Peel	de Pascual-Teresa *et al*., 2000
Flavan-3-ols	Catechin	Juice, Peel	de Pascual-Teresa *et al*., 2000
Flavan-3-ols	Epicatechin	Juice, Peel	de Pascual-Teresa *et al*., 2000
Flavan-3-ols	Epigallocatechin 3-gallate (ECGC)	Juice, Peel	de Pascual-Teresa *et al*., 2000
Flavonols	Quercetin	Juice, Peel	Artik, 1998
Flavonols	Kaempferol	Peel	van Elswijk *et al*., 2004
Flavonol glycosides	Rutin	Peel, Juice	Artik, 1998
Flavonol glycosides	Kaempferol 3-*O*-glycoside	Peel	van Elswijk *et al*., 2004

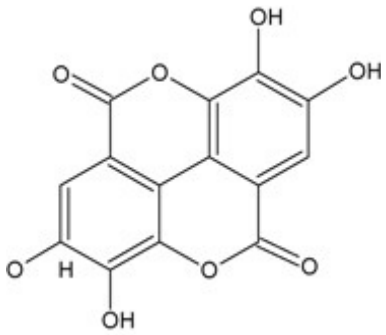
Table 2.2 Chemical constituents of pomegranate (*Continued*)

Chemical class	Compound name	Plant part	References
Flavonol glycosides	Kaempferol 3-*O*-rhamnoglycoside	Peel	van Elswijk *et al*., 2004
Flavones	Luteolin	Peel	van Elswijk *et al*., 2004
Flavone glycosides	Luteolin 7-*O*-glycoside	Peel	van Elswijk *et al*., 2004
Flavone glycoside	Naringin	Peel	Kim *et al*., 2002
Anthocyanidins	Delphinidin	Peel	Noda *et al*., 2002
Anthocyanidins	Cyanidin	Peel	Noda *et al*., 2002
Anthocyanidins	Pelargonidin	Peel	Noda *et al*., 2002
Ellagitannins	Punicalin	Peel, Leaf, Bark, Root	Tanaka *et al*., 1986, Gil *et al*., 2000
Ellagitannins	Punicalagin	Peel, Leaf, Bark, Root	Tanaka *et al*., 1986, Gil *et al*., 2000
Ellagitannins	Corilagin	Peel, Leaf	Satomi *et al*., 1993, Nawwar *et al*., 1994b
Ellagitannins	Casuarinin	Peel	Satomi *et al*., 1993

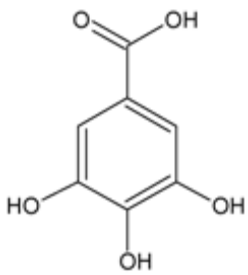
Table 2.2 Chemical constituents of pomegranate (*Continued*)

Chemical class	Compound name	Plant part	References
Ellagitannins	Gallagyldilacton	Peel	Satomi *et al*., 1993
Ellagitannins	Pedunculagin	Peel	Satomi *et al*., 1993
Ellagitannins	Tellimagrandin	Peel	Satomi *et al*., 1993
Ellagitannins	Granatin A	Peel	Tanaka *et al*., 1990
Ellagitannins	Granatin B	Peel	Tanaka *et al*., 1990
Pelletierine alkaloids	Peelletierine	Peel, Bark, Root	Neuhofer *et al*., 1993, Vidal *et al*., 2003

Hydroxybenzoic acids



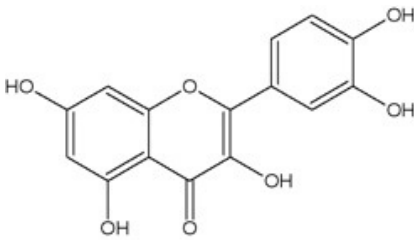
Ellagic acid



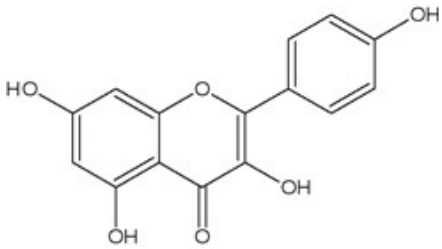
Gallic acid

Figure 2.4 Structure of some interested polyphenolic compounds

Flavonols

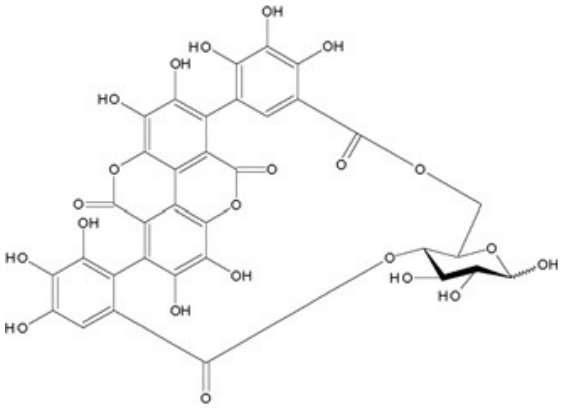


Quercetin

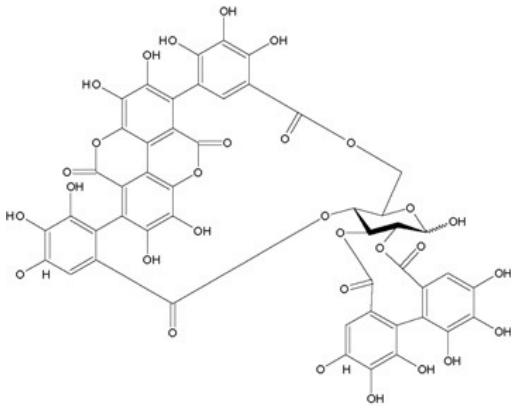


Kaempferol

Ellagitannins



Punicalin



Punicalagin

Figure 2.4 Structure of some interested polyphenolic compounds (continued)

2.3.1.1 Seed

Pomegranate seed oil (PSO) comprises 12-20% of total seed weight. The oil consists of approximately 80% conjugated octadecatrienoic fatty acids, with a high content of *cis* 9, *trans*11, cis 13 acid (i.e. punicic acid), synthesized in situ from nonconjugated octadecadienoic fatty acid, linoleic acid (Hopkins and Chisholm, 1968; Hornung *et al*., 2002), itself about 7% of PSO. The fatty acid component of PSO comprises over 95% of the oil, of which 99% is triacylglycerols. Minor components of the oil include sterols, steroids, and a key component of mammalian myelin sheaths, cerebroside (Tsuyuki *et al*., 1981). Seed matrix includes lignins (Dalimov *et al*., 2003), fusion products of cell wall components and hydroxycinnamic acids, and potently antioxidant lignin derivatives (Wang *et al*., 2004).

2.3.1.2 Juice

Anthocyanins, potent antioxidant flavonoids, provide pomegranate juice with its brilliant color, which increases in intensity during ripening (Hernandez *et al*., 1999), and declines after pressing (Perez-Vicente *et al*., 2002; Miguel *et al*., 2004). Minerals in the juice and seed include Fe, relatively prevalent, but not in so high concentrations as in watermelon, and Ca, Ce, Cl, Co, Cr, Cs, Cu, K, Mg, Mn, Mo, Na, Rb, Sc, Se, Sn, Sr, and Zn (Waheed *et al*., 2004).

2.3.1.3 Pericarp (peel, rind, hull are synonymns)

Both flavonoids and tannins are more abundant in the peels of wild-crafted compared to cultivated fruits (Ozcal and Dinc, 1993). Complex polysaccharides from the peels have been studied and partially characterized (Jahfar *et al*., 2003). The presence of alkaloids (e.g., pelletierine) in the peel is equivocal, positive by Dragendorff assay, but negative by Mayer assay (Vidal *et al*., 2003).

2.3.1.4 Leaf

Unique tannins occur in pomegranate leaves, as well in peel. Leaves also contain glycosides of apigenin, a flavone with progestinic (Zand *et al*., 2000) and anxiolytic (Paladini *et al*., 1999) properties. With respect to chemical elements, N is high in medium age, K in young age; Ca and Fe in old leaves. In July and August in the Northern Hemisphere, N and K are both

low during flowering and fruit-setting, N further declines during fruit maturity, along with Mg, Fe and Zn (Munde *et al*., 1980, 1981).

2.3.1.5 Flower

The flowers contain compounds also found in peels (e.g. gallic acid) and seed (e.g. ursolic acid), and quite possibly unique, distinctive compounds as well (Huang *et al*., 2005c). Further study is in process to elucidate the chemistry of these flowers that have also been ethnomedically employed.

2.3.1.6 Tree bark and roots

Extracts prepared from the rougher parts of the tree also have potent physiological effects and a long medical history. Their chemistry is notable against that of other tree parts mainly for the extensive presence of alkaloids (Neuhofer *et al*., 1993).

2.3.2 Medicinal properties;

The root bark as well as stem barks of the plant is astringent and antihelmintic. The dried flowers are used in haematuria, hemorrhoids, haemoptysis and dysentery. The powdered flowerbuds are used in bronchitis. The seeds are considered to be stomachic and the pulp as cardiac and stomach. The fruit rind is valued as an astringent and green leaves are made into a paste and applied in conjunctivitis (Ross *et al*., 2001).

Treatment;

- Conjunctivitis: Prepare a paste from the green leaves by washing and grinding on a stone grinder, and then apply to the eyes.

- Diarrhea: About 3 inches of bark is put into 4 cups of water which is boiled, reduced to 1 cup and then strained and taken. *Dosage*: Half a cup in the morning and then again in the evening till cured. Alternatively prepare a decoction of fruit peel. Roast the fruit, crush and then take out the juice.

- Vomiting: Half a cup of pomegranate leaves are crushed, put in 1 cup of boiling water for 15 minutes and then drunk. *Dosage*: 1 cup once only.

- Dysentery: A combination of rind of fruit and bark is an efficacious remedy, to be taken internally. Also the juice of the pulp is advised. The rind and bark can be prepared by grinding into a paste and making an infusion, which is filtered before consuming.

- Intestional Worms: Ripe fruit skin is dried in the shade and then crushed to prepare a fine powder obtained by straining through a muslin cloth. *Dosage*: 1 teaspoon of the powder is taken with water in the morning and evening for 3 days. Or fresh bark, 1 part fresh bark with 20 parts of water, boil till reduces till half, filter, 5 ml half hourly 4 times on an empty stomach and then give castor oil.

- Weakness: The fruit pulp is crushed in a cloth and juice obtained. To 1 cup of juice, 2 cups of sugar are added. This is boiled to a thick syrup consistency. *Dosage*: 2 teaspoons of the syrup are taken in the morning and evening until cured.

- Abscess: A 3 inch long strip of bark is rubbed on a stone to make a paste. The paste is applied in the morning and evening until cured.

- Astringent: The plant has a cooling effect on the body and is thus good for relieving burning sensations.

2.3.3 Biological activities of pomegranate

Antioxidant activity

It has been reported that the aqueous extract of the fruit peel showed antioxidant activity when evaluated by DPPH radical scavenging assay, 5-lipoxygenase assay and chemiluminescence assay with IC_{50} value of 0.094 ± 0.001, 0.198 ± 0.013 and 0.944 ± 0.031 mg/ml, respectively. In addition the ethyl acetate extract of pomegranate fruit peel also showed

antioxidant activity with IC_{50} value of 8.492 ± 0.042, 0.245 ± 0.008 and 6.93 ± 0.189 mg/ml, respectively (Ricci *et al*., 2006).

The antioxidant properties of the pomegranate fruit peel extract were investigated as compared with the pulp extract. The contents of total phenolics, flavonoids, proathocyanidins and ascorbic acid were also measured. The results showed that pomegranate fruit peel extract had markedly higher antioxidant capacity than the pulp extract in scavenging or preventive capacity against superoxide anion, hydroxyl and peroxyl radicals as well as inhibiting $CuSO_4^-$ induced LDL oxidation (Li *et al*., 2006).

The pomegranate fruit extract, orally administered to rats at dose of 10 mg kg^{-1} day^{-1}, showed potential antiperoxidative effect (Sudheesh and Vijayalakshmi, 2005).

Antibacterial activity

It has been reported that pomegranate extract exhibited inhibitory effect against *Shigella flexneri* with MIC value of 1-4 mg/ml (Alanis *et al*., 2005).

Methanolic extract of pomegranate exhibited inhibitory effect against *Staphylococcus aureus* (Braga *et al*., 2005).

Both aqueous and ethanolic extracts of pomegranate were highly effective against *Escherichia coli* O157:H7 with the best MIC and MBC values of 0.09, 0.78, and 0.19, 0.39 mg/ml, respectively (Voravuthikunchai *et al*., 2004).

It has been reported that methanolic extract of *P. granatum* possessed strong *in vitro* antibacterial activities against *Escherichia coli* and *Staphylococcus aureus* (Melendez and Capriles, 2005).

The methanolic extract of *P. granatum* exhibited inhibitory effect against *Proteus vulgaris* and *Bacillus subtilis* with MIC value of 1.5 and 6.0 mg/ml, respectively (Prashanth *et al*., 2001).

Cell proliferation promotion

Pomegranate seed oil, juice, peel and seed cake, were shown to stimulate keratinocyte proliferation in monolayer culture. In parallel, a mild thickening of the epidermis (without the loss of ordered differentiation) was observed in skin organ culture. The same pomegranate seed oil that stimulated keratinocyte proliferation was without effect on fibroblast function. In contrast, pomegranate peel extract (and to a lesser extent, both the fermented juice and seed cake extracts) stimulated type I procollagen synthesis and inhibited matrix metalloproteinase-1 (MMP-1; interstitial collagenase) production by dermal fibroblasts, but had no growth-supporting effect on keratinocytes (Aslam *et al*., 2006).

Inhibition of gastric mucosal injury

It has been reported that administration of 70% methanolic extract of pomegranate fruit peel (250 mg/kg and 500 mg/kg) shows percentage of inhibition of 22.37, 74.21, 21.95 and 63.41 in aspirin- and ethanol-induced gastric ulceration, respectively (Ajaikumar *et al*., 2005).

Immunomodulatory activity

It has been reported that pomegranate fruit peel powder (PGFRP) at the dose of 100 mg/kg orally as aqueous suspension was found to stimulate the cell-mediated and humoral components of the immune system in rabbits. PGFRP elicited an increase in antibody titer to typhoid-H antigen. It also enhanced the inhibition of leucocyte migration in leucocyte migration inhibition test and induration of skin in delayed hypersensitivity test with purified protein derivative (Ross *et al*., 2001).

Anti-diabetic activity

It has been reported that methanol extract of pomegranate flowers (at a dose 500 mg/kg, daily, 6-week oral administration) inhibited glucose loading-induced increase of plasma glucose levels in *Zucker diabetic fatty* rat, a genetic animal model for type 2 diabetes, but not in *Zucker lean* rats (Huang *et al*., 2005b). In addition, pomegranate flower extract exhibited a potent inhibitory effect on α-glucosidase activity with IC_{50} value of 1.8 μg/ml, *in vitro*. The inhibitory

effect depended on the concentration of enzyme and substrate, as well as on the length of pretreatment with the enzyme (Li *et al*., 2005).

Toxicity studies of pomegranate

It has been reported that whole fruit hydroalcoholic extract of pomegranate at doses of less than 0.1 mg per embryo are not toxic in chick embryo model. The LD_{50} of the extract, determined in OF-1 mice of both sexes after intraperitoneal administration, was 731 mg/kg. Confidence limits were 565-945 mg/kg (Vidal *et al*., 2003).