CHAPTER II

LITERATURE REVIEW

Tea

Botanical Properties

Sciencetific name	:	*Camellia sinensis* (L.) O. Kuntze
Related name	:	*Camellia thea Link.*; *C. theifera* Griff.; *Thea sinensis* Linn.
Common name	:	Tea, Thea
Family	:	Theaceae

Figure 1 The picture of green tea [15]

General properties

Tea is a small evergreen shrub cultivated to a height of 7 to 8 feet, but it is growing wild up to 30 feet high and has many branches. Tea bark is rough and grey. It has dark green and lanceolate or elliptical leaves with short stalks and blunt apex. Leaf's base is tapering and have shortly serrate. The young leaves are hairy but the older one

is glabrate. It has the solitary flowers but two or three of them stay together on short branchlets. It is in the leaf axils. The short stalks have a few small bracts that wide 1 to 1 1/2 inches. The tea flower has five sepals and imbricates. The sepal is ovate or rounded. It is smooth and blunt. The petals are usually have five or up to nine. It is unequal and rounded. The stamens are indefinite and adhered to the base of petals. The anthers are large. The ovary is small and having downy hair. The ovary is having three cells and has three or four pendulous ovules in each cell. Furthermore, the ovary has slender stigmas. The fruit is smooth, rounded and flattened that have three-celled capsule and seed solitary in each cell [15].

Part of use

Young leaves, nuts and leaf buds

Tea Fermentation

First, the freshly plucked leaves are withered over night in the trough to reduce their moisture content to almost half. Withering is an important process in producing the fragrance of tea. It renders the leaves more pliable for rolling in the next process. During the rolling process, polyphenol oxidase (PO) and catechins, which exist separately in the tea leaf, mixed. The catechins are located in vacuole in the palisade layer of tea leaves, whereas the enzyme is located in the epidermal layer. During the process of fermentation, enzymatic oxidation occurs with catechins forming complex groups of compounds. These oxidized products represent the reddish-brown colors of black tea [1, 4]. Additionally, the tea types are classified by the preparation process that explained in Figure 2.

Many kinds of tea are produced, although these can be classified principally into three types: green (unfermented), oolong (semi-fermented), and black (fully fermented). Green tea is made by inactivating the enzymes in the fresh leaves, either by firing or by steaming, to prevent the enzymatic oxidation of catechins. Processed products are described as roasted green tea and steamed green tea (such as Sencha in Japan).Black tea is made by a polyphenol oxidase catalyzed oxidation of fresh leaf catechins, termed

fermentation. This fermentation process results in the oxidation of simple polyphenols, i.e. tea catechins, to more complex condensed molecules which give black tea its typical color and strong, astringent flavor. Oolong tea is prepared by firing the leaves shortly after rolling, and then drying the leaves. The oxidation is ended by the firing process; hence oolong tea is called semi-fermented tea. The characteristics of oolong tea are between black and green tea[1, 2].

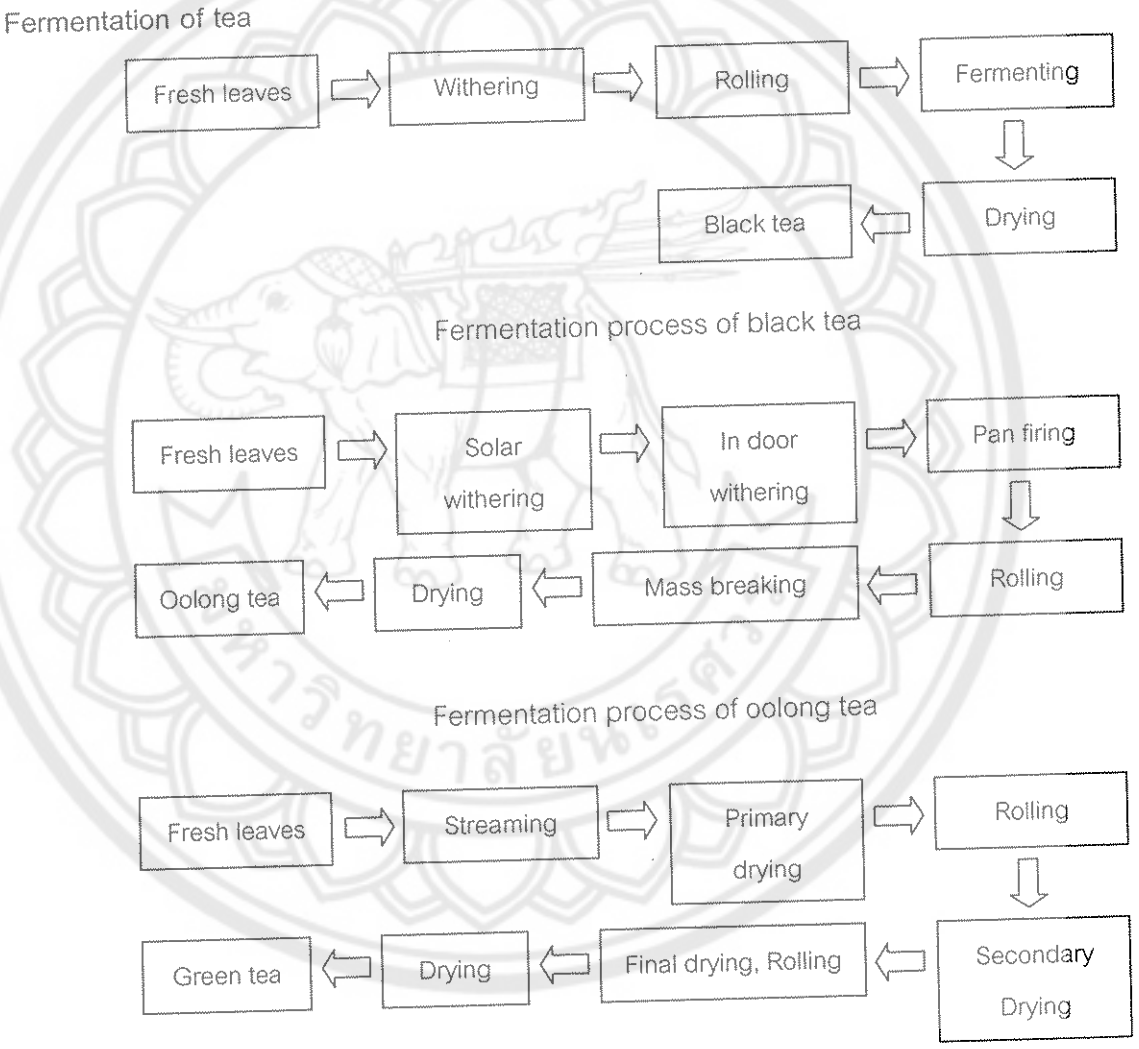


Figure 2 The fermentation process of various types of teas [1]

Brewing of green tea

When preparing green tea, filtered cold water is always used. It is passed to a rolling boil. Next, allow the water to cool to a temperature somewhere between 90-100 C° before pour it over the tea leaves. (Boiling water usually takes between 30 and 60 seconds to cool to this temperature). Boiling water should never be used to prepare green tea. Water at this temperature will brew the leaves and destroy the tea and its flavor, creating a bitter tasting tea. Approximately 90 C° is perfect for green teas taste. The amount of tea needed per pot or cup is less than one might expect. Approximately 2 grams of tea per cup make a perfect tasting tea (1 teaspoon for a 6 to 8 oz. cup). Leaves can be used for more than one infusion, which this is a common practice throughout the world. Full-leaf teas always produce more than one cup per teaspoon when made correctly[16].

The cup or pot should be rinsed with hot water to heat the vessel before preparing the tea. To reduce the amount of caffeine, pour just enough hot water over the leaves to cover them and let the tea sit for approximately 20 seconds before pour off the water. Then, cover the leaves with enough water to make a perfect cup or pot of tea and allow the tea to steep[1, 16].

Green tea applications

Green tea and their applications are always using for support health and decrease risk factor of disease that can be concluding follow as:

Antioxidant application

Green tea and its constituent catechins are best known for their antioxidant properities, which has led to their evaluation in a number of diseases associated with reactive oxygen species (ROS), such as cancer, cardiovascular and neurodegenerative diseases. Several epidemiological studies as well as studies in animal models have shown that green tea can afford protection against various cancers such as those of the skin, breast, prostate and lung[1, 6-8, 17, 18]. Also, several reports were confirming the active substances of green tea had antioxidant activity stronger than vitamin C and E[1, 2, 19].

Antimicrobial activity

The polyphenols in green tea can prevent teeth from decaying by inhibiting the biological activities of the cariogenic streptococci, Streptococcus mutans and Streptococcus sobrinus. The minimum inhibitory concentration (MIC) of tea polyphenols against these cariogenic bacteria was found to be 0.25 ± 0.12 mg/ml. Tea extracts not only prevent the growth of *S.mutans* but also determine its adhesion and inhibit glycosyl transferase activity. It is believed that under normal circumstances tea will reduce the acid on the tooth enamel for the whole time that starch is trapped in the teeth, and this is how it exerts its anti-caries effect[1, 2]. Besides antibacterial activity, recent report indicated the result of catechins from green tea that could be inhibiting the influenza virus especially EGCG and ECG. The report showed that the influenza virus was decreased by the suppression of viral RNA synthesis from the catechins compound[20].

Anti-cancer

Anti-carcinogenic activity of tea polyphenols was also reported. This activity had an effect on various parameters that induced carcinogenic in rats and mice. Free radicals induce the degeneration of DNA synthesis and changing the gene expression which affect to the occurring of cancer and abnormal cell[5]. Catechins, especially EGCG from green tea are the potent antioxidant agent that reduce the free radical and lower the risk of cancer [5, 6, 8, 21]. Recent reports revealed the efficacy of EGCG which suppress the growth of EBV (Epstien Barr Virus), the EBV are well-known as the cause of cancer. The report believed that the suppression of EBV by EGCG might be reducing the risk of cancer. The mechanism of EGCG suppressed the EBV growth was not clear but expect that hampered the viral RNA synthesis.

Adverse effect of green tea

Although regular consumption of green tea has high health benefit a few studies showed the data of toxicity of green tea. The recent report result the disturbance of gastrointestinal in some people. Some researchers believed that green tea can disturb the digestion and absorption of gastrointestinal tract. Several studies were trailed about the toxicity in animals with short term studies. Those showed minor skin irritation in rat

and guinea pig and eye irritation in rabbit [22]. However the long term effect such as teratogenicity or reproductive toxicity was not found in vitro studies [23].

Catechins Compound

Green tea polyphenols are mainly flavonoids, which subdivided into flavones, flavanones, isoflavones, flavonols, flavanols and anthocyanins. Their common chemical structure is the heterocyclic oxygen ring of a molecule presented in Fig. 3

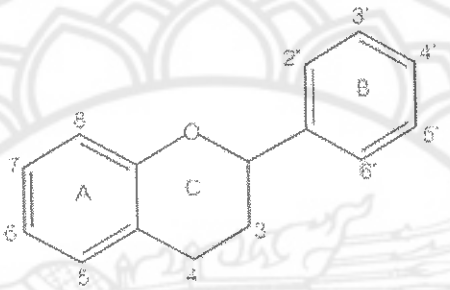
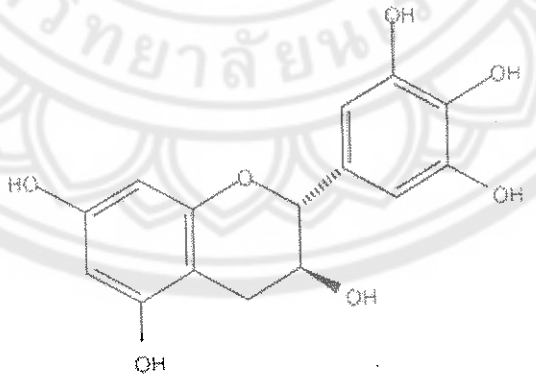


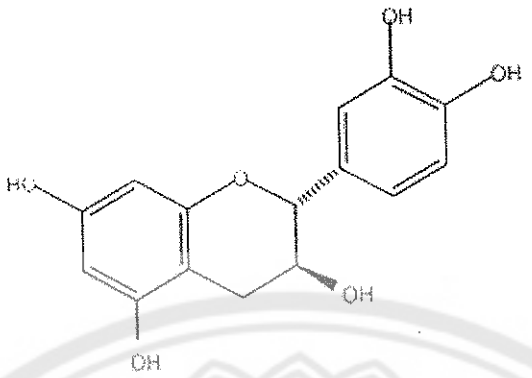
Figure 3 Basic structure of flavonoids [2]

Tea polyphenols include groups of compounds of different chemical structure with variable biological properties. Green tea leaves contains six major catechins: (+)-catechin (C), (-)-epicatechin (EC), gallocatechin (GC), (-)-epicatechin gallate (ECG), (-)-epigalocatechin (EGC), (-)-epigalocatechin gallate(EGCG). Chemical structures of the catechins are shown in Fig. 4 [24]

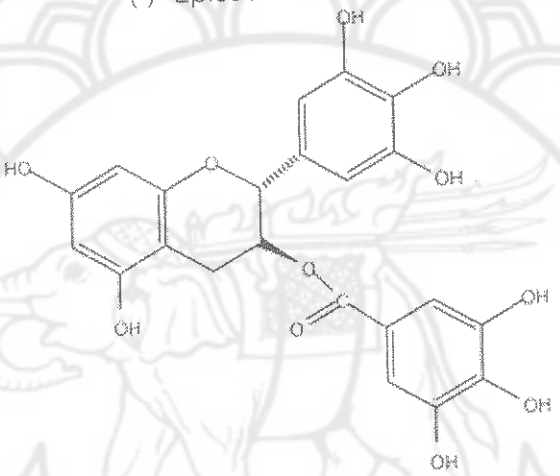


(-)- Epigallocatechin

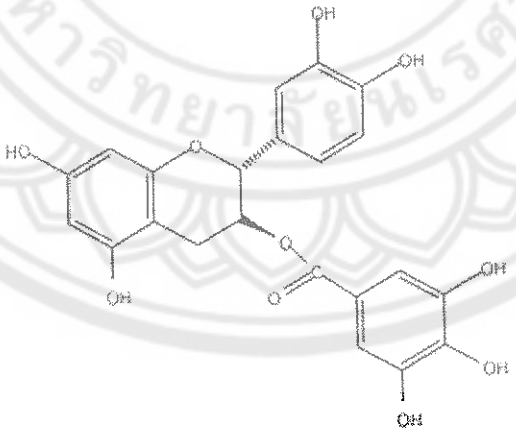
Figure 4 Chemical structure of catechins [24]



(-)- Epicatechin



(-)- Epigallocatechin



(-)- Epicatechin gallate

Figure 4 (Cont.)

Chemically catechins are water soluble, colourless compounds and impart astringency to tea infusions. It was proved that during black tea production, catechins are lowering the bitterness[2, 24]. The ratio content of individual hot water extracted green tea catechins was show in Table 1 [25].

Table 1 The chemical contents of green tea (Extracted by hot water)

Active Ingredient	Content (g/Kg dry leaves)
Caffeine	36.0
Catechins	
Epicatechin gallate	15.2
Epigallocatechin	46.0
Epigallocatechin galate	129.0
Epicatechin	0.9
Flavonols	
Myriscetin	0.8
Quercetin	1.8
Kaempherol	2.6

From Table 1, catechins have high content (191 g/kg dried leaves) when compared with other compound in green tea leaves. However, the differences in tea leaf constituents are due to many factors. It was stated that essential factors are: tea variety, collecting season, leaves age, climate, geochemical background of soil and harvesting, cultivation practices, environmental pollution, drying conditions as well as technological processes during tea production analytical methods, and kind of solvent used for extraction[2, 25-27].

The results from many investigations showed potential antioxidant properties of tea polyphenols. The tea catechins can act as antioxidants by donation of hydrogen atom, as acceptors of free radicals, interrupter of chain oxidation reactions. Several

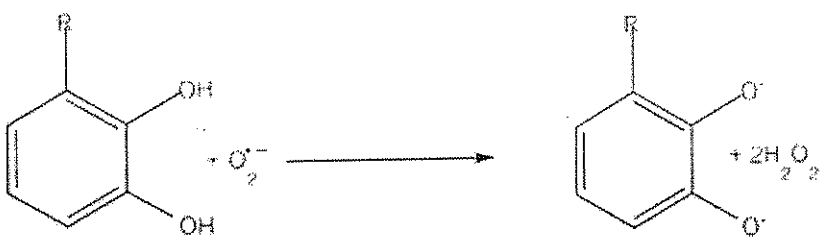
report suggested that annexation of hydroxide groups to catechin molecules is probably the main factor causing their strong antioxidant properties[2, 5, 10]. From the data of Table 1, the important component of green tea is EGCG that have the high content ratio than other catechins. According to chemical structures which show the potent antioxidant activity, EGCG processes a lot of hydroxyl group. Therefore, EGCG have the main problem that is very unstable in normal condition which almost certainly due to the three vicinal hydroxyl groups at positions 3', 4' and 5' in EGCG being more vulnerable to destruction (producing semiquinone free radicals)[11]. Thus autoxidation are occurred. Other than autoxidation reaction, the hydrolysis reaction also appears when EGCG contact with water or high humidity due to the broken of acyl group in EGCG by hydrolysis reaction[10].

Catechins Degradation and Mechanism

Catechins are susceptible when expose to water or heat, the degradation reactions are induced by autooxidation and hydrolysis reaction. The initial step of autoxidation would be the oxidation of catechins by O_2 itself as written in Eq.1

[Eq.1]

Next, the $O_2^{\bullet-}$ would also oxidized catechins as written in Eq.2. The $O_2^{\bullet-}$ is the stronger oxidant than oxygen. Therefore, $O_2^{\bullet-}$ would oxidize catechins faster than O_2. The autoxidation rate is enhanced in the presence of $O_2^{\bullet-}$.



[Eq.2]

The semiquinone intermediate generated in Eq.1 and Eq.2 can work as the better electron donor to O_2 than catechins. The semiquinone and oxygen produce $O_2^{\bullet -}$ as written in Eq.3.

[Eq.3]

From Eq.3 it generate $O_2^{\bullet -}$ that oxidized the fully reduced state. Thus, $O_2^{\bullet -}$ and semiquinone work as catalysts. The autoxidation of catechins proceeds via radicals chain processes that show in Figure 5 [10].

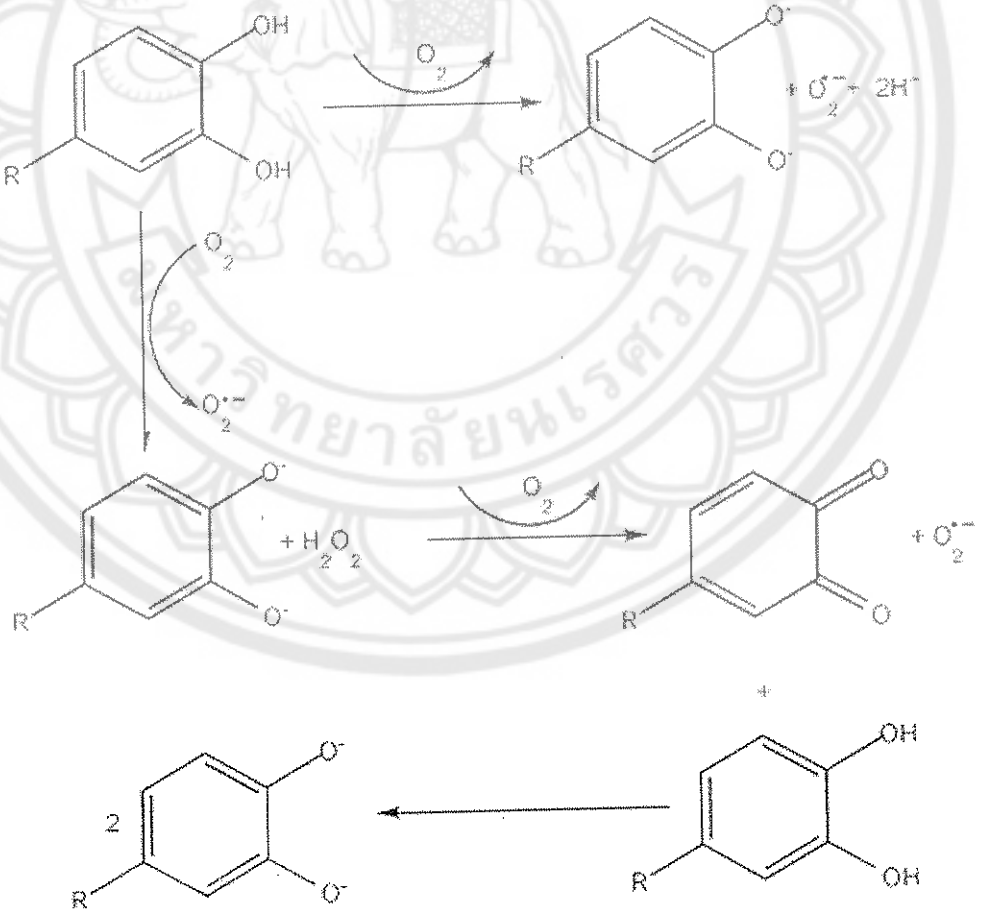


Figure 5 Proposed mechanism of autoxidation of catechins.

Catechins Stability

Several reports indicated the proper condition for maintain the catechins compound in the susceptible environment. Generally, catechins compound are more stable in low pH and low temperature. Furthermore, some reports showed that the EGCG was more stable when adding the water soluble polymer in the formulation such as Carbopol. Normally, the main mechanisms of catechins protection is reducing free water and protect the substance from light and heat that lower the occurring of autoxidation and hydrolysis reaction[13]. The reducing of autoxidation reaction always used the antioxidant agent; several reports confirmed that antioxidant agents could be protecting the EGCG from degradation. They could significantly increased the EGCG shelf-life when compared to the EGCG formulation without antioxidant[28]. BHT (Butyl hydroxytoluene) was the best reported antioxidant agent for the EGCG protection, the maximum efficacy was obtained when used the BHT concentration of about 0.05% (w/w)[12, 28]. However, the using of antioxidant agent was limited because almost antioxidant agents are oil soluble. Furthermore, few studies were showing that EGCG stability in aqueous solution of abundance of sodium or copper was lower when increase those substances[12, 29]. However, the studies on the stability of catechins in the long term are lower.

Chitosan

General properties

Chitosan, or ß (1, 4) 2-amino-2-deoxy-D-glucose, is a hydrophilic biopolymer obtained by hydrolyzing the aminoacetyl groups of chitin. The chitin is the main component of shells, crab, shrimp and krill, by an alkaline treatment. After deacetylation of chitin, the obtained chitosan is dissolve in acid and then filtered. The precipitate is wash and dried to get amine free chitosan which chemical structure is show in Figure 4. Chitosan is the linear polyelectrolyte in acidic pH. It is soluble in variety of acid which show positive charge and interacts with polyanionic counter ions [30].

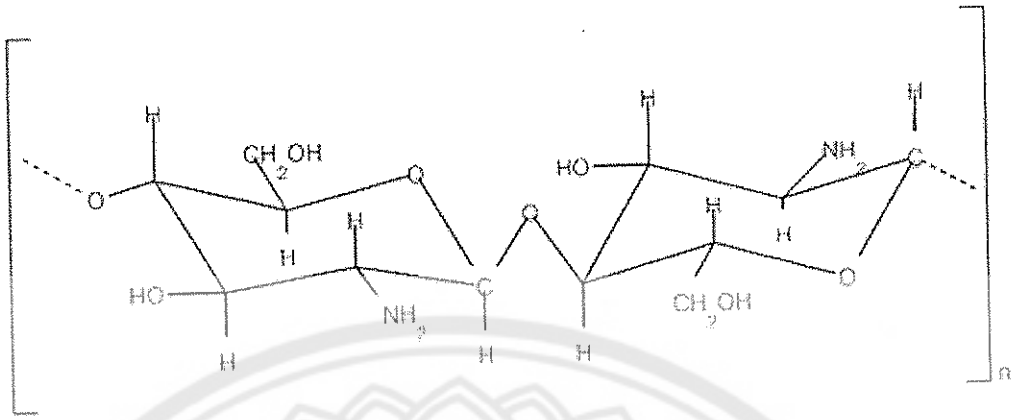


Figure 6 Chemical structure of chitosan

Chitosan is soluble in acidic solution but insoluble at pH>6.5 and in most organic solvent. It has gel forming properties in low pH range and has high positive charge density. Nowadays, many drug delivery systems are derived from chitosan because the advantage about biodegradable as well as an ability to increase membrane permeability both *in vitro*[31, 32] and *in vivo* [33]. It is also degraded by lysozyme in serum. From a biopharmaceutical point of view, Chitosan has the potential of serving as an absorption enhancer across intestinal epithelial from its mucoadhesive and permeability enhancing property.

Chitosan preparation

Chitosan is commonly prepared by deacetylating ∞-Chitin using 40-50% aqueous alkali at 100-160 C^{o} for a few hours. The resulting chitosan has a degree of deacetylation up to 95%. For complete deacetylation, the alkali treatment can be repeated. Nowadays the ∞-Chitin can be deacetylated at a much lower temperature, the reaction near 80 C^{o} is adequate for deacetylation as well as for the suppression of coloration processes, that give colorless chitosan products[30].

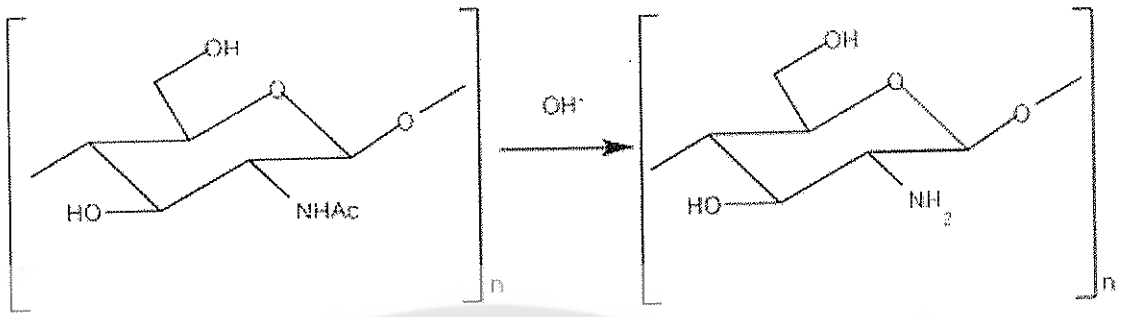


Figure 7 Deacetylation reaction of chitin

Deacetylation of chitosan

The N-acetyl group of chitin can be removed by alkali hydrolysis under heterogeneous conditions. Deacetylation is effect by heating a suspension of chitin flakes, in strong aqueous bases such as sodium and potassium hydroxide at 100-160 C^{o} to give chitosan with a degree of deacetylation between 70-95%. During the deacetylation process, the degradation of the main chain occurs as show by considerable decrease in the molecular weight [30].

Determination of degree of deacetylation

The degree of deacetylation of chitosan is one of the most important factors for specifying chitosan. Many methods has been reported for assessing the degree of deacetylation including elemental analysis, hydrolysis of acetamide groups, titration of free amino groups, dye adsorption and spectroscopic such as IR, UV and NMR. IR spectroscopy is a convenient way for determining the degree of acetylation. The IR spectroscopy is determined by using the absorbance ratio of A_{1655}/A_{3450} (absorbance at 1655 cm^{-1} for amide-I and at 3450 cm^{-1} for OH group inchitosan) base on the base line method was first examined[34]. The absorbance of chitosan is used to calculate the degree of deacetylation (DDA) using following equation:

$$DDA = [1-(A_{1665}/A_{3450})/1.33]x100]$$

where 1.33 represent the ratio of A_{1655}/A_{3450} for fully N-acetylated chitosan [34].

Molecular weight of chitosan

The molecular weight of chitosan is the important factors for chitosan characterization. Recent report showed that chitosan molecular weight affected chitosan microsphere characterization such as particle size, swelling and release profile[34]. Chitosan is soluble in aqueous acid solution. The molecular weight is determined by gel permeation chromatography or viscometry. However the using of viscometry is the simple and rapid method for determination of molecular weight. The constant ∞, K in the Mark-Houwink equation have been determined in 0.1 M acetic acid and 0.2 M sodium chloride solution. The intrinsic viscosity is express as

$$[n] = KM^{\infty} = 1.81x10^{-3} M^{0.93}$$

The charged nature of chitosan in acid solvent and chitosan's propensity to form aggregation complexes require care when applying these constants. Furthermore, converting chitin into chitosan lowers the molecular weight[30, 34, 35].

Chitosan and microparticle

The microparticle is well-known for the high advantage of drug delivery system that show the efficiency of the increasing of bioavailability and target drug delivery as well as increasing of drug stability and drug permeation. Many reports are available on the preparation of chitosan microspheres. Many methods used in the development of microparticulate polymeric drug delivery devices can also be used to prepare chitosan microspheres. The methods for chitosan microparticle preparation are summarized in Table 2 [36].

Table 2 Chitosan-microparticle prepared by different methods for various kinds of drugs

Methods of Preparation	Drug
Emulsion cross-linking	theophylline, cisplatin, pentazocine, phenobarbitone, theophylline, insulin, 5-fluorouracil, diclofenac sodium, griseofulvin, aspirin, diphtheria toxoid, pamidronate, suberoylbisphosphonate, mitoxantrone, progesterone prednisolone, interleukin-2, propranolol-HCl
Coacervation, precipitation	cimetidine, famotidine, nizatidine, vitamin D-2, diclofenac sodium, ketoprofen, metoclopramide-HCl, bovine serum albumin, ampicillin, cetylpyridinium chloride, oxytetracycline, betamethasone
Spray drying	
Ionic gelation	felodipine
Sieving method	clozepine

From the Table 2, the chitosan microparticle could be preparing with several methods. Each method is suitable for different drugs that intend to be incorporate in chitosan particle according to their properties. The methods are briefly explained following as

Emulsion cross-linking

This method utilizes the reactive functional amine group of chitosan to cross-link with aldehyde groups of the cross-linking agent. In this method, water-in-oil (w/o) emulsion is prepared by emulsifying the chitosan aqueous solution in the oil phase. Aqueous droplets are stabilized using a suitable surfactant. The stable emulsion is cross-linked by using an appropriate cross-linking agent such as glutaraldehyde[37] and polyanion[33, 38] to harden the droplets. Microspheres are filtered and washed repeatedly with n-hexane followed by alcohol and then dried. By this method, size of the particles can be controlled by controlling the size of aqueous droplets. However, the

particle size of final product depends upon the extent of cross-linking agent used while hardening in addition to speed of stirring during the formation of emulsion. However, a few study were modified the system from the water-in-oil emulsion to oil-in-oil emulsion, which changed the water in aqueous phase by organic solvent that miscible in water such as actetone [38], this procedure was proper for hydrophilic drug, the high encapsulation was obtained.

Coacervation, precipitation

This method utilizes the physicochemical property of chitosan since it is insoluble in alkaline pH medium, but precipitates/coacervates when it comes in contact with alkaline solution. Particles are produced by blowing chitosan solution into an alkali solution like sodium hydroxide, NaOH-methanol or ethanediamine using a compressed air nozzle to form coacervate droplets. Separation and purification of particles was done by filtration/centrifugation followed by successive washing with hot and cold water[39].

Spray drying

Spray-drying is a well-known technique to produce powders, granules or agglomerates from the mixture of drug and excipient solutions as well as suspensions. The method is based on drying of atomized droplets in a stream of hot air. In this method, chitosan is first dissolved in aqueous acetic acid solution. Drug is then dissolved or dispersed in the solution and then, a suitable cross-linking agent is added. This solution or dispersion is then atomized in a stream of hot air. Atomization leads to the formation of small droplets, from which solvent evaporates instantaneously leading to the formation of free flowing particles. Anil K. prepared ampicilin-chitosan microsphere by spray drying method that compared with solvent evaporation method. Microspheres was prepared by spray drying were spherical with a smooth and distorted morphology. Particle size of the un-cross-linked microspheres varied between 4 and 5 μm, while cross-linked microspheres ranged from 2 to 10 μm [38].

Ionic gelation

The use of complexation between oppositely charged macromolecules to prepare chitosan microspheres has attracted much attention because the process is

very simple and mild. Tripolyphosphate (TPP) is a polyanion, which can interact with the cationic chitosan by electrostatic forces. Many researchers have explored its potential pharmaceutical usage. In the ionic gelation method, chitosan is dissolved in aqueous acidic solution to obtain the cation of chitosan. This solution is then added dropwise under constant stirring to polyanionic TPP solution. Due to the complexation between oppositely charged species, chitosan undergoes ionic gelation and precipitates to form spherical particles [34, 38, 40]. However, TPP/chitosan microparticles formed have poor mechanical strength, thus limiting their usage in drug delivery [40].

Sieving method

A recent report has developed a simple, yet novel method to produce chitosan microparticles. In this method, microparticles were prepared by cross-linking chitosan to obtain a non-sticky glassy hydrogel followed by passing through a sieve. A suitable quantity of chitosan was dissolved in 4% acetic acid solution to form a thick jelly mass that was cross-linked by adding glutaraldehyde. The non-sticky cross-linked mass was passed through a sieve with a suitable mesh size to get microparticles. The microparticles were washed with 0.1 N NaOH solution to remove the un-reacted excess glutaraldehyde and dried overnight in an oven at 40 Co. Clozapine was incorporated into chitosan before crosslinking with an entrapment efficiency up to 98.9%. This method is devoid of tedious procedures, and can be scaled up easily. Microparticles were irregular in shape, with the average particle sizes in the range 543–698 μm. The in vitro release was extended up to 12 hrs, while the in vivo studies indicated a slow release of clozapin [41].

The chitosan microparticle preparation was explained so far those were the parts of many methods of chitosan microparticle preparation, the summation of those methods were shown in Figure 8 [42]. The method selection was depended on the physicochemical properties of substance that was entrapped into the particle.

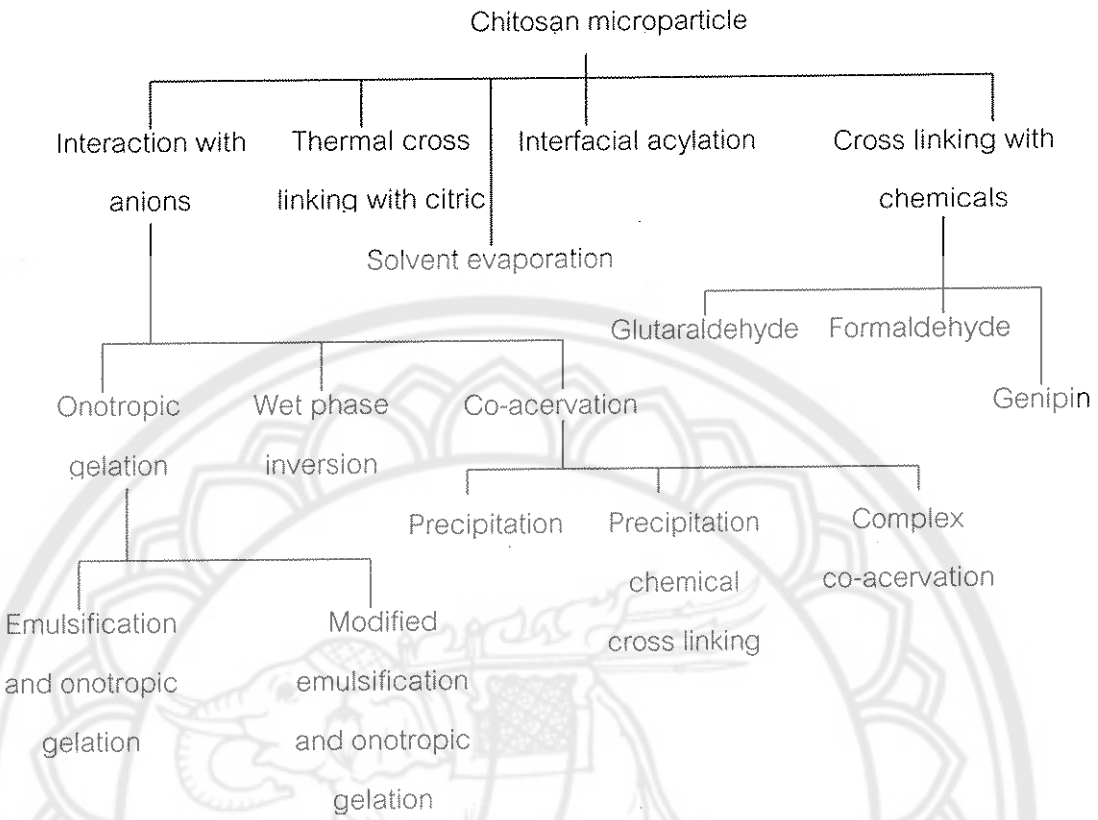


Figure 8 Methods for preparation of chitosan microparticle

Chitosan and polyphenolic compound

Some repots shown the relation between chitosan and polyphenolic compound. The study indicated that those chitosan and polyphenolic compounds were having interaction. The study was aim to encapsulate polyphenolic compound, which extracted from olive leaves, into chitosan microsphere. The microsphere was prepared by spray drying method. The result shown that chitosan was bound with polyphenolic compound by covalent bounding. Those bounding were confirmed by differential scanning calorimetric method. The thermogram in Figure 9 shown the shift in thermal transition that indicated the encapsulation and interaction of polyphenolic compound with chitosan matrix in chitosan microsphere [39]. Furthermore, the bounding of chitosan and polyphenolic compound was observed by FTIR spectroscopy those were shown in Figures 10-11. The result showed that the FTIR of chitosan-loaded polyphenolic compound microspheres shown a small peak at 1700cm^{-1}, which was not presence in

the chitosan-non loaded microspheres. This indicates interaction between the hydroxyl/carboxyl/aldehyde groups of the polypheniloc compound and the amine functionality of the chitosan molecule [43]. Therefore, the bounding between chitosan and polyphenolic compound could increase the encapsulation efficiency of polyphenolic compound in chitosan particle.

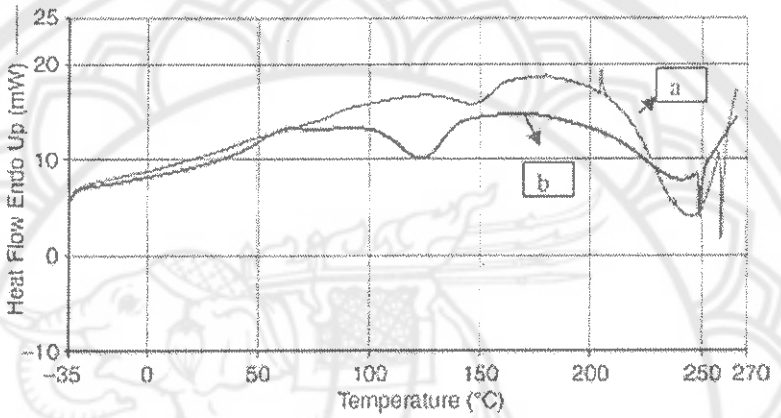


Figure 9 DCS of placebo (a) and polyphenolic-loaded (b) chitosan microsphere prepared by spray-drying technique

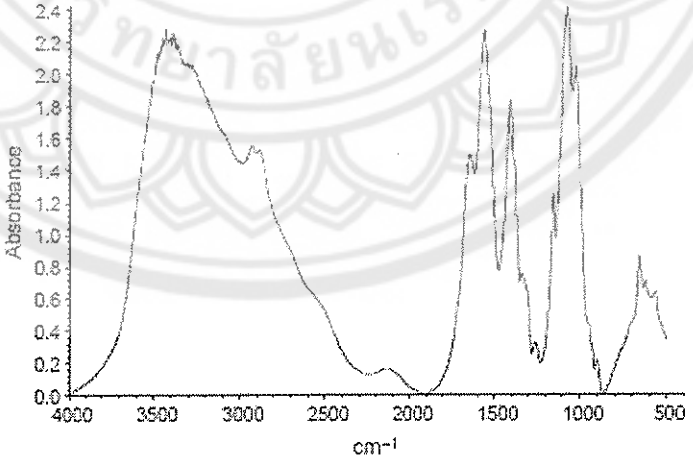


Figure 10 FTIR of placebo chitosan microsphere prepared by spray-drying technique

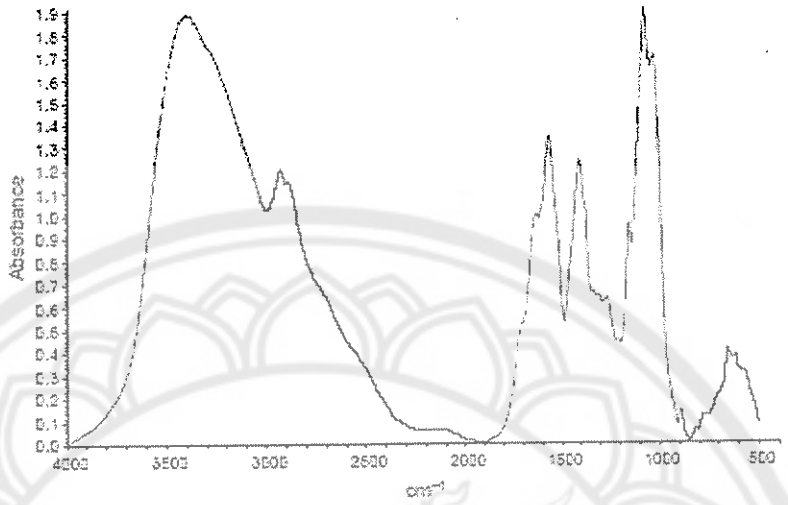


Figure 11 FTIR of polyphenolic-loaded chitosan microsphere prepared by spray-drying technique