# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Definition of free radicals

A free radical is a molecule or just a single atom with an unpaired electron, conventionally symbolized by a radical dot“ X˙ ”[8].

## 2.2 Sources of free radicals

Free radicals can be formed in three ways: 1) by the hemolytic cleavage of a covalent bond of a normal molecule 2) by the loss of a single electron from a normal molecule 3) by the addition of a single electron to a normal molecule[9]. Some examples of free radicals are shown in Table 2.1[10].

**Table 2.1** Examples of free radicals

| Free radical | Formula |
|---|---|
| Hydrogen atom | $H^{\cdot}$ |
| Superoxide | $O_2^{\cdot -}$ |
| Hydroxyl | $^{\cdot}OH$ |
| Alkoxyl | $RO^{\cdot}$ |
| Peroxyl | $ROO^{\cdot}$ |
| Nitric oxide | $NO^{\cdot}$ |
| Nitrogen dioxide | $NO_2^{\cdot}$ |
| Thiyl | $RS^{\cdot}$ |
| Trichloromethyl | $CCl_3^{\cdot}$ |

**2.3 Reaction of free radicals with biomolecules**

Free radicals and active oxygen species attack lipids, sugar, proteins, and DNA and induce their oxidation, which may result in oxidative damage such as deterioration of foods, membrane dysfunction, protein modification, enzyme inactivation, and break of DNA strands and modification of its bases. Lipid peroxidation of polyunsaturated fatty acids proceeds in three stages. It begins with an initiation step followed by a propagation step and finally a termination step. The propagation step is particularly damaging since it involves a chain reaction until the last step of termination occurs[11]. DNA and proteins are likely to be less susceptible than lipids to free radical attack. Damaged DNA is repaired by a wide range of enzymes that recognize the abnormalities in DNA and remove them by excision, resynthesis and rejoining of the DNA stand[12]. Proteins can be oxidized by reactive oxygen species at –SH (sulfhydryl) groups. Numerous amino acid residues are attacked by hydroxyl radicals[13].

**2.4 Definition of antioxidant**

An antioxidant is "any substance that, when present at low concentration compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate"[14].

**2.5 Sources of antioxidants**

Antioxidant defenses comprise:

(a) Agents that catalytically remove free radicals and other 'reactive species'. Examples are the enzymes superoxide dismutase (SOD), catalase, peroxidase and 'thiol-specific antioxidants'.

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

(b) Proteins that minimize the availability of pro-oxidants such as iron ions, copper ions and heam. Examples are transferrins, haptoglobins, haemopexin and metallothionein.

(c) Proteins that protect biomolecules against damage by other mechanism, e.g. heat shock proteins.

(d) Low molecular mass agents that scavenge ROS and RNS. Examples are glutathione, α-tocopherol and bilirubin and uric acid. Some low molecular mass antioxidants come from the diet, especially ascorbic acid and α-tocopherol[15].



Most antioxidants are obtained in diet from natural sources, especially from food of plant origin. Vegetables, fruits, and the others are the best sources of these natural antioxidants. These antioxidants include vitamin C, vitamin E, carotenoids and polyphenol compounds. These essential micronutrients are antioxidants and can act by either interfering with the propagation stage of free radical generation or act directly as free radical scavengers. Vitamin C (ascorbic acid) is water soluble. Ascorbic acid has been found to react with hydrogen peroxide ($H_2O_2$), hydroxyl

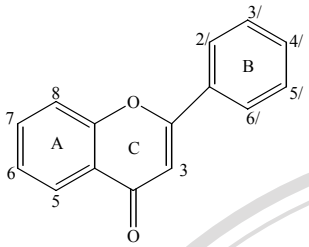
radical ($^{\cdot}OH$), peroxyl radical ($ROO^{\cdot}$) and singlet oxygen ($^1O_2$), to form the semidehydroascorbate radical and dehydroascorbate. Ascorbic acid also has the ability to regenerate the ability of lipid-soluble antioxidants, such as α-tocopherol and β-carotene, by interacting with biological membranes at the aqueous-lipid interphase. Vitamin E (α-tocopherol), the major lipid-soluble antioxidant in cellular membranes, protects against lipid peroxidation and prevents the loss of membrane fluidity. Vitamin E functions by donating hydrogen to fatty peroxyl radicals, thereby halting lipid peroxidation. Vitamin E has been touted to be the most effective antioxidant for reducing lipid peroxidation. α-Tocopherol supplementation was shown to decrease the severity of atherosclerosis and promote the regression of diet-induced atherosclerotic lesion[16]. The carotenoids including β-carotene, γ-carotene and lycopene, are lipid soluble, and are therefore carried within lipoprotein particles. β-Carotene, a pigment found in all photosynthetic plants, is an efficient quencher of singlet oxygen, $^1O_2$ and can function as an antioxidant.

In recent years phenolic compounds have attracted the interest of researchers because they show promise of being powerful antioxidants that can protect the human body from free radicals. Polyphenols are one such class of compounds. They occur in a variety of fruits, vegetables, nuts, seeds, flowers, bark, beverages, and even some manufactured food, as a component of the natural ingredients used. Phenolic compounds cannot be produced by the human body and thus must be taken in mainly through the daily diet. Knowledge about the nutritional and therapeutic role of dietary phenolic antioxidants is essential for the development of functional foods, which refers to the improvement of conventional foods with added health benefits. On the other hand, detailed chemical composition of foods considered to be functional is

needed, and the main goal of the chemistry of natural compounds is screening for promising biologically active substances of plant origin. Recent evidence has raised the possibility that major diseases may be prevented by the simple expedient of improving the dietary intake of antioxidants. Antioxidants, which are among their major components, have been proposed as the principle active agents for reducing the risk of chronic disease. These are phenolic compounds with several biological functions including antioxidant activity. Phenolic compounds, or polyphenols, constitute one of the most numerous and widely-distributed groups of substances in the plant kingdom, with more than 8,000 phenolic structures currently known. Polyphenols are products of the secondary metabolism of plants. The expression "phenolic compounds" embraces a considerable range of substances that possess an aromatic ring bearing one or more hydroxyl substituents. Most of the major classes of plant polyphenols are listed in Table 2.2, according to the number of carbon atoms of the basic skeleton. The structure of natural polyphenols varies from simple molecules, such as phenolic acids, to highly polymerized compounds, such as condensed tannins[17]. One of the major groups of phenolic compounds is the flavonoids, which are important in contributing to the flavour and colour of many fruits and vegetables and products derived from them such as wine, tea and chocolate. Flavonoid molecules consist of two aromatic rings linked by three carbon aliphatic chains (C6-C3-C6) which is condensed to form a pyran ring (Figure 2.1)[18].

**Table 2.2** The major classes of phenolic compounds in plants

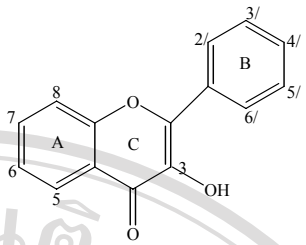
| Number of carbon | Basic skeleton | Class | Examples |
|---|---|---|---|
| 6 | C6 | Simple phenols | Catechol, Hydroquinone |
| | | Benzoquinones | 2, 6-Dimethoxybenzoquinone |
| 7 | C6-C1 | Phenolic acids | Gallic acid, Salicylic acid |
| 8 | C6-C2 | Acetophenones | 3-Acetyl-6-methoxybenzaldehyde |
| | | Tyrosine derivatives | Tyrosol |
| | | Phenylacetic acids | p-Hydroxyphenylacetic |
| 9 | C6-C3 | Hydroxycinnamic acids | Caffeic, Ferulic |
| | | Phenylpropenes | Myristicin, Eugenol |
| | | Coumarins | Umbelliferone, Aesculetin |
| | | Isocoumarins | Bergenon |
| | | Chromones | Eugenin |
| 10 | C6-C4 | Naphthoquinones | Juglone, Plumbagin |
| 13 | C6-C1-C6 | Xanthones | Mangiferin |
| 14 | C6-C2-C6 | Stilbenes | Resveratrol |
| | | Anthraquinones | Emodin |
| 15 | C6-C3-C6 | Flavonoids | Quercetin, Cyanidin |
| | | Isoflavonoids | Genistein |
| 18 | $(C6-C3)_2$ | Lignans | Pinoresinol |
| | | Neolignans | Eusiderin |
| 30 | $(C6-C3-C6)_2$ | Bioflavonoids | Amentoflavone |
| n | $(C6-C3)_n$ | Lignins | |
| | $(C6-C3-C6)_n$ | Flavolans (condensed tannins) | |



(a) Flavones

Fesetin-3, 7, 3′, 4′-tetra-OH

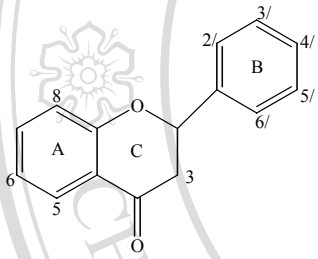
Robinetin-3, 7, 3′, 4′, 5′-penta-OH



(b) Flavonols

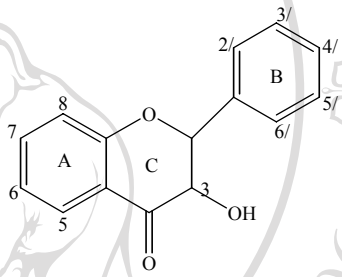
Myricetin-3, 5, 7, 3′, 4′, 5′-hexa-OH

Morin-3, 5, 7, 2′, 4′-penta-OH



(c) Flavanones

Naringin-7-rhamnoglucose, 5, 4′-di-OH



(d) Flavanonols

Taxifolin-3, 5, 7, 3′, 4′-penta-OH

**Figure 2.1** Chemical structures of flavonoids

The main structural features of flavonoids required for efficient radical scavenging in Figure 2.2 could be summarized as follows:

(i) An ortho-dihydroxy (catechol) structure in the B ring, for electron delocalization.

(ii) A 2, 3 double bond in conjugation with a 4-keto function, provides electron delocalization from the B ring.

(iii) Hydroxyl groups at positions 3 and 5, provide hydrogen bonding to the keto group[19].

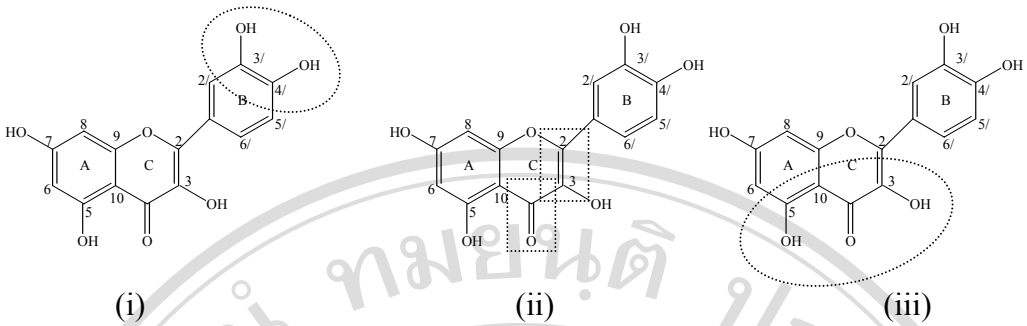



**Figure 2.2** Structures for flavonoids to be an effective antioxidant

## 2.6 Research on antioxidants in some medicinal plants

Over the last 10 years, a wealth of information has emerged from studies of the chemical and pharmacological properties of phenoloic compounds present in fruits and vegetables. Although not generally considered as nutrients, a growing body of evidence suggests that dietary phenolics may contribute to chemoprevention of a variety of human diseases, including coronary heart disease and certain cancers. The antioxidant activity of these compounds is believed for their beneficial health effects. Many studies have demonstrated the antioxidant activities of plant phenolic compounds, and some of the most relevant are briefly described here. María et al., 2000 evaluated antioxidant activity of pomegranate juices by four different methods (ABTS, DPPH, DMPD and FRAP) and compared to those of red wine and green tea infusion. The results showed higher antioxidant activity of commercial juices compared to the experimental ones. In addition, anthocyanins, ellagic acid derivatives, and hydrolysable tannins were detected and quantified in the pomegranate juices[20]. Moreover, *in vivo* models on antioxidant activity of pomegranate peel was studied by Kotamballi et al. Histopathological studies of the liver were also carried out to

determine the hepatoprotection effect exhibited by the pomegranate peel extract against the toxics effects of $CCl_4$. The results showed that the methanol extracts of pomegranate peel support the hepatoprotective by restoring the normal hepatic architecture[21]. In the same year, Yasuko et al. evaluated antioxidant activity of freeze-dried preparations of a 70% acetone extract of peel, seed coats and juice from pomegranate. The pomegranate extracts exhibited scavenging activity against $OH^{\cdot}$ and $O_2^{\cdot-}$. The results found delphinidin, cyanidin, and pelargonidin obtained and showed antioxidant activity in pomegranate fruits[22]. Moreover, another pharmacological studied also antimutagenic activity was studied by Negi et al., 2003. The results showed the pomegranate peel extracts exhibited strong antimutagenicity[23]. Punicalgin from pith and carpellary membrane of pomegranate fruit was isolated on silica gel column, follow by HPLC and identified based on UV, IR, NMR, GC-MS and MALDI-MS[24, 25].

Takashi et al., 1992 found six new complex tannins, guajavin A and B, psidinins A, B and C, and psiguavia, together with a variety of condensed hydrolysable and complex tannins. There have been isolated from the bark of *Psidium guajava* L. (Myrtaceae). On the basis of chemical and spectroscopic evidence, the structures of guajavins and psidinins were stablished to consist of a (+)-gallocatechin unit and a hydrolysable tannin moiety linked C- glycosidically, while psiguavin was found to be a novel metabolite probably derived from eugenigrandin A through successive oxidation, benzylic acid-type rearrangement, decarboxylation and oxidative coupling of the gallocatechin B-ring and one of the aromatic rings in the hydrolysable tannin moiety[26]. In addition to above components from *P. guajava*, proanthocyanidins was extract from the leaves of guava with water and ethanol[27]. The

extract from leaves of *P. guajava* was found to inhibit paw oedema induced by carrageenan in rats and induced by acetic acid in mice, and exhibited an antipyretic effect. Oral administration of the extract reduced intestinal transit time and prevented castor oil-induced diarrhea in mice[28]. Not only guava leaf components studied but also the pulp and peel fraction of guava fruit showed high content of dietary fiber (48.5-49.42%) and polyphenol (2.62-7.79%). The antioxidant activity of polyphenol compounds was studied, using three complementary methods: (i) free radical DPPH· scavenging, (ii) ferric reducing antioxidant power assay (FRAP), and (iii) inhibition of copper catalyzed *in vitro* human low density lipoprotein (LDL) oxidation[29]. After, Takashi et al. found six new complex tannins from the bark of *P. guajava*. Ten years later, Sabira et al. isolated and identified two triterpenoids: guavanoic acid and guavacoumaric acid from leaves of *P. guajava*[30]. Modern proof of the traditional use can be found in modern studies. The methanolic extract of *P. guajava* (leaves) showed significant inhibitory activities against the growths of two isolates of *Salmonella, Shigella* spp. (*Shigella flexneri, Shigella virchow* and *Shigella dysenteriae*) and two isolates of the enteropathogenic *Escherichia coli*. The results have confirmed the effectiveness of this medicinal plant as an antidiarrhoeal agent. Guava sprout extracts by 50% diluted ethanol showed the most effective inhibition of *E. coli*, while those in 50% acetone were less effective. It is concluded that guava sprout extracts constitute a feasible treatment option for diarrhoea caused by *E. coli* or by *S. aureus* produced toxins, due to their quick therapeutic action, easy availability in tropical countries and low cost[31]. The anti-inflammatory and analgesic activities of 70% ethanolic extract of *P. guajava* was investigated in rats using the carrageenin-induced hind paw oedema model. Extracts which exhibited anti-inflammatory activity

were screened for analgesic activity using the Randall-Selitto method in rats. The extracts were administered at a dose of 300 mg/kg; p.o. Aspirin (300 mg/kg, p.o.) was employed as the reference drug. *P. guajava* leaves, showed significant anti-inflammatory activity with percentage inhibitions of 58.27%[32]. In the lagoon area of coastal Ivory Coast young twigs of *P. guajava* serve as chew- sticks. In southern Nigeria the twigs are used as chew sticks and the presence of bioactive compounds comprised of saponins, tannins, flavonoids, alkaloids is responsible for their effectiveness. Chewing sticks when used without toothpaste are very efficient, effective, and reliable for cleaning teeth. The teeth of chewing sticks users are usually strong, clean, fresh, and devoid of dental plaques and carries[33].

*Thunbergia laurifolia* in ethanolic extract showed antioxidant activity determined by $ED_{50}$ varied from 20-99 μg/ml[34]. Two iridoid glucosides, 8-epi-grandifloric acid and 3'-*O*-β-glucopyranosyl-stilbericoside were isolated with seven known compounds, benzyl β-glucopyranoside, benzyl β-(2'-*O*-β-glucopyranosyl) glucopyranoside, grandifloric acid, (E)-2-hexanyl-β-glucopyranoside, hexanol β-glucopyranoside, 6-C-glucopyranosylapigenin and 6, 8-di-C-lucopyranosylapigenin constituted in aerial part of *Thunbergia laurifolia*[35]. Chromatographic separation of n-hexane fraction, by silica gel and gel filtration revealed the presence of phytol, stigmasta-5, 22-dien-3-ol and some known compounds. The antioxidant activity in each fraction was determined by DPPH radical scavenging activity assay. The ethanol extract did not show any cytotoxic activity against NIH/3T cells by MTT colorimetric method[36].

Irene et al., 2002 isolated and characterized an antimutagenic constituent from *Mentha cordifolia* leaves. The $CHCl_3$ extracts showed antimutagenicity against

tetracycline[37]. Besides non polar extraction, water-soluble extracts from the mentha species showed potential antioxidative property. These properties included iron (III) reduction, iron (II) chelation, 1, 1-diphenyl-2-picrylhydrazyl radical scavenging, and the ability to inhibit iron (III) ascorbate-catalyzed hydroxyl radical-mediated brain phospholipids peroxidation[38].

*Andrographis paniculata* exhibited antioxidant and hepatoprotective action by activity of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase[39]. The root and aerial parts of *Andrographis paniculata* were reported the isolation and structure determination of a new flavone and a novel 23-carbon terpenoid[40].

Cevallos-Casals and Cisneros-Zevallos, 2002 studied the antioxidant activities and color potential of extracts from a highly pigmented from *Hylocereus undatus* for promoting its use in natural colorant markets. The *Hylocereus undatus* has significant phenolic content and antioxidant activity[41].

Active anti-ulcerogenic ingredients from unripe banana by solvent were fractionated and identified flavonoid leucocyanidin by HPLC[42].

The linear positive relationship between the antioxidant activity and total phenolic acid contents and Iranian basils possesses valuable antioxidant properties for culinary and possible medicinal use[43].

The analgesic and free radical scavenging properties of an aqueous extract from the husk fiber of *Cocos nucifera* L. (Palmae) by *in vivo* and *in vitro* models were evaluated. The topic treatment of rabbit with the *Cocos nucifera* extract indicated that it does not induce any significant dermic or ocular irritation. *In vitro* using the DPPH assay demonstrated that this plant also possesses free radical scavenging properties[44].

Tan and Matthew investigated the antioxidant activities from three local fruits durian (*Durios zibethinus*), rambutan (*Nephelium lappaceum*) and mangosteen (*Garcinia mangostana*) by ABTS and DPPH assays. The TEAC and $IC_{50}$ values of the mangosteen extract displayed the greatest antioxidant activity, followed by rambutan and durian[45].

Anchana et al., 2004 screened the antioxidant activity by β-carotene bleaching method and antioxidant compounds of some edible plants in Thailand. The *Gymnema inodorum* showed the highest antioxidant activity followed by *Piper sarmentosum* and *Mentha arvensis*, respectively[46].

However, the antioxidant studies in plants by other authors have been spaciously and continuously studied so far but this research will be concentrated on the structural determination of antioxidant composition from certain Thai plant in order to increase the value added application. With this purpose, in this research, the study will be focused firstly on the in vitro antioxidant and cytotoxic bioactivity of the crude extracts, secondy on the purification of the fractionated bioactive extracts and eventually with structure identification of the active compounds.