CHAPTER II

LITERATURE REVIEW

1. Herbal tea

Many epidemiological studies have been emphasized on naturally occurring plant materials, and among these, tea plant has recently attracted much attention and globally accepted as medicinal herbs due to its remarkable and versatile health benefits. Some of the salient points on tea such as origin, botanical characteristics, types, environmental condition for tea plantation, harvesting and manufacturing processing are noted as follows.

1.1 Origin of tea

Tea plant is believed to have originated in the western Yunnan region of China on the border between Myanmar and China. Around the seventh century, tea cultivation spread from China to Japan and then subsequently spread to other Asian countries and later to Europe. Tea plant has long been cultivated, and consumed widely in Asia, particularly in China and Japan (McCaleb et al., 1999).

1.2 Botanical characteristics of tea

Tea plant belongs to the family Theaceae; *Camellia sinensis*. It is a perenial plant, evergreen shrub or tree that can grow to a height of 30 feet. The plant is heavily branched with young leaves, pruned and maintained at the height of 2 to 3 feet. Leaves and buds are the useful parts of the tea plant. Leaves are shiny dark green in colour, alternate, oval to oblong in shape with serrated edges. However, it can differ in colour, shapes, and sizes depending on the various types, species and environmental conditions. For tea production, leaves are preferentially picked as young shoots where older leaves are considered to be of inferior quality (Brown 1999). The top two leaves and the buds are prized for their fullness of its flavour contents. Flowers are white in colour, fragrant and appear in cluster or singly.

1.3 Types of tea

There are three types of tea, all coming from the same plant, but differing in the way of processing; namely, black tea, oolong tea and green tea. Black tea is fully fermented tea, oolong tea is partially fermented tea and green tea is unfermented tea (Murray, 1995). Black tea is mostly produced in Africa, India, Sri Lanka and Indonesia while green tea is produced in far East countries such as China, Japan, Thailand and Myanmar. Moreover, the popularity of different teas also differ in different regions. Although black tea is popular among western countries, green tea is popular among Asian countries.

1.4 Favourable condition for tea plantation

Tea plants can grow under the direct sunlight or partial shade with slightly acidic soil. An annual rain fall of 50 inches per annum, with the average temperature of 30°C and an altitude of 7000 feet above sea level are an ideal environmental conditions for tea plantations (McCaleb *et al.*, 1999). The difference in environmental conditions and varieties of tea species create various quality of active compounds in tea.

1.5 Harvesting

The manner and time of harvesting also differ between each region, which also play an important role in differentiating and establishing the quality of tea. Each area has its own way of harvesting. Even though modern technique has been developed some area still carry on the traditional ways such as picking by hand or knife.

Harvesting mostly take place three times per year, the first flush of the year is in the late April. The first spring leaves called first flush, are considered the highest-quality leaves. After the first flush leaves are picked, another one grows, called second flush, and later on, an autumn flush appears. The first harvested leaves are highly regarded as the highest quality leaves and contain more polyphenols than others. Moreover, the first top two leaves are graded to be the best quality with the leaves further down the stem considered to be of inferior quality.

1.6 The manufacturing process of tea

The method of processing plays an important role in differentiating teas and also contributed to the difference in tea constituents. For instance, the constituents of green tea are similar to the fresh tea leaves, but those in black tea differ due to the way of processing. However, there are different ways for processing different types of tea, the basic stages involved are steaming or drying by exposure to air after plucking, then followed by rolling and final firming.

Steaming process plays a major role for green tea preparation which is a distant step from black tea. Immediately after plucking, leaves are subjected directly to high temperature steaming which inactivates enzymes; polyphenol oxidase action and ceases the oxidation process. This can also preserve the polyphenol content as in fresh tea leaves.

In black tea processing, harvested leaves are fully oxidised due to exposure to air but those of green teas are unoxidised due to the steaming process at high temperature. The contrast in the process of black tea is that the fresh leaves are allowed to wither by exposure to air which is favouring the fermentation by polyphenol oxidase enzymes. Consequently, the large quantities of polyphenols present in the leaves are oxidised to phlobaphenes, forming black colour with characteristic aromatic odour.

In both processes, final drying is followed by using machine or pan-fried to remove excess moisture content from the leaves. Rolling and twisting into variety of shapes are then carried out during drying.

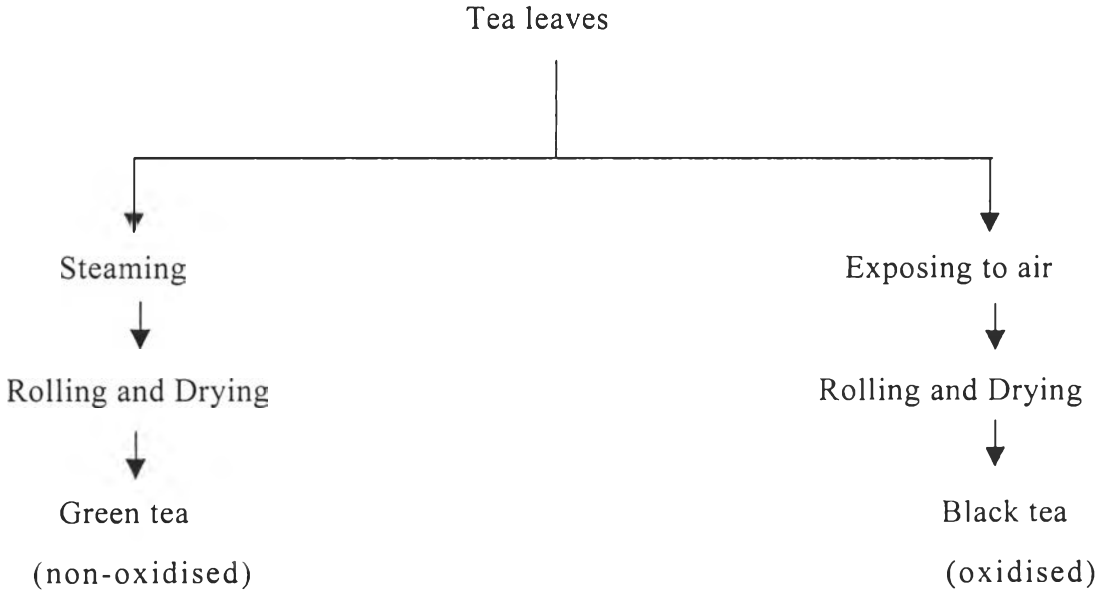


Figure 1. Schematic presentation of the way of processing of green and black tea

1.7 Chemical composition of tea

There are great differences among the tea constituents even though they are manufactured from the same fresh tea leaves and thus it differentiates the types of tea (Zhang *et al.*, 1999). Tea leaves comprise of polyphenols (flavonoids), alkaloids (caffeine, theophylline and theobromine) and to the lesser extent of metals (calcium, iron and maganese) (McCaleb *et al.*, 1999). The major constituent of green tea and black tea are as shown in figure 2.

The major polyphenolic fraction present in green tea is the group of flavonoids, commonly known as green tea catechins, which exhibited many biological functions including antioxidant and anticarcinogenic activity. Tea is the best and rich dietary source of polyphenols; catechins and account for about 30% of the dry weight of the water-extractable materials (Hong *et al.*,2001). In contrast, oxidized black tea has been shown to have lower amounts of catechins than non-oxidized green tea.

Polyphenols catechins present in green tea are epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate

(EGCG). EGCG is the main component of catechins in green tea with vast range of activity and is the major catechin present in green tea. In black tea, the major polyphenols are in the form of theaflavins and thearubigins. Caffeine content is about one-third in green tea when compared with black tea (Brown, 1999; Dufresne *et al.*, 2001). As a matter of fact, there are no perfect products that are universally accepted without any constraint, so even in green tea the small quantity of caffeine content which should not be underestimated.

A typically brewed green tea beverage contains 30% - 42% catechins, whereas in the black tea, catechins count for 3% - 10% by dry weight (Lambert and Yang, 2003). According to Lin and Liang (2000), the amount of catechins in the various tea extracts was in the order of green tea>oolong tea>black tea. This outcome is due to the consequence of different manufacturing processes.

Green tea catechins as a mixture in alkaline solutions (pH>8) were extremely unstable and degraded almost completely in a few minutes, whereas in acidic solution (pH<4) they were very stable. On the other hand, green tea catechins are pH-dependent i.e. the lower the pH the greater the stability. In alkaline solution EGCG and EGC are equally instable but EC and ECG are relatively stable (Zhu *et al.*, 1997). From the study of Stach *et al.* (2001) demonstrated that the concentration of free catechins in tea decreased with time due to the instability of catechins in solutions with neutral or basic pH values but increase in caffeine content.

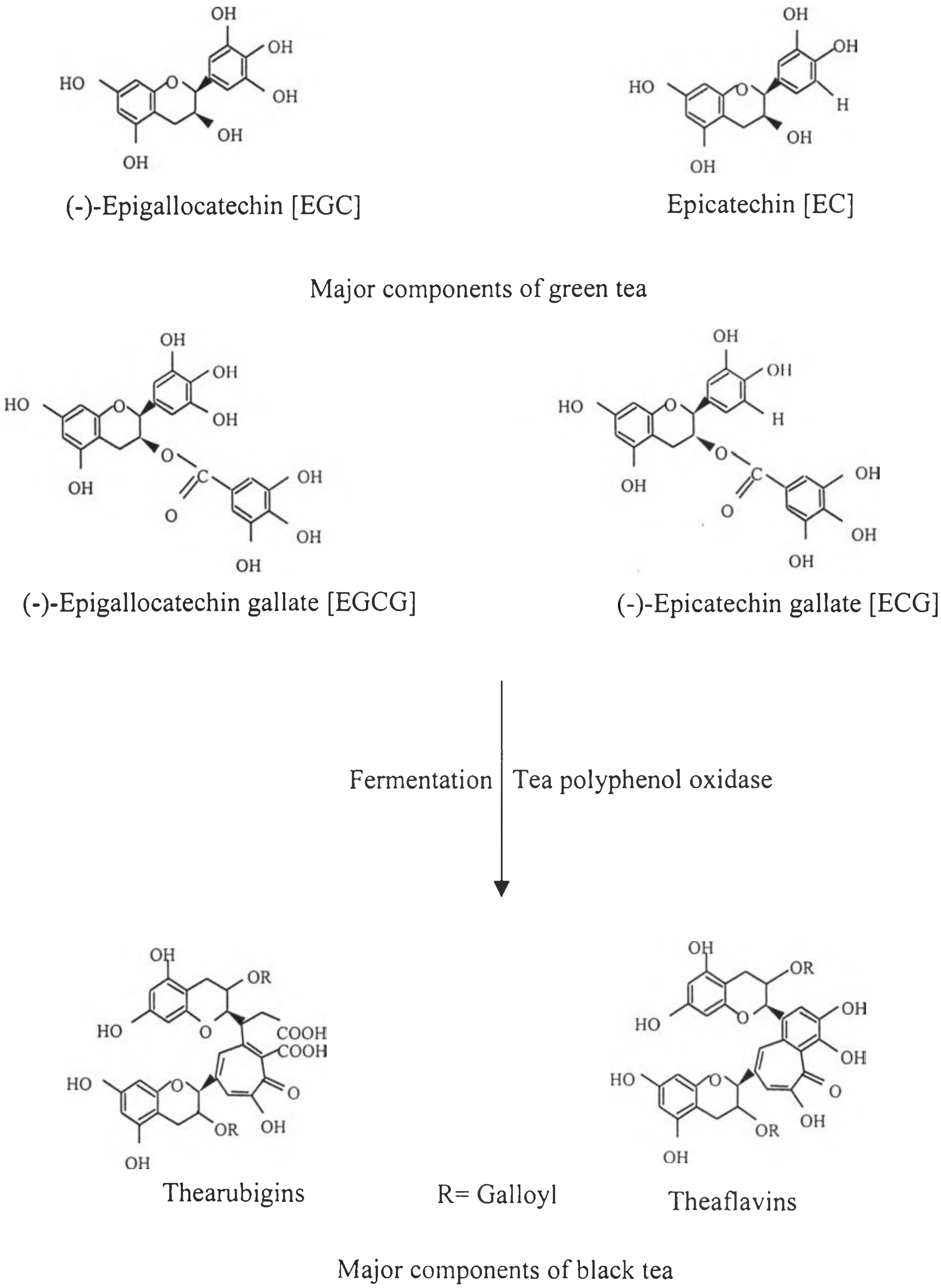


Figure 2. Major components of green tea and black tea (Yang *et al.*, 1993)

2. Green Tea

Green tea is the one of the most popular beverages in Asian countries which is now consumed worldwide. It is second only to water in popularity as a beverage (Lambert *et al.*, 2003) especially among Asian. Green tea is nontoxic and readily available to the general population (Imai *et al.*, 1997). Furthermore, it is also one of the most healthy and effective tonics in our diet and its therapeutic benefits of tea have long been recognized. All the interesting points of green tea are due to the presence of polyphenols. However, the presence of polyphenols in green tea could depend on its origin and this study is made to substantiate this claim. Green tea has been prized for centuries in the East for its flavour and delightful fragrance as well as its myriad of the properties.

2.1 Background of green tea from three different Asian sources

2.1.1 Japanese green tea

In the late 12^{th} century, the Japanese Zen priest Eisai brought some seeds of the green tea plant, *Camellia sinensis*, as a source of medicine from South China where he had learned the therapeutic effects of green tea in addition to Zen (Fujiki *et al.*, 2002). Many Eastern religious drink tea to stay alert during long meditations.

Green tea plays an essential role in Japanese daily life for almost 800 years. Obviously, Japanese green tea is one of the most popular beverages in Japan. If you are visiting someone's house in Japan, you will probably be offered a cup of green tea. The ancient tea ceremony of Japan has been honored for many generations and continues to play an important role in the spiritual life of many Japanese people (McCaleb, 1999).

The climatic condition of Japan is most suitable for tea production and it has the highest green tea production both in terms of quality and quantity. Virtually, all teas grown in Japan end up as green tea with almost no oolong or black tea. Tea production in Japan is the most significant in Shizuoka prefecture, located south and west of Tokyo and constitutes 41% of tea plantations in the whole country.

There are many grades of green tea produced in Japan depending on the time of harvesting and quality of the green tea leaves. The bud or the bud plus up to two top leaves are plucked during harvesting. Harvesting take place two or three times per year and there is a marked difference in quality between leaves from the first harvest and the second harvest. The leaves that are from the first harvest are the highest grade and they also believed that first two leaves of the plant contain the highest quantities of polyphenols and also taste better. The first harvest starts in late April or early May every year in Japan. After the leaves are picked, steaming is done immediately to prevent fermentation. It is then dried after steaming and when dried enough, rolled into a variety of shapes until it is completely dried.

Generally, there are many varieties of green tea and the most popular kinds of tea drunken in Japan are gyokuro, sencha, maccha, houjicha and bancha depending on the quality of the products. *Gyokuro* is the most superior green tea and famous for its rich sweet flavour. As it is raised, it is protected from the direct sunlight. Only the youngest flush leaf is picked during harvesting, to produce gyokuro. *Sencah* is the most common green tea and it is raised without cover for the sun. *Bancha* is rough tea which is made from lower grade tea leaves and it is inexpensive. *Matcha* is made by grounding steamed dried tea leaves into powder with a stone mortar which has a quite bitter taste and mostly used for tea ceremonies. *Houjicha* is made by roasting the leaves (brown coloured tea). Over the different kind of green tea in Japan, about 75% of all tea harvested in Japan is graded as Shincha. Shincha is the most common green tea in Japan. Shincha or sencha is referred to as 'new tea' where shin means new and cha means tea.

After the concept of cancer chemoprevention was introduced in Japan, more and more emphasis on polyphenols derived from medicinal plants, mostly from green tea as possible cancer preventive agents. Various scientists have subsequently reported on the preventive effects of green tea against human cancers. Based on geographical correlation studies, Fujiki *et al.*, (2002) reviewed the two stages of cancer prevention with green tea beverage: before cancer onset and following cancer treatment for cancer patients in Japanese population. Studies among cancer patients prove that consumption of green tea of over 10 cups per day delayed the onset of cancer and reduced the relative risk of cancer studied in different organs. Considering with the cancer patients undergoing cancer preventive treatment, the main component of green tea, epigallocatechin gallate (EGCG) gave synergistic effect with cancer preventive drug (sulindac).

2.1.2 Myanmar green tea

Tea plants are mostly grown in the Shan plateau in the Shan state of Myanmar (northern part of Myanmar) which has favorable conditions for tea plantations and Shan races of Myanmar live there to earn their living by producing tea. These plants are 200 years old and native Shan national tea growers descend from generation to generation. Myanmar green tea plant is mainly *Camellia sinensis* and belong to Theaceae family. Other then that there exist other plants like *Camellia caudata* and *Camellia kissis* (Hundley, 1961). Leaves and sprouts from tea plant cultivated in Shan plateau, are picked and made into various tea products.

The leaves are mostly picked up by hand. Harvesting of tea plants takes place three times per year and depending on the time of harvesting named “Shwephyioo”, “Shwefihnaung” and “Shwephyimoelut”. The best kind of tea is plucked in early April, which is Shwephyioo. There are 3 types of tea products made from same plant but differ in their way of processing. The different types of Myanmar tea products are black tea (Ah Cho Chaut), green tea (Ah Chan Chaut) and last one is Myanmar famous pickled tea (Letphet).

The way of processing green tea after harvesting is roughly the same as others. First harvested leaves, are steamed with water vapor for five minutes and later steamed green-tea leaves are laid and spread in the sun and the leaves are rolled and mixed by hands. After that, the dried tea leaves are available for green tea lovers. They believed that leaves dried in a single day provide the best quality with good scent, taste and color.

Since ancient times most of the Myanmar citizen prefers the green herbal plain tea with a peculiar taste of both bitterness and sweetness and traditionally believed green tea consumption and long life. Green tea always involves not only on Myanmar dining table but also at Myanmar National's green tea chat which is a very important occasion when family and relatives gather intimately. Friendliness, collectiveness and intimacy are the mainstay built into this kind of chat.

2.1.3 Thai green tea

The natural habitat of tea plant is south of the Yangi River, east from the Zhejiang Province of China, to Assam-Burma in the west, including the hills of Thailand and Vietnam (Huang, 1999). Tea production was born in China and spread to Japan as well as many other countries including Thailand.

Tea plant *Camellia sinensis* which is evergreen thrives that cultivated in the climate of the high mountain regions of the world. This plant is also available to grow in mountainous part of northern Thailand. In Thailand, Chiang Rai (northern Thailand) province is known as the biggest tea growing area and most of the places were forest and plentiful with wild tea. Thailand ends at Chiang Rai which is the mountainous province that touches border with Myanmar (Shan state) and Laos. One of the village of northern Thailand; Mae Salong is also well-known place for its tea plantation. Mae Salong is the famous Chinese-Thai village which perched on the border, a mere mountain pass away form Myanmar. One can imagine of this area's main tea plantation by seeing two giant chinese tea pot sculpture; a recent addition to the landscape by a tea plantation owner (Malcolm, 2003).

Beside the tea plant *Camellia sinensis*, other mixed varieties are also develop in this area. From the tea plantation in Chiang Rai, different kind of tea products are available in the market in the form of green tea, oolong tea and

Jasmine tea. The Agriculture Department in Thailand has urged tea planters to go organic and to expand their tea market.

In the north part of Thailand, people widely drink green tea after meal or during day-work. For the elderly, after meal they mixed green tea with hot water, drink and smoke local-made cigarettes called "Buri Shiya" simultaneously in order to release their tension and weariness from field work. The local believe that drinking green tea will help them release stress from work, awaken from sleepiness and so make them industrious.

3. Polyphenol contents in green tea

3.1. Phytochemicals background

Historically, most of the plant food (including fruits and vegetables) has been used as medicinal agents due to the number of phytochemicals (bioactive non-nutrient plant chemicals) present in varying levels of different plants. They are natural compounds with none or minimum toxicity of low molecular weight. The primary action of all these substances are mostly concerned with their antioxidant activities. Moreover, the search for cheap, renewable and abundant sources of antioxidant compounds is gaining worldwide interest. These components are likely to exert some health benefits by working together in an additive or synergistic manner and have been linked to reduction in risk of chronic disease. Among these several phytochemicals in plant food, a lot of attention has been focused on plant polyphenols due to their positive health benefits.

3.2. Plant polyphenols

Plants polyphenols are a group of chemical compounds, the most numerous and widely distributed in the plant kingdom which are secondary plant metabolites, responsible for the colour formation. They are abundant in diet and have a wide range of structure and functions. Basically, the structure possesses an aromatic ring bearing one or more hydroxy substitute. They have been classified into major groupings distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic skeleton.

Plant polyphenols possess several common biological and chemical properties, namely, antioxidation activity, the ability to scavenge both active oxygen species, to chelate metal ions, the potential for autooxidation, and the capability to modulate certain cellular enzymes activities (Robards *et al.*, 1999). Morover, the consumption of polyphenols in the diet is associated with a lower incidence of diseases, particularly of degenerative diseases, such as cancer, heart disease and improved human health and well being.

3.3 Flavonoids

Among the polyphenols, flavonoids are large class of compounds, ubiquitous in plants, one of the most common and naturally occurring compounds with low molecular weight (Rice-Evans *et al.*, 1997). They are widely distributed in fruits, vegetables and beverages. The basic structure is (C_6-C_3-C_6), the two aromatic rings link through three carbon that usually formed an oxygenated heterocycle (Leighton, 1999).

Flavonoids can be subdivided into groups depending on the structural variations within the rings; such as unsaturation and oxidation of the three-carbon segment, the number and nature of substitute groups attached to the rings (Robards *et al.*, 1999). They are (1) flavonols (eg. Quercetin and kaempferol); with the 3-hydroxy pyran-4-one C ring, (2) flavones (eg. Apigenin and chrysin); lacking 3-hydroxyl group, (3) flavanols (eg. catechins); lacking the 2,3-double bond and the 4-one structure, (4) isoflavones (eg. Genistein); in which the B ring is located in the 3 position on the C ring (Rice-Evans *et al.*, 1997) (figure 3)

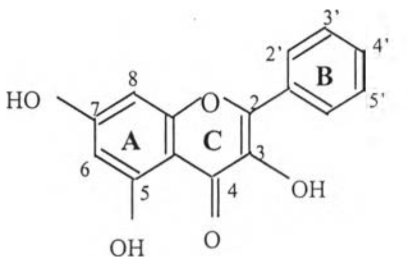
Diet rich in flavonoids are flavones (quercetin) found in apples, onions and broccoli, flavanols (catechins) in green teas and red wine, flavanones primarly found in citrus fruits, and anthocyanins present in cherries, berries and grapes. Flavanols and flavonols are widely distributed and quantitatively dominant among others flavonoids. They are important flavonoids in oxidation defense mechanism.

3.4 Green tea catechins

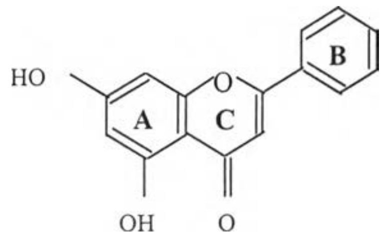
The catechins are abundantly distributed in green tea. Flavonoids mostly occurred as a glycosylated forms, but in catechins groups, they are present as free forms rather than glycosylated forms which is one of the distinct points from other flavonoids. Many epidemiological studies have been made and shown that green tea has antibacterial, anticarcinogenic and radical-scavenging activities which can be attributed due to the high content of flavonoids; catechins present in it. Many biological activities of green tea are mostly related to the structural activity of catechins. The presence or absence of galloyl group in ortho-dihydroxy or trihydroxy group of B ring in the basic catechin structure is still a controversial issue as regard the biological activities (see figure 3).

4. Antioxidant activity of green tea polyphenols

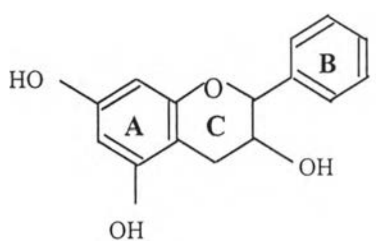
In the biological system, the oxidation/antioxidation balance is highly regulated. However, under certain circumstances, there is an imbalance between the radical-generating and radical-scavenging systems, and consequently increase in free radical production or reduce in antioxidant defense mechanism or sometimes both the phenomena take place simultaneously. The impairment of the antioxidant activity is considered to be critically involved in all events of cancer, cardiovascular, inflammatory disease and aging. Therefore, much attention has been paid on the regulation of oxidation.



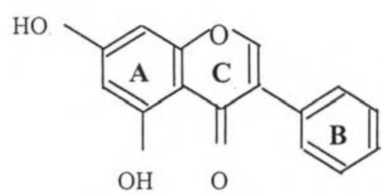
Flavonol



Flavone



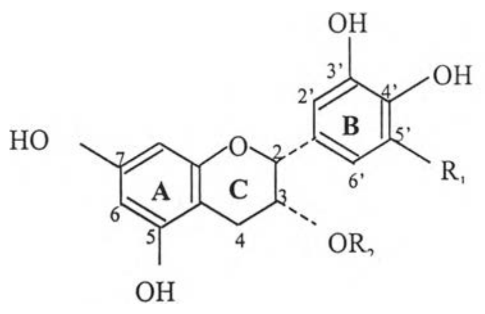
Flavan-3-ol



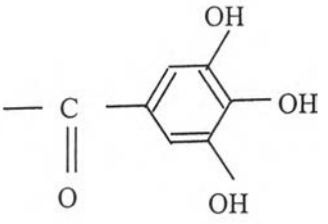
Isoflavone

Figure 3. Structures of the flavonoids (Rice-Evans *et al.*, 1997).

The basic structure consists of the fused A and C rings, with the phenyl B ring attached through its 1' position to the 2-position of the C ring (number from the pyran oxygen).



Catechin



Galloyl (G)

		R_1	R_2
(-)-Epigallocatechin gallate	[EGCG]	OH	G
(-)-Epicatechin gallate	[ECG]	H	G
(-)-Epigallocatechin	[EGC]	OH	H
(-)-Epicatechin	[EC]	H	H

Figure 4. Structures of green tea polyphenols (Lambert *et al.*, 2003)

Focus on the antioxidant activity of naturally occurring materials has been gradually increased. Several studies have shown that plant polyphenols consumption is able to bolster biological resistance against free radicals, retards the process of degenerative diseases, such as cancer, cardiovascular diseases. Among these, many researchers have proved that green tea polyphenols have the strong antioxidant activity, not only scavenge free radicals directly but also enhance the effectiveness of the body's natural antioxidant systems.

4.1 Oxidation mechanism

Oxygen is the most prevalent element on Earth consisting large amount in atmosphere. It plays a pivotal role for all aerobic organism, by generating energy and activate enzymes for normal body metabolic functions. Briefly, O_2 itself is relatively unreactive, however, during respiration, on a cellular level, some oxygen molecules are converted into 'free radicals' as a consequence of oxidation.

Oxidation is the chemical process and is the part of the normal metabolism in which oxygen adds to and withdraws energy from carbon-based molecules resulting in loss of electrons from an atom or ion. Indeed, this reaction is useful for the synthesis of nucleic acids, hormones and proteins. Beside this reaction, reactive oxygen species (ROS) are evolved as by products.

4.2 Free radicals

As the result of the radical chain reaction, reactive oxygen species (ROS) are evolved as by products. Free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNA) are molecules or ions with one or more unpaired electrons. They are unstable, highly reactive molecules and capable of reacting with each other (Fenton reaction) or with other molecules to equilibrate its charge and to form more or less reactive molecules. It is believed that free radicals are one of the causes of many diseases.

In biological system, free radicals are continuously produced in the body as the result of the normal metabolic processes from mitochondria, phagocytes, inflammation and enzyme action. External environmental stimuli such as toxic substances, microbial attacks, ozone, UV radiation, cigarette smoke, or intensive exercise are another sources of free radicals formation (Dufresne *et al.*, 2001). Indeed, free radicals are the part of the immune system which intercept the challenge of invaders like microbes and viruses, but in certain condition they tend to attack the body by altering the cell membranes, tamper with DNA, accumulate oxidised LDL which lead to coronary heart disease and in worst case may lead to cancer and cell death. Although regular exercise builds up body defense systems, an increase in demand and utilization of oxygen, increases the free radicals formation.

Both the deleterious effects of ROS (reactive oxygen species) and RNS (reactive nitrogen species) contribute to the onset of cancer, cardiovascular disease and impairment of the immune function (Bidlack *et al.*, 1998) and are gaining recognition as a key phenomenon in chronic diseases. ROS/RNS formation are of great concern and interlink with oxidative stress. Oxidative stress is induced by an overproduction of reactive oxygen species (ROS), improper balance between the formation and destruction of free radicals in the organisms. Consequently, it leads to a disruption of cellular functions by modifying activity over DNA expression and energy production (Dufresne *et al.*, 2001).

The ROS comprises those of oxygen-centered radicals such as superoxide anion ($O_2^{\cdot}$), hydrogen peroxides (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and peroxynitrite ($ONOO^{\cdot}$).

(a) Superoxide anion ($O_2^{\cdot}$)

Superoxide anion is the most common intracellular radical. Generally, *in vivo* $O_2^{\cdot}$ radicals can be generated by phagocytic cells, macrophages during defending against foreign organism. It can also formed upon one-electron reduction of oxygen mediated by enzymes such as NADH–NADPH oxidases. However, $O_2^{\cdot}$ radical life span depends on the prescence of enzyme superoxide dismutase (SOD), which catalyzes to H2O2 and molecular oxygen.

$$O_2^{\cdot} \quad + \quad SOD \longrightarrow H_2O_2 \quad + \quad O_2$$

(b) Hydroxyl radical ($OH^{\cdot}$)

Hydroxyl radicals are most reactive and toxic radicals. They are usually more susceptible to react with various compounds such as enzymes, carbohydrate, protein and lipids than superoxide anion radical ($O_2^{\cdot}$). It can attack lipids and produces lipid peroxides. They are produced by metal-catalyzed reaction of superoxide and hydrogen peroxide.

(c) Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is formed by oxidase enzyme. It is not directly damaging, however, it is easily converted to hydroxyl radical in the present of transition metals such as Fe^{2+}, Cu^{+}.

$$H_2O_2 + Fe^{2+} \longrightarrow OH^{\cdot} + OH^{-} + Fe^{2+}$$

(d) Peroxynitrite ($ONOO^{-}$)

Peroxynitrite ($ONOO^{-}$) is produced by the reaction of superoxide and nitric oxide which plays a significant role in various inflammatory disorders. Under physiological condition, NO is the stable free radical, and plays an important role in regulating the renal and heart function by acting as vasodialator and neurotransmitter. However, as a results of oxidative stress, NO rapidly reacts with $O_2^{\cdot}$ resulting in $ONOO^{-}$ and its metabolite $OH^{\cdot}$. They are extremely reactive and directly induced SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications (Nakagawa *et al.*, 2002).

4.3 Lipid peroxidation reaction

Lipid peroxidation is a free-radical mediated propagation of oxidative insult to polyunsaturated fatty acids involving several types of free radicals, and termination occurs through enzymatic means or by free radical scavenging by antioxidants. Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) appear to contribute to the pathology of cardiovascular disease. Lipid and protein oxidation are also correlated with increases in atherosclerosis and diabetic complications and a reduction of the immune function.

The basic mechanism can be distinguished in three distinct steps: initiation, propagation and termination.

(a)Initiation

$$RH\ (Lipid) \xrightarrow{metal} R^{\cdot} + H^{\cdot}$$

Formation of lipid radical $R^{\cdot}$ is mediated by traces of metals, light, and UV radiation.

(b) Propagation

$$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}\ \text{(peroxy radicals)}$$

$$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot}$$

Propagation of free-radical oxidation process occurs in the case of lipids by chain reactions that consume oxygen and yield new free-radical species. The products of $R^{\cdot}$ and $ROO^{\cdot}$ can further propagate free-radical reactions.

(c) Termination

$$ROO^{\cdot} + ROO^{\cdot} \longrightarrow ROOR + O_2$$

Free radicals are structurally unstable due to bonding-deficiency, and whenever possible they tends to be in stable form. However, reduction in unsaturated lipids level lead the unstable radical to bind with each others resulting in the stable non-radical compound. In other words, it terminates the propagation sequence of chain reaction.

4.4. Antioxidant mechanism

An antioxidant can be broadly defined as any compound that delay or prevents oxidation of a substrate. On the other hand, antioxidants are molecules that interact with the 'free radicals' thereby neutralizing them, which results in protecting normal tissue and DNA from potential damage. Because of the seriously damaging potential of reactive oxygen species, cells depend on elaborate defence mechanisms to effectively neutralise or metabolise these toxic intermediates and to prevent significant free radical injury. Fortunately, the normal body mechanism has its own antioxidants to neutralize 'free radicals'.

In biological system, a complex antioxidant defense system normally protects cellular system against the injurious effects and the cellular damage caused by free radical production. Cells possess enzymatic and non-enzymatic internal defense systems for protection against ROS, and consequently prevent cellular damage. For instances, enzymatic antioxidants are produced by certain enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase whereas non-enzymatic antioxidants are antioxidant vitamins (vitamin C, vitamin E) and some trace elements like zinc, copper and selenium.

The first line of defence against the superoxide radicals are the superoxide dismutase enzymes. They catalyses the reduction of superoxide radical to H_2O_2. H_2O_2 formed during superoxide dismutation which is highly toxic however it can be removed by catalase. This whole mechanism is necessary for cell survival.

$$O_2 + e^- \xrightarrow{\text{Glutathione peroxidase}} O_2^- \xrightarrow{\text{Superoxide dismutase}} H_2O_2 \xrightarrow{\text{Catalase}} H_2O + O_2$$

Generally, natural antioxidant system can effectively neutralize the radical or oxidized product up to a certain limit, even though massive oxidative stress and aging induced by an overproduction of reactive oxygen species (ROS) leads to a disruption of cellular functions. Under these circumstances, there is imbalance between oxidants and antioxidants necessitating the addition of exogenous antioxidants. Therefore, diet rich in antioxidant such as vitamin C, vitamin E, vitamin B_2 and B_6, beta-carotene and flavonoids have played an important role. Moreover, considerable attention has been emphasized on naturally occurring materials that can protect against ROS and their antioxidant activities have been identified.

4.5 Measurement of antioxidant activity

The antioxidant activity of the tested samples can be evaluated with different tests for different mechanisms. Many strategies have been developed and well established to evaluate the antioxidant activities of the tested samples in terms of detecting the free radicals, antioxidant activities and oxidative damage. To determine the oxidative damage, detection upon lipid peroxidation and DNA damage have been established.

Free radicals can be detected by electron spin resonance, fluorescence and chemiluminescence. Electron spin resonance (ESR) spectroscopy is well known method to measure the free radicals directly in conjunction with spin trapping agents such as dimethylpyrroline-N-oxide (DMPO), which is hydrophilic compounds, traps free radicals in an aqueous environment. However, this technique had limited application (Robards *et al.*, 1999). In another method fluorescence probe, for instance, dichlorofluorescin diacetate was used and oxidative process in living cells can be visualized.

Many different experimental methods have been developed for the determination of antioxidant activity. Most widely used methods are typically based on the generation of radical species thereby determining the disappearance of these radicals in the addition of antioxidants and free radical scavenging activity can then be determined. Most of the reliable methods that have been developed to determine the radical scavenging activity involved the measurement of the disappearance of the challenged free radicals for instance, superoxide radical, hdroxyl radical, 2,2'-azino-bis (3-ethylbenzenthiazoline-6-sulphonic) cation radical ($ABTS^{\cdot+}$), the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) or other radicals. Effectiveness is measured by monitoring the extent of reduction in oxidation with the help of chemicals, instruments or sensory methods under standard conditions. However, all these methods should not be time-consuming, but sensitive enough to screen the radical scavenging activity of the tested samples which may be the important factor that needs to be considered.

DPPH radical is not a naturally occurring radical, and is relatively stable compared to the highly reactive superoxide and hydroxyl radicals primarily responsible for oxidative damage in biological systems. Any molecule that can donate an electron or hydrogen to DPPH can react with it and thereby bleach the DPPH absorption (Naik *et al.*, 2003).

Some others well known methods for the determination of antioxidant activities are total radical trapping antioxidant parameter assay (TRAP), trolox equivalent antioxidant capacity assay (TEAC) and hypoxanthine/xanthine oxidase assay. Trolox equivalent antioxidant capacity assay (TEAC) is the spectrophotometric radical scavenging analysis with Trolox as standard. Trolox is a synthetic commercial product very similar in chemical structure to vitamin E, however it exhibits as water-soluble vitamin E analog. In this assay the stable free radicals ($ABTS^{\cdot}$ or $DPPH^{\cdot}$) which react with antioxidant was used.

Another strategy has been developed for measuring the total antioxidant activity (TAA) of the sample which has the ability to scavenge free radicals generated in the aqueous and lipophilic phases. Generated radical is coupled to oxidation of a substrate and the inhibitory effect of substrate measured which is based on the detection of the radicals or the products of oxidation. This ability to scavenge specific radicals may be targeted as, for instance hydroxyl radicals or nitric oxide radicals.

Recently, one approach to quick assessment of total antioxidant activity was established. Randox (UK) has launched the world's first kit for the measurement of the total antioxidant status of an individual. Randox total antioxidant status kit was commercially available for easy and quick assessment of the antioxidant activities. This method is most suitable to determine antioxidant activity of water soluble substances. The basic principle is the chromogenic indicator ABTS which has high water solubility and chemical stability which is incubated with a peroxidase (metmyoglobin). This peroxidase substrate which, when oxidised in the presence of H_2O_2 generates a metastable radical. The formation of the ferrylmyoglobin radical (from reaction of metmyoglobin with hydrogen peroxide) is then free to react with ABTS to produce the $ABTS^{®.+}$. radical cation. This has realatively stable blue-gren colour, which is measured at 600 nm. The accumulation of the ABTS cation radical can be inhibited by the presence of an antioxidant in the reaction medium, to an extent and on a time scale dependent on the antioxidant activity (Robards *et al.*, 1999). Trolox, water soluble vitamin E analogue is used as the standard.

Methods of expressing the results are as varied as the approaches to measurement. EC_{50} has been determined as the amount of antioxidant necessary to decrease, by 50% the initial stable radical concentration by monitoring the decrease of its absorbance at 517 nm or time taken to reach the stable state to EC_{50} concentration (T_{EC50}). Another method of expressing results employs the Trolox Equivalent Antioxidant Activity (TEAC). This measure the concentration of the Trolox solution with an equivalent antioxidant potential to a 1.0 mM solution of the substance under investigation.

4.6 Green tea polyphenols as antioxidant

Many phytochemicals have been labeled as antioxidants. Basically, the mechanisms involve in 3 different ways; (1) acts as preventive antioxidant which reduce the rate of initiation of free radicals, (2) acts as chain-breaking antioxidant which interact rapidly with the radicals after chain-reaction is initiated, and converted to the stable radicals and inhibits the propagation phase, (3) repair compound to its original state or degrade them to nonfunctional compounds (appotosis) where enzymes reaction are also involved (Bidlack *et al.*, 1998). Most of the flavonoids showed the scavenging activity with various intensities.

Green tea has been represented as the powerful radical scavenger, studied by both *in vivo* and *in vitro* results. The propensity of green tea polyphenols flavonoid to inhibit free-radical mediated events is governed by its chemical structure. Since these compounds are based on the flavonoid nucleus, the number, position and types of substitutions influence radical scavenging and chelating activity.

Both the configuration and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity. Free radical scavenging capacity is primarily attributed to the high reactivities of hydroxyl substituents. The B-ring hydroxyl configuration is the most significant determinant of scavenging of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to relatively stable flavoniod radical. Moreover the radical-scavengering activities of polyphenols greatly depends on their molecular weight. For instance, increase in molecular weight and increase in phenolic hydroxy groups in the compound, resulted in an increase in radical-scavengering effects studied on 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion (O_2^-) radicals. The activities may vary depending on the character of the radicals to be scavenged.

One of the major component of green tea polyphenol; EGCG has a high molecular weight (458.4) and altogether 8 numbers of phenolic hydroxy group present in contributed to the strongest radical scavenging activity than other catechin derivatives (Hatano *et. al.*, 1989). Similar effect was reported with additional remarks on stronger radical scavenging activity of green tea polyphenols than ascorbic acid and alpha tocopherol (Yoshida *et. al.*, 1989).

All the determination of catechin reactivity toward oxidants are due to the presence of 3'- and 4' hydroxy groups and further addition of 5'-hydroxy groups in the B-ring. Moreover, the presence of galloyl moiety adds to its activity. Nanjo *et al.* (1996) reported both trihydroxyphenyl B ring and the 3-galloyl moiety of EGCG has highest involved in reactions with the diphenylpicrylhydrazyl (DPPH) radical, in the order of EGCG>EGC>ECG>EC. The inhibitory activity of tea polyphenols on 12-O-tetradecanoylphorbol-13-acetate (TPA-induced ornithine decarboxylase induction is in the order of

EGCG>EGC=ECG>EC (Agarwal *et al.*, 1992). However, Valcic *et al.* (1999) shown that the principal site of EGCG molecule is the trihydroxyphenyl B ring, rather than the 3-galloyl moiety, in the reaction with peroxyl radicals generated by the initiator 2,2'-azobis(2,4-dimethulvaleronitrile) (AMVN).

Nakagawa *et al.* (2002) studies have shown that green tea catechins directly scavenged NO and O_2^-, consequently preventing $ONOO^-$ formation. All this events are mostly concerned with the galloly group and *O*-trihydroxy structure in the B ring which enhanced the scavenging activity of green tea catechins. All these results may suggest the relationship between the structure and the effect of catechins. The ortho-dihydroxyl group in the B ring of the catechins is necessary for their biological effects; the ortho-trihydroxyl group in the B-ring and/or the galloyl group at the 3-position increase the effects of catechins.

Moreover, tea catechins are excellent antioxidant agents against lipid peroxidation and their antioxidant capacity has been demonstrated in several *in vitro* and *ex vivo* systems. Many phenolic compounds, in particular catechins, are good antioxidants and they are functional as antioxidants of relatively low concentrations. While at higher concentrations, since they themselves are susceptible to oxidation, they can behave as pro-oxidants.

The mechanism of action of green tea catechine against lipid peroxidation occured mostly in two ways; one is by reducing reactive O_2 species and/or lipid peroxy radicals by H-atom donation from free hydroxyls on the B-ring; giving phenoxy radicals and another way is by metal-chelating by phenolic OH groups (Bidlack *et al.*, 1998).

Various studies have been described on the antioxidative action of the green tea polyphenol against lipid peroxidation. Sugihara *et al.* (2001) have studied the effects of tea catechins on metal-induced lipid peroxidation by using three metal ions; ferrous (Fe), copper (Cu) and vanadium (v) ions in rat hepatocytes. The findings demonstrated that tea catechins have the lipid peroxidation activities in the order; EGCG>ECG>EGC>EC. Liu *et al.* (2000)

studied in human low density lipoprotein with water-soluble initiator 2,2'-azobis (2-amidinopropane hydrochloride) (AAPH), shown that the activity sequence of tea catechin are as follows; EC>EGCG>ECG>EGC. Moreover, theflavins persent in black tea inhibited Cu^{2+}-mediated LDL oxidation and the results demonstrated that black tea theaflavins possess at least the same antioxidant potency as catechins present in green tea (Leung *et al.*, 2001). All these findings contributed that consumption of green tea and reduce the risk of cardiovascular disease.

5. Cytotoxicity effects of green tea

Tea is consumed world wide as a beverage and many health benefits including prevention of cancer, have been ascribed to consumption of tea. The cancer-preventive activity of tea has been the subject of numerous investigations and is well documented for cancer of all sites in animals investigated so far. Most of the studies have been conducted with animals, mostly with rodents, to get a better understanding of the effect of tea components in the living organism. Many evidences have shown that tea protects against wide range of cancer by inhibiting the UV irridiation, chemically induced tumorigenesis and participate in all stages of carcinogenesis. Studies have indicated that green tea polyphenol and its constituent had been shown to inhibit carcinogenesis in the skin and lungs. Many researches confirmed the significant carcinogenesis effect of green tea extracts and its components, in particular EGCG in different organs such as colon, prostate, oesophagus, stomach, liver and memory gland (Agarwal, 2000; Hong *et al.*, 2001; Tusubono *et al.*, 2001). Extensive studies on the mechanisms of cancer prevention by tea polyphenols, especially (-)-epigallocatechin-gallate (EGCG), which is believed to be the most potent ingredient among the polyphenolic compounds isolated from green tea, has revealed their influence on biochemical events related to tumor promotion and progression. Tea polyphenols shown the inhibitory activity during the initiation, promotion and progression status of carcinogenesis (Jung *et al.*, 2001; Lambert *et al.*, 2003).

No efforts have been spared to prevent incidence of cancer. Top priority has been given to prevent recurrence of cancer with patient undergoing treatment and also with people who had survived cancer. The need for alternative treatment regarding cancer patients is thus becoming a top priority. Moreover, green tea has several advantages over other chemopreventive agents, the most important being the absence of toxicity, documented by its long history of extensive and safe consumption by the Japanese and Chinese people. The purpose of this review is to discuss the potential skin cancer cytotoxicity effect of three different Asian green teas.

5.1. Cancer

Cancer is a disease where cells grow out of control, highly invasive or metastatic, erode and destroy normal tissue. Notably, cancer is the second most common cause of death and dubbed the 'killer disease'. Carcinogenesis can be regarded as an accumulation of genetic or biochemical cell damage which involves multisteps; initiation, promotion or progression stage (Gerhauser *et al.*, 2003). The mechanism involves multiple stages of biochemical and molecular alteration in target cells. The driving forces behind the development of cancer are damaged genes. Different types of cancer occur at any ages, anywhere in the body and are attributed to the different causes, different symptoms and require different types of treatment. Although it can be avoided from body mechanism in young age, it can occur significantly when getting old.

Cancer chemoprevention can be defined as the prevention, inhibition or reversal of carcinogenesis. Chemotherapy or radiation treatments had been widely used for cancer treatment. But there are disadvantages such as hard to differentiate between normal cells and tumor cells, high incidence of malignant changes especially in proliferation tissue and increase risk of cancer. Therefore, any possibility of finding a readily available natural substance to prevent cancer warrants closer examination. Moreover, elevated ROS levels can initiate DNA damage, for instance the formation of oxidized DNA, and also involved in tumor promotion, and ultimately lead to carcinogenesis.

5.2. Skin cancer

At the present time, skin cancer is the most common type of human cancer when compared with others, attributing up to one third of all new cancers. There are two types of skin cancer namely melanoma and non-melanoma. The incidence of skin cancer has been increasing due to the recreational exposure to sunlight (280-320 nm) and depletion of the ozone layer.

5.3. Melanoma skin cancer

Malignant melanoma is the most dangerous and deadly form among all skin cancers. There are less cases of melanoma as compared to non-melanoma. But the incidence and increase in mortality rate of melanoma are the highest among all cancers (Stratton *et al.*, 2000). Melanoma skin cancer originates from the melanocytes, which are located between the epidermis and dermis of the skin. They are the pigment producing cells and become melanoma if they are cancerous. This pigment helps to protect deeper layer of the skin from the harmful effects of the sun (Einspahr *et al.*, 2002). Even though melanoma can be cured by surgical incision in early stage, it cannot be cured in case of distant metastasis stage. Melanoma is the most common cancer in woman and it is second to breast cancer in women ages 30 to 34 years.

Melanoma may appear without warning or it may begin in or near a mole or dark spot on the skin. The early stage of melanoma called cutaneous melanoma. It has better chance to cure by surgical means if detected early. However, when it spreads internally; it become metastatic melanoma which can be found in lymph nodes and others parts of the body; such as liver, lungs or brain, and finally it can be fatal. Since the treatment at latest stages of melanoma is very limited, there is an increasing need for effective chemoprevention strategies.

5.3.1.Risk and causes

Exposure of human skin to the sunlight and in particular to the ultraviolet band of the spectrum, is the common risk factor, which significantly increases the development of melanoma skin cancer. The deleterious effects of the ultraviolet radiation have been attributed largely to the generation of free radicals, such as superoxide and hydrogen peroxide. It is claimed that 50-70 % of melanoma is caused by sun exposure.

Moreover, a little mark on the skin could be a major causing factor for melanoma. Melanoma mostly starts from the pre existing moles. Moles are common pigmented skin lesions. There are two types, normal and atypical moles. The 'atypical moles' are larger than normal moles and are uneven in colour with irregular border. When they changed in colour, size and shape, they have high tendency to become malignant. As a result, people having lots of moles have higher than average risk of malignant melanoma.

Another risk factor known for malignant melanoma is people with the family history of maligant melanoma in which they have the same sort of skin type. It doesn't mean, however, that they can definitely get melanoma, it means that they have more chance to develop and need to pay attention on the skin under the sun.

Fair skin contains less protective pigment than dark skin people, therefore the people with the fair skin have increased risk of developing melanoma than those with dark skin. However, it doesn't mean that dark skin people have more melanocytes than fair skin people, their melanocytes are more active making more pigment.

5.3.2.Prevention and treatment of melanoma

The good news for skin cancer is that it can be prevented and early detection can be done easily. Causes of melanoma can be prevented by avoiding over-exposure to the sun and other sources of ultraviolet radiation, by wearing protective clothing or using other additional supplements such as naturally occurring agents with none or minimal toxicity. Checking the skin changes weekly is highly advisable. For the treatment of melanoma, there are various options such as surgery, radiotherapy, and chemotherapy (Pawlik *et al.*, 2003). Some scientists are now studying even up to the molecular level, such as gene therapy research, however all these are in the very early stages of clinical trials.

5.4.Effect of green tea on skin cancer and other cancer

Many experimental researches in laboratory animals demonstrated that tea components had an inhibitory effect on carcinogenesis at a number of organ sites (Lin *et al.*, 2000). Since the last decades, convincing evidences of the powerful green tea polyphenolic antioxidant activities that afford protection against the wide range of cancer studies both *in vivo* and *vitro* were found in research data around the world. The mechanisma of carcinogenesis involve multiple alterations in target cells and tea components are effect in the all stages of cancer.

EGCG which is the major component of green tea, inhibits tumor invasion (Jung, *et al.*, 2001) and metastasis lung in the present of melanomas in mice (Liu, *et al.*, 2001). In addition, both green tea and black tea extracts dose-dependently inhibited the proliferation and invasion of a rat ascities hepatoma cell line of AH109A without effecting the normal cell line (Zhang et al., 1999).

Consumption of tea on a regular basis has been associated with reduced risk of several forms of cancer in human populations and mouse models. There is the strong evidences linking green tea use to reduction in cancer risk in parts of Asia, especially in Japan and China. Population studies have demonstrated that green tea consumption may be one of the reasons why the occurrence of cancer is lower in Japan. Several case-control studies on human, as well as many animal studies, also suggested that green tea polyphenols possess significant chemopreventive properties and have demonstrated inhibitory effect against tumor formation and growth. Results found a negative association between green tea consumption and cancer incidence in Japanese population, especially among females drinking more than 10 cups of tea a day (Imai *et al.*, 1997). It thus confirmed the effect of green tea in preventing cancer. Moreover, the fact that green tea consumption was not associated with the risk of gastric cancer was confirmed by a population based prospective study in Japan (Tsubono *et al.*, 2001).

The inhibitory action of tea and tea components against chemically induced carcinogenesis has been demonstrated in animal models. Many experimental researches demonstrated that tea components had an inhibitory effect on carcinogenesis, which have been attributed to the biological activities of the polyphenol fraction in tea. Inhibitory effects of tea catechins on hepatocarcinogenesis in rat were investigated and the results suggest that tea catechins have a chemopreventive action against hepatocarcinogenesis (Matsumoto *et al.*, 1996)

One study ascertained that the topical application of green tea prevents UVB-induced DNA damage in the skin studied *in vivo* (Katiyar *et al.*, 2001). Agarwal *et al.* (1992) suggested that green tea polyphenols, specifically EGCG, provide anti-tumor-promoting effects against a wide spectrum of skin tumor promoters and and also protect against UVB-induced photocarcinogenesis (Ichihashi *et al.*, 2000). Furthermore, polyphenols, particularly flavonoids not only exert antioxidant protective effects on DNA and gene expression but also inhibit the initiation, promotion and progression of tumors and inducing apoptosis (Leighton *et al.,* 1999). Even though caffeine content is high in green tea, both green tea constituents and caffeine may have been an important constitutent in inhibiton effects of UV-induced carcinogenesis and lung carcinogenesis (Chung *et al.*, 1998).

Many experimental researches in animal models and in vitro cell line demonstrated that tea components had an inhibitory effect on carcinogensis with different cell lines. Green tea has not only effect on the skin cancer but also works on other types of cancer. The work of Agarwal (2000) indicated that one of the green tea component, EGCG, dose-dependently inhibit the prostate cancer.

Study of purified green tea polyphenol on human colon tissue results in altering the risk of colon cancer (Hong *et al.*, 2001). Fujiki *et al.* (2002) reported that tea polyphenol inhibited the growth of human lung cancer cell lines.

Valcic *et al.* (1996) demonstrated that green tea polyphenols inhibited the growth of four selected human tumor cell lines, including MCF-7 breast carcinoma, HT-29 colon carcinoma, A-427 lung carcinoma and UACC-375 melanoma. This finding proved that EGCG was potent against breast, colon and melanoma cell lines and other components.

Based on origin of tea, there can be difference in ECGC content and this difference in EGCG content could give rise as to the effect of cytotoxicity. Thus an in-depth study on the changes involved on different origin of tea as regards the cytotoxity effect should be studied.