ANTI-RANCIDITY EFFECT OF EDIBLE THAI LOCAL PLANT EXTRACTS IN O/W EMULSION SYSTEM AND THEIR BIOLOGICAL ACTIVITIES

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ABSTRACT

Thirty edible Thai local plants in Thailand were evaluated antioxidant activities. Ten plants were selected to evaluate the potential for retarding lipid oxidation in 30 % soybean oil in water emulsion and study their biological activities for use as natural antioxidants. Firstly, crude extracts at 200 and 500 µg/ml concentrations added into o/w emulsion. Lipid oxidation analyses; Peroxide value, Thiobarbituric acid reactive substances, *p*-anisidine, Oil stability index (Rancimat) were used to evaluate. It was found that most plant extracts tend to inhibit lipid oxidation when the concentration of extracts increased. However, *P. odorata* and *E. trifoliatum* extracts still showed as pro-oxidant. *C. formosum* and *G. sphaerogynum* extracts showed the highest effectiveness for retarding lipid oxidation. Therefore, they were chosen to study effects of pHs and storage temperatures on oxidative stability of emulsion. Emulsion added both plant extracts adjusted pH to 3, 5, and 7 and kept at 25, 35, and 45 °C respectively. At pH 3, emulsions were found increases in PV, TBARS, *p*-anisidine over than the control at all storage temperatures. Whereas, emulsion added extracts at pH5 could retard lipid oxidation especially *G. sphaerogynum* extract had the most potential against lipid oxidation. Hexanal of emulsion added both plant extracts at pH 5 were evaluated by GC-MS in the first day and last day of all storage temperatures. The *G. sphaerogynum* extract had the lower hexanal content than the control and *C. formosum* extract.

The reaction rate constant and shelf life evaluation were evaluated. The k value of all samples increased with the temperature. It was noted that, emulsion added *G. sphaerogynum* extract adjusted to pH 5 and stored at 25°C was the best condition for extending shelf life to 280 days. Then, the biological activities of 10 plant extracts including phytochemical contents, antioxidant

activities, cytotoxic activities and inflammatory activities were examined. Each plant extract was prepared at the concentration of 2000 µg/ml. For the phytochemical contents it was found that *G. sphaerogynum*, *C. sphaerica*, *F. auriculata and P. odorata* extracts had the highest in total phenolic content, tannin, saponin and flavonoid respectively. Antioxidant activities, the highest in ABTS assay was found in *G. sphaerogynum* extract, *C. inermis* extract had the most in DPPH and FRAP assay whereas the most potential in TBARS assay were found in *C. sinensis*, *C. inermis* and *F. auriculata* extracts.

Cytotoxic activities, anti-cancer activity were tested with colon cancer (HT-29), liver cancer (HepG2) and oral cancer (KB) cell lines by MTT assay. It was found that *P. odorata*, *S. wallichii*, *C. sinensis* and *V. sprengelii* extracts exhibited high antiproliferative activity. However, four plant extract could destroy the Vero cell line (normal cells). Then, DNA fragmentation by gel electrophoresis was examined. Cells treated with *S. wallichii*, *P. odorata, V. sprengelii* and *C. sinensis* extracts showed a smear pattern when compared with the DNA ladder. The *S. wallichii* and *P. odorata* extracts had the highest potential in DNA fragmentation for HT-29 and HepG2, respectively. In addition, the results showed that, all plant extracts affected on normal cell line (TK6). Two plant extracts were confirmed with TK6 by apoptosis observation. Both extracts induced apoptotic cells in TK6 cell line at 500 and 1000 µg/ml concentrations of extracts. It was also those observed in the control cells. Finally, anti-inflammatory activities of 10 plant extracts were evaluated by using lipoxygenase and hyaluronidase assay. It was found that, anti-inflammatory effects of *C. sphaerica* and *S. wallichii* extracts showed high activity in lipoxygenase inhibition while all samples had lower activity in hyaluronidase inhibition.

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Chapter 1

Introduction

Processed foods containing lipid is called food emulsions including oil-in-water or water-in-oil emulsions. Therefore, lipid oxidation reaction is a main deterioration cause to change the quality of food emulsions such as losses in important nutrients, formation of toxic products (such as aldehydes and ketones), changes in appearance and texture, and development of rancidity that leads to the short product shelf-life (Frankel, 1998). Moreover, food emulsion, particularly oil-in-water emulsion such as milk, salad dressing, mayonnaise, sauces, creams, etc. (McClements and Decker, 2000) can occur oxidation reaction rapidly because of their large surface areas facilitating interactions of the lipids and water- soluble prooxidants.

To retard oxidation reaction, synthetic antioxidant like BHA, BHT, and TBHQ, are widely used to break chain-reaction in food systems (Nazni and Dharmaligam., 2013; Guo *et.al.*, 2006). However, synthetic food additive may cause problems about safety related to their metabolism, possible absorption and accumulation in body organs and tissues (Linderschmidt *et al.*, 1986; Tappel, 1995). Currently, the food industry has focused in replacing the use of synthetic by natural antioxidants. Several researches have reported with the use of natural antioxidants extracts including grape seed, rosemary, blackseeds, green tea to protect emulsions and other foods from oxidation (Gibis and Weiss, 2012; Ramful *et al.*, 2011; Samotyja and Malecka, 2007). *Eugenia pollicina* leaf extract inhibited lipid autooxidation in both soybean oil and olive oil (Ramful *et al.*, 2011). *Rheum rhizome* extract significantly delayed lipid oxidation by thiobarbituric acid reactive substance analysis (Chang and Kim ,2018).

Oxidative stress occurs from reactive oxygen species (ROS), are produced from cigarette smoke, air pollutants, UV radiation, a high, polyunsaturated fatty acid diet, and inflammation, etc. The scientific literatures were shown that oxidative stress played important roles in the development of a wide range of disease (Valadez-Vega et al., 2013). Several plants have been obtained much attention to be sources of bioactive substances containing phenolics, flavonoid, coumarins, stilbenes, hydrolysable and condensed tannins, lignins and lignans (Naczk and Shahidi, 2006). Those substances are showing a wide range of activities for the prevention of diseases like antioxidants, anti-mutagens, anti-carcinogens, skin protection against UV-mediate oxidative damage and also anti-propagation of lipid oxidative chain reaction (Valadez-Vega *et*

al.,2013). In recent years, natural products have used more tendency for antioxidant and antiproliferative properties. Thus, if natural products can inhibit oxidation in emulsion and also show high biological activities for health, it will become a good choice for consumers.

In the North of Thailand, many local plants are growing. Some plants were reported about antioxidant, antirancidity, antimicrobial and used as folk medicine. *Camellia sinensis*, *Careya sphaerica*, *Castarnopsis inermis*, *Cratoxylum formosum*, *Eleutherococcus trifoliatus*, *Ficus auriculata*, *Glochidion sphaerogynum, Schima wallichii* and *Vaccinium sprengelii* reported about antioxidant activities. However, there is no information about activities against lipid oxidation in emulsions and also their biological activities. These plants may be used in the development new antioxidant strategies. This research focused on lipid oxidation in oil in water emulsion because they were found in several foods and easily occurred lipid oxidation. Therefore, this research emphasized on studying the effects of 10 edible Thai local plants extracts for oxidative stability in oil in water (O/W) emulsion, and their biological properties for using as alternative natural antioxidant.

Objectives

1. To study effect of different concentrations of 10 edible Thai local plant extracts on oxidative stability of 30% O/W emulsion system
2. To study effects of pHs and storage temperatures on oxidative stability of 30% O/W emulsion system
3. To evaluate biological activities including antioxidant capacities, anti-cancer activities, anti-inflammatory and phytochemical contents of 10 edible Thai local plant extracts

Chapter 2

Literature Reviews

2.1 Emulsions

An emulsion consists of two liquids phases (usually oil and water), with one distributed in another phase. Emulsions are normally divided into 2 systems. A system that consists of oil droplets dispersed in an aqueous phase is called an "oil-in-water" (or O/W) emulsion. Another system is water droplets dispersed in an oil phase is called a "water-in-oil" (or W/O) emulsion (Figure 2.1). This review focuses on lipid oxidation in O/W emulsion because it is commonly found in several foods such as cream, milk, mayonnaise, sauces, soups, and beverages etc. (McClements, and Decker, 2000). A large contact surface of lipid droplets makes them easily susceptible to lipid oxidation reaction between the lipids and water-soluble contained prooxidants by influencing the location and reactivity of prooxidative transition metals, lipid hydroperoxide, minor lipid components, free radical scavengers and metal chelators (Frankel, 1998).

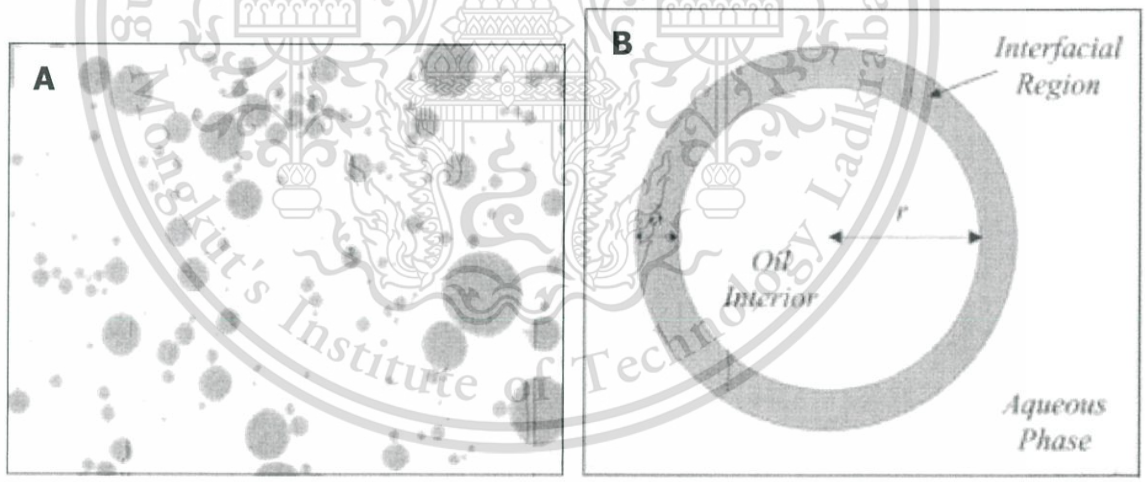

Figure 2.1 O/W in-water emulsion (A) The dark areas are emulsion droplets and the light ares are the aqueous phase (B) o/w emulsion consist of 3 phases; the droplet interior, the interfacial region and the aqueous phase. (McClements and Decker, 2000)

The stability of emulsion is very important for the quality of food and there are two main deteriorative causes: physical and chemical changes (McClements and Decker, 2000). Physical change derives from an alteration in the spetial distribution or structural organization of

the molecules resulting in separation of two phases called creaming, flocculation and coalescence etc. On the other hand, chemical changes are commonly oxidation and hydrolysis leading to unacceptable flavor and nutritional loss. This deteriorative cause is a great concern in food industry.

2.2 Lipid oxidation

Lipid oxidation is a reaction used to explain the chemical changes between lipids and oxygen-active species (Nawar, 1996; Min, 1998; Frankel, 1998). The mechanism of lipid oxidation in food depends on the type of the reactive species presented and their physicochemical environments (Fritsch 1994; Decker 1998a). Lipid oxidation mechanism Lipid oxidation mechanism can be devided into 3 steps: initiation, propagation, and termination (Nawar, 1996; Frankel, 1998; Min, 1998). Many researches report about lipid oxidation in O/W emulsions and liquid colloidal systems mention that it causes from the interaction between lipid hydroperoxides situated at the droplet surface and transition metals within the aqueous phase which is the most causes of deterioration of lipid (Yoshida and Niki, 1992; Mei *et al.*, 1998a, 1998b). Transition metals are able to change unsaturated lipid molecule into alkyl radicals (for example $Fe2^{+}$+ RH $Fe3^{+}$+ R+ H^{+}). However, this chemical reaction occurring is not considered to be important factors in promoting lipid oxidation due to very slow (Reische and others 1998).

When lipid hydroperoxide (ROOH) decomposed, it became highly reactive peroxyl (ROO) and alkoxyl (RO) radicals by transition metals (Eq. 1 and 2) or other prooxidants in emulsion from was the most important mechanism for the acceleration of lipid oxidation. Those radicals attach with unsaturated lipids (LH) within the droplets or at the water-oil interface area, result in the formation of lipid radicals (L˙ and LOO˙) (Eq. 3 to 5). The lipid oxidation chain reaction propagates as these lipid radicals immediately react with other lipids nearby (Eq. 6). Some of the occurance of lipid radicals may be terminated when they reacted with other radicals (Eq. 7) (Mcclements and Decker, 2000).

$Fe^{3+} + ROOH \longrightarrow ROO^{\cdot} + H^{+}$ (1)

$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + RO^{\cdot} + OH^{-}$ (2)

$ROO^{\cdot} + LH \longrightarrow ROOH + L$ (3)

$RO^{\cdot} + LH \longrightarrow ROH + L$ (4)

$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$ (5)

$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$ (6)

$LOO^{\cdot} + LOO^{\cdot} \longrightarrow$ nonradical products (7)

Synthetic antioxidants, such as BHT, BHA, or TBHQ, are commonly used for inhibiting lipid oxidation as chain-breaking antioxidants in food emulsion systems (Decker, 1998b; Reische *et al.*,1998). These synthetic food additives are highly effective in retarding lipid oxidation; however, there are some problems concerning with safety and toxicity on the body.

Incorporate antioxidant in food emulsion is one of the most effective against lipid oxidation reaction (Reische *et al.*, 1998). Antioxidants act by various mechanisms, including control of oxidation substrates in foods such as oxygen and lipids, control of pro-oxidants like the reactive oxygen species and pro-oxidant metals, and stop activity of free radicals (Decker,1998b; Frankel,1999). Incorporate antioxidant in food emulsion is one of the most effective against lipid oxidation reaction (Reische *et al.* 1998). Antioxidants act by various mechanisms, including control of oxidation substrates in foods such as oxygen and lipids, control of pro-oxidants like the reactive oxygen species and pro-oxidant metals, and stop activity of free radicals (Decker, 1998b; Frankel, 1999). For this reason, many researchers had studied the effectiveness of natural antioxidants for inhibiting lipid chain reaction in emulsions. Di Mattia *et al.* (2009) found that phenolics, gallic acid, catechin and quercetin showed their protective effect of lipid oxidation in emulsions. Plant extracts such as sage, thyme, lemon and catnip extracts had effected on the oxidative stability of sunflower oil and emulsion (Abdalla and Roozen, 1999). Ramful *et al.* (2011) reported *Eugenia pollicina* leaf extract demonstrated a potential to maintain oxidative stability of emulsion system. The hydrophilic antioxidant of *Melissa officinalis* extract was very efficient in o/w emulsion (Poyato *et al.*, 2013).

2.3 Lipid oxidation analysis

Many methods are used for measuring lipid oxidation in foods. However, there is no pattern or standard method for detecting all oxidative changes in all food systems (Shahidi and Wanasundara, 2002). Therefore, it is necessary to choose the appropriate methods for application. The monitoring lipid oxidation in foods can be classified into five groups based on what they measure: the absorption of oxygen, the loss of initial substrates, the formation of free radicals, and the formation of primary and secondary oxidation products (the primary oxidation products are hydroperoxides and secondary oxidation products are aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids, and epoxy compounds) (Dobarganes and Velasco, 2002). These include titration for peroxide value, spectrometry for conjugated dienes and trienes, TBARS value, anisidine value (*p*-AV), and carbonyl value, Rancimat and Oxidative Stability Instrument (OSI) method for oil stability index and headspace oxygen uptake method for oxygen absorption, chromatographic analysis for changing of reactant.

2.3.1 Peroxide values

Lipid oxidation involves the continuous formation of hydroperoxides as primary oxidation products that may change to several of volatile and nonvolatile secondary products (Dobarganes and Velasco, 2002). The formation rate of hydroperoxides is more than their rate of decomposition during the initial stage of lipid oxidation, and this becomes reversed at later stages. Hence, the peroxide value (PV) is an index of the initial stages of lipid oxidation (Riuz *et al.*, 2001). However, one can evaluate whether a lipid is in the increase or decrease portion of the hydroperoxide concentration by monitoring the amount of hydroperoxides as a function of time (Shahidi and Wanasundara, 2002). Chromatographic techniques can be described the total amount of hydroperoxide and the amount of specific hydroperoxides contain in oil and fat samples (Dobarganes and Velasco, 2002). The PV indicates the total hydroperoxide content. It is the most common quality indicators of fats and oils during production and storage (Antolovich *et al.*, 2002). Several methods have been developed for determination of PV, among which the iodometric titration, ferric ion complex measurement spectrophotometry, and infrared spectroscopy are most frequently used (Yildiz *et al.*, 2003).

2.3.2 Thiobarbituric Acid Reactive Substances (TBARS)

Malonaldehyde (MDA) is a secondary oxidation product which occurs during lipid oxidation. It is a minor component of fatty acids with 3 or more double bonds, is occured from the degradation of polyunsaturated fatty acids. It is usually used as an indicator of the lipid oxidation process. In this assay, the one molecule of MDA is reacted with 2 molecules of thiobarbituric acid (TBA) reagent under heated acidic condition to form a pink MDA-TBA complex that is measured by spectrophotometer at 530–535 nm. The TBARS assay is a well-recognized, established method for quantifying these lipid peroxides, although it has been limited for its reactivity on other compounds more than MDA (Yaki,1998).

2.3.3 Anisidine Value (*p*-AV)

The anisidine value (*p*-AV) method determines the content of aldehydes (2-alkenals and 2,4-alkadienals), is formed during the decomposition of hydroperoxides. It is based on the color changing of reaction *p*-methoxyaniline (anisidine) and the aldehydic compounds (Doleschall *et al.*,2002). The reaction of 1 g of fat sample in isooctane solution (100 ml) with *p*-anisidine (0.25% in glacial acetic acid) under acidic condition gives the yellowish products that absorb at 350 nm. It is defined as absorbance of a solution. The color is quantified and converted to *p*-AV.

This method is more sensitive to unsaturated aldehydes than to saturated aldehydes because the colored products from unsaturated aldehydes are better absorbed at this wavelength (Pokony *et al.*, 2001). However, it correlates well with the amount of total volatile substances (Doleschall *et al.*, 2002). A good correlation between *p*-AV and flavor scores and PV has been found. In addition, some reviews have mentioned that *p*-AV has been comparable only within the same oil type because initial AV varies according to oil sources (Guillen and Cabo.,2002). For instance, oils with high levels of polyunsaturated fatty acids might have higher AV even when they are fresh (White *et al.*,1995).

2.3.4 Oil stability index (OSI)

Volatile organic acids, mainly acetic acid and formic acid are generated as secondary volatile oxidation products during lipid oxidation at high temperatures, simultaneously with hydroperoxides (Schwarz *et al.*,2001). In addition, other secondary products, including alcohols and carbonyl compounds, can be changed to carboxylic acids (Kiritsakis *et al.*,2002). The oil

stability index (OSI) method monitored formation of volatile acids by changing in electrical conductivity when oxidizing oils passed through water (Gordon *et al.*,2001). The OSI value is defined as the point of maximal change of the rate of oxidation, indicated to the increase of conductivity. In the Rancimat assay, a flow of air is bubbled through a heated oil (usually 100 °C or above). For marine oils, temperatures about 80 °C are often used. During accelerated oxidation, volatile compounds are formed in distilled water resulting in increasing of the water activity. The change of conductivity is plotted automatically and the induction period of the oil or the time is taken to reach a level of conductivity is recorded (Kiritsakis *et al.*,2002). The Rancimat assay is commonly used continuous monitoring of the oxidation process.

2.3.5 GC-MS

Headspace analysis by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) has been widely used to identify and quantify volatiles formed by the oxidative deterioration of oil or emulsions. It is very suitable for direct determination of individual compounds that affects on rancid flavors (e.g., low molecular weight aldehydes) at very low levels (Robards *et al.*, 1998). The volatile compounds from oil samples were extracted and concentrated by SPME and analyzed by a GC/MS system. After the equilibration time, volatie compounds completely absorbed at the fibre. It was placed in the injection port of the GC system where it was desorbed in splittless mode. Then, the desorbed compounds were separated on chromatograph with mass spectrometer detector. The results were calculated and compared with standard curve (Battimo *et al.*,2007).

Many researches have been studied about the potential of edible Thai local plant extracts to inhibit lipid oxidation in bulk oil and water in oil (w/o) emulsion. Areekul and Phomkivon (2011) studied the effect of 10 Thai local plant extracts in soybean w/o emulsion. It was found that at higher concentration of extracts showed the higher anti-oxidation. *Schima wallichii* extract showed the highest anti-rancidity in both 30 and 70% w/o emulsion. Kaewdana (2013) studied the potential of 15 Thai local edible plant extracts to inhibit oxidation in soybean oil and palm oil by conjugated diene (CD), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS). In soybean oil, the extracts at 3 different concentrations (75, 200, and 500 ppm), *Castanopsis inermis*, *Glochidion sphaerogynum* and *Mosia dianthera* extracts with 500 ppm showed their potential for anti-rancidity similar to BHT (200 ppm). Whereas, *Micromelum*

minutum extract was exhibited anti-rancidity only in CD and PV. In palm oil, it was examined at extract concentration (200, 300 and 500 ppm). The results revealed that 3 extracts of *Gymnema inodorum*, *M. minutum* and *Basella Alba* at the same concentration of 500 ppm and the extract of *G. inodorum* at 350 ppm had higher their anti-rancidity potential than BHT (200 ppm). As a result, it was suggested that different plant extracts showed different oxidative stability. In some cases, the same plants species could be different effectiveness in different systems. In addition, it still depends on concentrations of extracts and methods tested.

2.4 Factors affecting lipid oxidation in emulsion

2.4.1 Emulsion droplet interfacial area

Emulsion droplets in foods vary from 0.2 mm to 100 mm . The surface area of oil deoplets contact with the aqueous phase varies greatly (McClements and Decker, 2000) . However, most authors reported that only emulsion droplet size surface has a little impact on oxidation rates (Sun and Gunasekaran, 2009). Actully, lipid oxidation rates do not only change greatly in oil-in-water emulsions with huge variations in surface area. It was suggested that the large surface area of oil droplets in emulsion emulsions never limited the rates of lipid oxidation reaction.

2.4.2 Emulsion droplet interface

All emulsion droplets have an oil-water interfacial layer which is the borders between lipid and aqueous phases. The chemical composition of this layer is complex because it contains emulsifiers and other surface active materials such as antioxidants and minor lipid components (e.g., sterols and triacylglycerol hydrolysis products) as well as mineral ions (McClements and Decker, 2007). The thickness of the interfacial layer is usually in the range of 1-40 nm. The thickness of layer can be changed; it depends on the type of surface active materials in the emulsion. Since lipid oxidation rates are often influenced by the interactions of aqueous phase prooxidants (i.e. metals) with lipid phase oxidizable substrates (i.e. hydroperoxides), the physical properties of the emulsion droplet interface strongly influence oxidation kinetics.

2.4.3 Emulsion droplet charge

Another important factor on oxidative stability in oil in water emulsions is ability of the droplet interact to attract or push cationic metals (Boon *et al.*, 2008; Haahr and Jacobsen, 2008). The type of emulsifiers (e.g., cationic, anionic or neutral) in order to stabilize the emulsion, charge emulsion component that absorbed on the emulsion droplet surface (Shaw *et al*, 2007) or emulsion pH that impacts emulsifier charge (Djordjevic *et al*, 2004) influence on oxidative stability of O/W emulsions.

Many studies have been reported about role of droplet charge of lipid oxidation in protein stabilized emulsions. Donnelly *et al.* (1998) studied the rate of lipid oxidation in oil -in water emulsion stabilized by either Tween 20 or whey protein isolate. Emulsions were adjuised at pH 7 and pH 3, the isoelectric point of the protein (IEP pH 5). In the emulsions stabilized with the nonionic surfactant, at pH 3 showed the faster lipid oxidation rates than those at pH 7. As the electrical charge of the droplets stabilized by nonionic surfactants did not change obviously with pH, the observed difference in oxidation rates occurred from iron is more water-soluble at the lower pH.

Several researchers found that emulsions stabilized by anionic surfactants oxidize very quickly due to the electrostatic attraction of cationic transition metals while cationic surfactants decrease lipid oxidation rates by electrostatically repelling metals from the lipids. In this case, the rate of lipid oxidation was fastest when the pH is higher than the pI of the protein and the emulsion droplet is negatively charged. However, the density of the droplet charge does not always relate with oxidative stability. It depends on different types of protein to use stabilize emulsion because some proteins have beneficial properties that impact oxidation. They can scavenge free radicals and their ability to increase thick emulsion droplet interfaces that could against water phase prooxidants from interacting with lipids in the emulsion droplet core (Hu *et a*l., 2002).

2.4.4 Emulsion droplet interfacial thickness

Emulsion droplet interfacial thickness depends on the size and uniform of the emulsifier head and tail group or by conditions where biopolymers can form layers around emulsion droplets (Shaw *et al.*, 2007). The large size of emulsifiers acts as a barrier that decreases interactions between lipids and aqueous phase prooxidants (Silvestre *et al.*, 2000). This

was observed in studied of Chaiyasit *et al.* (2000), Brij 76 or Brij 700 emulsifiers were used for emulsions stabilized in salmon oil-in-water. The greater area of hydrophilic head group of Brij 700 (10 times larger Brij 76) showed slower lipid oxidation rates.

2.4.5 Emulsion droplet interface permeability

Emulsifier-packing density of O/W interface affects from physical and chemical properties of surfectants may influence on diffusion of oxygen, free radicals, and prooxidants at the interfacial layer (Villiere *et al.*, 2005). Some proteins layers do not have the ability to inhibit lipid oxidation suggests that these layers are still highly porous allowing prooxidants to diffuse through the emulsion droplet interface where they can react with lipids.

2.4.6 Antioxidants

Many factors such as reactivity, antioxidant concentration, patitioning between oil-water and interfacial phases, interactions with other food components, involved environmental conditions like pH, ionic strength and temperature influence on the ability of antioxidant to against lipid oxidation in food emulsions (Frankel, 1998).

In the hypothesis, non-polar antioxidants are more effective in oil-in-water emulsions than polar antioxidants because they are preserved in the emulsion droplet where oxidation is most prevalent. The hypothesis is compatible with Frankel *et al.* (1994), in emulsion systems containing stripped oil where mainly non-polar antioxidants such as a-tocophorol, ascorbyl palmitate, carnosol etc. are more effective antioxidants than their polar complements (Trolox, ascorbic acid, carnosic acid and rosmarinic acid). Researchers have also suggested that non-polar antioxidants are more potential in O/W emulsions when they accumulate at the oil-water interface which has a lot of oxidation. Antioxidants such as -tocopherol, a-tocopherol, TBHQ and propyl gallate are good surface active due to they can gather at oil-water interfaces (Chaiyasit *et al.*, 2007).

Antioxidants generally work by primary antioxidants or secondary antioxidants. A primary antioxidant known as a "chain-breaking" antioxidant is a substance which delays the initiation step or interrupts the propagation step of autoxidation by accepting free radicals (Reische *et al.*, 1998). Chain-breaking antioxidants respond with lipid and peroxyl radicals and change them to more stable, radical, or nonradical products.

Secondary antioxidants can delay lipid oxidation with a various mechanisms, including chelation of transition metals, reinforce of hydrogen to primary antioxidants, oxygen scavenging, and inactivation of reactive species (Reische *et al.*, 1998). Chelating transition metals in the aqueous phase of O/W emulsion is the most important role of each secondary antioxidant. The major factor to promote lipid oxidation is the presence of transition metals in emulsion. Especially, they are located close to droplet surfaces, because they are then in closer vicinity to the lipid substrate.

2.5 Shelf life evaluation

Shelf life is important on the quality and characteristic of foods, including raw materials, ingredients, and processed foods. It is described as the time during the food product and it will remain safe (chemical, physical and microbiological), retain desired sensory, according to any label declaration of nutritional data and acceptable to the consumer (Earle and Earle, 2008).

Sevaral factors affect on shelf life of foods. It can be classified to internal (food compositions) and external (environment) factors. Compositional factors are the properties of the final product including; water activity (a_w), moisture content, pH value, total acidity, type of acid, redox potential (Eh), available oxygen, nutrients, natural microflora or pathogenic counts, enzymes, chemical reactants etc. use of prolong in products (e.g., salt, preservative), and concentration of reactant, inhibitor, and catalyst. For environmental factors, they are factors related with food chain (processing, storage, distribution, transpotation) including time–temperature profile, relative humidity (RH), pressure, exposure to light (UV and IR) microbial counts during processing, storage and distribution, involved atmospheric composition within packaging, subsequent heat treatment (e.g., reheating or cooking before consumption) and distributor, retailer, and consumer handling (Phimolsiripol and Suppakul, 2016).

2.5.1 Kinetic Reactions

The simplest technique for shelf life testing is the kinetic reaction approach. To predict the shelf life, the kinetic data is used to evaluate how the deterioration process behaves as a function of time. It is the concept of quantification of the quality of food products based on reaction change (Corradini and Peleg, 2007). The kinetic equation may be expressed as:

$$\frac{d[C]}{dt} = K\,[C]^n \qquad (1.1)$$

k is the reaction rated constant, t is time, and n is order of reaction, C is the change in concentration of a component is determined. The quality factors [C] are usually quantifiable chemical, physical, microbiological, or sensory characteristics, such as the loss of a nutrient or quality of flavor or formation of an off- flavor.

There are three forms of the quality function in foods including zero, first, and second order reactions are revealed in Table 2.1 and Figure 2.2, which show the different profiles of the reaction orders. For zero-order reactions, the reaction rate does not depend on the concentration of a reactant. First-order reactions depend on a single reactant, and the exponent value is one. For second-order reactions, the reaction rate may be proportional to one concentration squared or to the product of two concentrations (Labuza and Riboh,1982).

Table 2.1 Quality function of reaction order

Reaction order	Quality function
0	$C_A - C_{AO}$
1	$\ln (C_A / C_{AO})$
2	$C^{-1}_A - C^{-1}_{AO}$

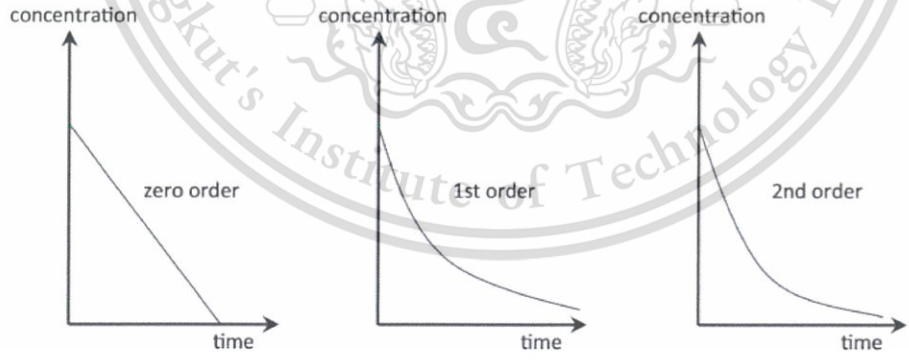

Figure 2.2 Profile of concentration changes in different reaction orders (Phimolsiripol and Suppakul, 2016)

Characteristic physicochemical, chemical, or microbial index cause from most reaction in foods resulted in shelf life loss. Difference of foods show the difference in reaction includes the following: zero order (e.g., frozen food overall quality, maillard browning) and first order (e.g., vitamin loss, oxidative color loss, microbial growth). Several researches have been applied to the kinetic model to explain the temperature dependence. The quality parameters can be chemical properties such as peroxide value in extra virgin olive oil (Calligaris *et al.*, 2006), vitamin C loss in citrus juice concentrate (Burdurlu *et al.*, 2006), physical property such as color loss in fresh-cut asparagus (Sothornvit and Kiatchanapaibul, 2009), weight loss in frozen bread dough (Phimolsiripol *et al.*, 2011).

2.5.2 Accelerated Shelf Life Simulation

The evaluation of actual shelf life is too long. Therefore, food industries need a short time for determining the shelf life of their products. Accelerated test techniques are chosen extensively for experiment. Any methods are able to evaluate product stability with obtained the data significantly shorter period than actual shelf life will use for accelerated shelf life simulation. (Steele, 2004).

The short time required to evaluate a shelf life. Therefore, the concepts of accelerated shelf life simulation include: (1) The hypothesis is that by storing food at a higher temperature, any effects that occur with the storage behavior and hence the shelf life may be noticeable in a shorter time. (2) The shelf life under normal storage conditions can be estimated by prediction using the data obtained from the accelerated determination.

When the accelerated shelf life testing is started, the compositional factors must be kept constant (Hough, 2010). For the most important, the microbial safety and quality parameters must be considered and determined as the first priority for shelf life of product. Then, key deteriorative reactions such as quality loss and consumer unacceptability are selected. In case of suitable packaging, the cost-effective packages are used except the concerned effect of packaging materials.

Next step, products should determine throughout experiment. The time and frequency of checking is fixed in each test temperature. Then, the samples will be calculated at each temperature Final step is that a kinetic study of the deterioration process at such levels of the accelerating factors is analyzed. However, frequency of sampling can be increased or decreased

when the rate of deterioration is too fast or too slow. The shelf life prediction can be calculated by constructing the shelf life plots to determine the shelf life under normal storage conditions using the extrapolating data or the kinetic model to predict shelf life at actual storage conditions.

2.5.3 Arrhenius Model

The Arrhenius model is a model that relates with the rate of reaction and temperature changing. This model is widely used in several food processing and storage tests as an influenced by temperature (Corradini and Peleg, 2007; Phimolsiripol *et al.*, 2008, 2011). The model is demonstrated by:

$$k = k_o \exp\left[\frac{E_a}{RT}\right] \quad (1.2)$$

where k_0 is the reaction rate constant, Ea is the energy of activation, R is the gas constant (1.9872 cal mol^{-1} K^{-1} or 8.3144 J mol^{-1} K^{-1}), and T is the absolute temperature (K)

The followings are five steps of the Arrhenius model for shelf life prediction; (1) Find the reaction order following the kinetic reaction concept in the section Kinetic Reactions. (2) Follow the Arrhenius relationship. (3) Plot the Arrhenius relationship. (4) Fit the curve using linear regression. (5) Slope of plot between ln k versus1/T is Ea/R.

2.6 Phytochemical of plants

Phytochemicals are non-nutritive chemical components containing in plants ('phyto' is the Greek word meaned plant) that exhibit protective or disease-preventing effects. They play important roles for preventing from treatment of chronic diseases such as heart disease, cancer, hypertension, diabetes and other medical conditions (Surh, 2003). Furtheremore, they have revealed to exhibit chemoprevention and chemotherapeutic effects in cell lines and in animal models of cancer and also some of them are in the clinical trial phase I and II (Bathaie *et al.*, 2015).

Phytochemicals produce colors and flavors to both pleasant and unpleasant plants when they are consumed. They indicate specific plants and parts of plants, and they usually increase in abundance during stressful events. Phytochemicals are beneficial for health when they are consumed.

2.6.1 Polyphenol

Polyphenol is a large group of phytochemicals found in plants (Figure 2.3).

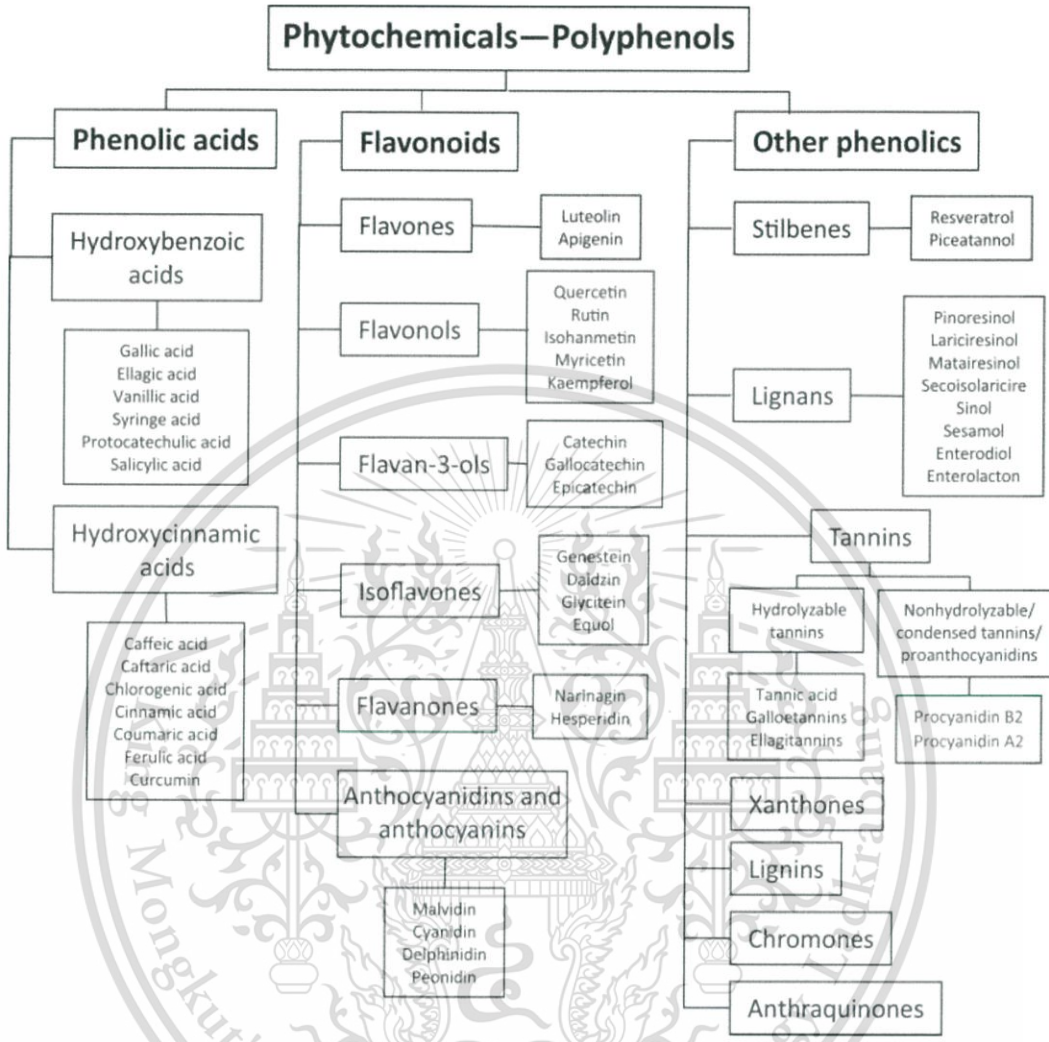

Figure 2.3 Types of polyphenols (Bohn, 2014)

Polyhydroxylated phytochemicals is a common of polyphenols structure. They can be divided in three main categories; flavonoids, phenolic acids, and stilbenoids. Phenolic compound is one of the most widespread of chemicals in the plants. It has more than 8000 compounds were isolated and described (Ramos, 2007).

Phenolic compounds are the major phytochemicals and have a role to play as the antioxidant activity of plant materials (Javanmardi *et al.*, 2003; Pizzale *et al.*, 2002). Antioxidants act in defending the body against free radical damages. They are able to retard or inhibit the lipid oxidation or other biomolecules. Therefore, they can protect or repair the damage cells caused from oxygen (Shahidi and Naczk, 2004). For instance, phenolic

compounds such as, quercetin and ellagic acid, are great antioxidants that are able to protect the body cells caused by reactive oxygen and nitrogen species (Sroka and Cisowski, 2003).

2.6.2 Flavonoids

Flavonoids are the largest group of phytonutrients, with more than 6,000 types, found in almost all fruits and vegetables. For carotenoids, they are responsible for the fresh colors in fruits and vegetables. The best-known flavonoids are quercetin and kaempferol are best- known (Szalay, 2015). The structure of flavonoids occurs as aglycones, glycosides and methylated derivatives. In plants, flavonoids aglycones (i.e., flavonoids without attached sugar) occur in a variety of structural forms. All contain fifteen carbon atoms in their basic nucleus: two six-membered rings linked with a three carbon unit which may or may not be a part of a third ring.

Flavonoids are the major active nutraceutical compounds in plants as the typical for phenolic compounds; they can act as the best potent antioxidants and metal chelators. They also have long been recognized to possess anti- inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic activities and cardiovascular diseases. The flavones and catechins have the most powerful flavonoids for protecting the body against reactive oxygen species (ROS). Free radicals and ROS have been associated in a large number of human diseases. In addition, quercetin, kaempferol, morin, myricetin and rutin, act as antioxidants, exhibited beneficial effects such as anti-inflammatory, antiallergic, antiviral, as well as anticancer activity. The scavenging activity of flavonoids has been reported to be in the order: Myrcetin > quercetin > rhamnetin > morin > diosmetin > naringenin > apigenin > catechin > 5,7-dihydroxy-3',4',5'-trimethoxy- flavone > robinin > kaempferol > flavone (Tapas *et al.*,2008).

2.6.3 Saponins

Saponins is one of phytochemicals comprise of an aglycone unit connected to one or more carbohydrate chains. The aglycone or sapogenin unit consists of either a sterol or the more common triterpene unit. In both the steroid and triterpenoid saponins, the carbohydrate side-chain is usually attached to the 3 carbon of the sapogenin. The foam is a remarkable property of saponins, it can dissolve in water. In aqueous solution, saponin molecules arrange themselves vertically on the surface with their hydrophobic ends oriented away from the water. As a result, it

can reduce the surface tension of the water, causing it to foam. For this reason, saponins are classified as surface active agents. Saponins have been found in many edible legumes (lupins, lentils, and chickpeas, as well as soy, various beans, and peas) (Savage, 2003).

Saponins are strongly bitter tasting in plants. They can form complexes with proteins and lipids (e.g. cholesterol) and possess a hemolytic effect. Saponins are only absorbed in small amounts, and their main effect is limited to the intestinal tract. Saponins can form complexes with zinc and iron, thus limiting their bioavailability (Chauhan *et al.,* 1992). Moreover, saponins show health-promoting effects like anti-carcinogenic, anti-microbial, cholesterol decreasing, immune modulating, as well as anti-inflammatory.

2.6.4 Tannin

Tannins are phenolic-based natural products. Tannins are a heterogeneous group of water-soluble polyphenolic compounds of high molecular weight (500–3000 Daltons) with as many as 20 hydroxyl groups and are presented in plants, foods and beverages (de Jesus *et al.*, 2012) and are seen mostly in the bark or wood of plants. They are also responsible for the astringent taste in many fruits and vegetables. Tannins are divided into two groups: hydrolysable tannins and condensed tannins (also named catechin tannins or proanthocyanidins). Hydrolysable tannins can be categorized into gallotannins, which provide sugar and gallic acid on hydrolysis, and ellagitannins, which on hydrolysis not only just sugar and gallic acid but also ellagic acid (Lamy *et al.*, 2016). These compounds are hydrolyzed by weak acids and decomposed by high temperatures to yield pyrogallol, a hepatotoxic and highly irritant compound (Jimenez *et al.*, 2014).

Condensed tannins (also known as proanthocyanidins) are flavonoid units linked by carbon–carbon bonds that are difficult to cleavage by hydrolysis. These compounds are responsible for many colors like intense pink, red, purple, or blue colors of many flowers, fruits, and leaves. Flavonoids, mainly anthocyanins, are responsible for the bright autumn colors in many kinds of plants. Proanthocyanidins are one of the most compounds present in food that consumed regulary. They are commonly found in many parts of plants; fruits, leaves, roots, seeds, wood and bark. More than 1000 derivatives have been identified to date, and these compounds continue to be of interest to researchers due to the wide range of applications and the numerous biological properties. Tannins exhibit several pharmacological effects, including antioxidant and

free radical scavenging activity as well as antimicrobial, anti-cancer, anti-nutritional and cardio-protective properties. They also have useful effects on metabolic disorders and prevent the onset of several oxidative stress related diseases (Smeriglio *et al.*, 2017).

2.7 Biological activities assay

2.7.1 Antioxidant activities

1) Total phenolic content (TPC)

Total phenolic content assay is based on Folin-Ciocalteu method. The FC reagent contains phosphomolybdic/ phosphotungstic acid complexes (Singleton *et al.*, 1995). The principle of assay, relies on transfer of electrons from phenolic compounds in alkaline medium to form a blue chromophore constituted by a phosphotungstic/ phosphomolybdenum complex. The maximum absorption depends on the concentration of phenolic compounds. The reduced Folin-Ciocalteu reagent is detected by a spectrophotometer by spectrophotometer in the range of 690 to 710 nm. The color intensity indicates the high content of phenolic compounds. The reaction temperature has been used to reduce the time necessary to attain the maximum color (T= 37°C). Generally, gallic acid is used as the reference standard compound (Magalhaes *et al.*, 2010) and results are expressed as gallic acid equivalents (mg/ml).

2) The DPPH method

DPPH• (2,2-diphenyl-1- picrylhydrazyl) is a stable free radical, owing to the delocalization of the spare electron on the whole molecule. Hence, DPPH• does not dimerize in the same way as most free radicals. The delocalisation on the DPPH• molecule determines from the occurence of a purple colour with a 520 nm absorption by spectrophotometer. When DPPH• reacts with a hydrogen donor, the reduced (molecular) form (DPPH) is generated, accompanied by the disappearance or reduced of the violet color. Therefore, the reduction of absorbance depends linearly on the antioxidant concentration. Trolox is used as a standard antioxidant (Thaipong *et al.*,2006; Pisoschi *et al.*, 2009). Another way, it can be calculated as the percentage of DPPH inhibition.

3) The TEAC method

The ABTS cation radical ($ABTS^{\bullet+}$) (Marc *et al.*,2004) had studied many analytical methods for antioxidant potential evaluation in foods which absorbs around 743 nm (giving a bluish-green colour). It is formed by the loss of an electron by the nitrogen atom of ABTS (2,2'-azino-bis(3- ethylbenzthiazoline-6-sulphonic acid)). In the presence of Trolox (or of another hydrogen donating antioxidant), the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization. ABTS can be oxidized by potassium persulphate (Thaipong *et al.*,2006) or manganese dioxide (Su *et al.*,2007), giving rise to the ABTS cation radical (ABTS•+) whose absorbance reduction at 743 nm was monitored. Trolox (Pellegrini *et al.*,2003) widely chosen as standard antioxidant. The effective antioxidants in many plants and fruits are based on the absorbance reduction of ABTS cation radical.

TEAC and DPPH assay are the most popular antioxidant methods due to their excellent reproducibility under the same conditions, but they also exhibit significantly differences in their response to antioxidant. The DPPH assay easy to prepare and does not require special preparation, while the ABTS radical cation must be produced by chemical reactions or enzymes (Arnao, 2000). Furthermore, another important difference between ABTS and DPPH are that ABTS can be dissolved in both of aqueous and organic media, almost antioxidant activity can be measured, due to hydrophilic and lipophilic nature of the compound contained in plant samples. On the other hand, DPPH can only be dissolved in organic solvent, especially in ethanol. It is an important limitation for measuring of hydrophilic compounds.

4) The FRAP method

The FRAP (ferric reducing antioxidant power) is based on the reduction of a ferric-ion by the antioxidants, of the complex ferric ion-TPTZ (2,4,6-tri(2-pyridyl)- 1,3,5-triazine). The binding of Fe^{2+} to the ligand creates a very intense of blue color by electron-donating antioxidants present within the sample in acidic medium. The absorbance can be determined from the amount of iron reduced and can be compared with the amount of antioxidants (Pellegrini *et al.*, 2003). Trolox or ascorbic acid is commonly used as standard references.

5) The TBARS method

TBARS is a standard marker for the lipid peroxidation induced oxidative stress in several oxidation products (Tsai and Huang., 2015). The method is based on the reaction of malondialdehyde (MDA) and TBA reagent in the glacial acetic acid medium. The method is precise, sensitive, and highly reproducible for quantitative determination of TBARS (Zeb and Ullah., 2016). The assay uses a Fenton-like system (Co(II) + H_2O_2), to induce lipid (e.g. fatty acid) peroxidation (Denev *et al.*,2010; Slavikova *et al.*,1998). α-linolenic acid is commonly used as a model substrate. It is mixed with the samples, as well as with the Fenton-like mixture, to produce lipid peroxidation. After the end of the incubation, the concentration of thiobarbituric acid reactive substances (TBARS) is measured as the marker of lipid peroxidation. Lipid peroxidation is expressed in nmoles of TBARS per 1 ml of mixture α-linolenic acid/analysed sample.

Several studies have been evaluated the antioxidant activities of a variety of plants. Wojdylo *et al.* (2007) studied antioxidant activities of 32 herbs by ABTS, DPPH and FRAP assay and total phenolic content that were measured using a Folin-Ciocalteu assay. It was found that different plants exhibited different antioxidant activities because different compounds contained in plants and also different methods testing. Singleton and Rossi (1965) mentioned that various phenolic compounds showed differently in each assay, depending on their number of phenolic groups. Some plants, which showed the highest scavenging of DPPH and ABTS did not show ferric reducing antioxidant power. In addition, some plants, especially the same family, showed similar a potent antioxidant activity.

Antioxidant activities have been evaluated in many methods. The limitation of many newer methods is the lack of substrate in the procedure. The combination of many methods can be explained the several of mechanisms in which results of antioxidant testing. The antioxidant activities, especially of antioxidants that are mixtures, multifunctional or are activities in complex multiphase systems, cannot be evaluated by only simple antioxidant test without other related variables that influence the results. Therefore, using the several test methods may be used to evaluate antioxidant activities for excellent results (Antolovich *et al.*, 2002).

2.7.2 Cytotoxic activity

Cytotoxicity and proliferation assays with cultured cells are popularly used for drug and chemical screening to detect whether the test samples have effects on cell proliferation or exhibit direct cytotoxic effects. Nowadays many researches focus on finding plant extracts for treatment. These assays are also used to evaluate toxicity and tumor cell growth inhibition of compounds during drug development because they are rapid, inexpensive and not necessary using animals. Furthermore, they are useful for testing numerous numbers of samples.

1) MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay is one of the method based on colorimeteric assay to evaluate cytotoxicity or cell proliferation (Mosmann,1983). This assay determines cell viability through examination of mitochondria of cells by measuring activity of mitochondrial enzymes like succinate dehydrogenase. MTT is reduced to a purple formazan form by NADH. This product can be quantified by absorbance at a specific wavelength.

This method is easy to use, safe, and having a high reproducibility, and is widely used to determine both cell viability and cytotoxicity tests. Meanwhile, MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Therefore, an organic solvent like imethyl sulfoxide (DMSO) or isopropanol is needed to solubilize the crystal before measuring the absorbance by spectrophotometer. Additionally, the limitation of assay is difficult to remove MTT formazan from cell culture media in the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error (Stone *et al.*,2009).

2) DNA fragmentation

One of the key hallmark of apoptosis is the cleavage of the genomic DNA into oligonucleosomal fragments represented by multiples size (180-200 bp) (Kasibhatla *et al.*,2006). Apoptotic signal pathways, which eventually cause DNA fragmentation, are largely mediated by the family of protease caspases. Caspases are a key mediator of DNA fragmentation. Caspases mediate apoptotic signal transduction by cleavage of apoptosis-implicated proteins and the caspases themselves (Kitazumi and Tsukahar, 2011). Cheung *et al.* (2003) reported that an early marker of cell death is the laddering of DNA during apoptosis following chromatin condensation, the DNA is degraded by a nuclease known as CAD (Caspase activated DNase). This was

identified as an active constituent of apoptotic or caspase-3-treated cell lysates (Enari *et al.*, 1998).

The agarose gel electrophoresis method is generally used for analysis DNA fragment separation and intact DNA fractions. In apoptotic cells specific DNA cleavage becomes evident as a typical ladder pattern due to multiple DNA fragments. Although this method is simple and generally able to provide good results, it is only qualitative because of its limitations in DNA recovery and solubilization. In order to obtain a cleaner DNA, other methods for DNA preparation are required (in some cases use of proteinase K for deproteinization is recommended) (Bossu,1999)

3) Apoptosis observation

Apoptosis, known as programmed cell death, is the process of eliminating unwanted or damage cells, and harmful cells in a clean and orderly manner during embryonic development, tissue homeostasis and immune regulation, as a primary mechanism for inhibition of cell proliferation. It is also preferred way for removing cancer cells. The process includes blebbing, cell shrinkage, and nuclear fragmentation. During the apoptotic cell death programme, a group of cysteine-dependent aspartate-specific protease, designated caspases is activated. When a cell for one or another reason cannot die by apoptosis, it may respond by another type of cellular death called necrosis. This cell death may occur after diverse injuring including hyperthermia, ischemia, physical injury or viral infection or appear as a result of exposure to cytotoxic cytokines or substances. (Los and Gibson, 2005)

Another program cell death is necrosis. The morphological change such as swelling of the cell and its organelles including most often the mitochondria are important characteristics of necrosis. The swelling is thought to occur due to the absence of the regulation of internal pressure and results from the loss of membrane integrity. The process most often terminates with rapture of cell and spilling of the cellular contents to the neighboring tissue and is usually associated with a strong inflammatory process. Dual staining with fluorescent. Annexin V and Propidium iodide (PI) has been used to identify apoptotic and necrotic cell death, in which Annexin V-positive (green color)/PI-negative staining is regarded as apoptosis and PI-positive (red color) staining as necrosis (late apoptotic) (Alabsi *et al.*, 2012).

2.7.4 Anti-inflammatory

Oxidative stress occurs from imbalance of production of reactive oxygen species (ROS) and body protection by antioxidant mechanisms. This process associated with inflammatory response. Inflammation is a defense response of the body to hazardous stimulation such as allergens and/ or injury to the tissues. On the other hand, uncontrolled inflammatory response is the main cause of a numerous of disorders including allergies, cardiovascular dysfunctions, metabolic syndrome, cancer, and autoimmune diseases imposing a huge economic burden on individuals and consequently on the society (Bagad *et al.*, 2013). There are various medicines for controlling and suppressing inflammatory crisis such as steroids, nonsteroid anti-inflammatory drugs. The immunosuppressant are the practical method of these medications which are associated with adverse effects while in practice the goal is to apply minimum effective dose by the highest capability with the least adverse effects. Therefore, oxidative enzymes that are known as the key role in inflammation are increasingly investigated in connection to cancer. Today, applying natural anti-inflammatory factors is instead of drug therapy to achieve increased pharmacological response and the lowest level of unwanted side effects.Lipoxygenases (EC 1.13.11.12) are widely distributed in

1) Lipoxygenases (EC 1.13.11.12) are lipid-peroxidizing enzymes involved in the biosynthesis of leukotriene from arachidonic acid and it is an inflammatory mediators also allergic reactions. These enzymes catalyzed by addition of molecular oxygen to unsaturated fatty acids such as linoleic and arachidonic acids (Porta and Rocha, 2002). In mammals, three major types of lipoxygenases have been identified, inserting dioxygen at C-5, C-12 and C-15 positions of arachidonic acid, respectively (Smith and Laneuville1994) due to the proposed role of lipoxygenase-derived metabolites (leukotriences and lipoxines) in a number of pathophysiological processes such as asthma, psoriasis, cancer metastasis and atherosclerosis (Handerson, 1994).

There is a research that is interested in finding the compounds that can inhibit the formation of these mediators through the lipoxygenase pathway in the arachidonate cascade (Gleason *et al.*, 1996). Kaladhar *et al.* (2014) reported that the good anti-inflammatory was found in ethanolic leaf extract of *A. indicum* (IC_{50}: 8.89 μg/ml) based on 5-Lipoxygenase (5-LOX) inhibition assay. Whereas, curcumin (Positive control) has shown IC_{50} as 8.14 μg/ml. Chung *et al.* (2009) found that active plant extracts were isolated from *Agelaea borneensis* (Connaraceae)

(bark), *Chisocheton polyandrus* (Meliaceae) (bark), *Garcinia cuspidata* (Guttiferae) (bark) and *Timonius flavescens* (Rubiaceae) (leaf) showed inhibitory activity of 70% or higher.

2) Hyaluronidases (EC 3.2.1.35) are enzymes that able to hydrolyze hyaluronic acid, one of the most abundant constituents of the extracellular matrix, which is ubiquitously distributed in body tissues (Trochon *et al.*, 2000). These enzymes have been associated to many biological functions, including allergy inflammation, migration of cancer cells, and permeability of the vascular system (West and Kumar, 1989). Therefore, the modulation of hyaluronidases by suitable inhibitors will be useful for normal homeostasis in the body (Ling *et al.*, 2003).

Manny researches have been reported about phytochemicals in plants which are able to suppress the production of inflammatory mediators from activated macrophages can act as potential anti-inflammatory agents. Nishida *et al.* (2014) reported that *Scilla scilloides* extract had potential on the lipoxygenase and hyalulonidase which are family of key enzymes that produce inflammative leukotriens in a number of pathophysiological process and key enzymes involved during allergy and other inflammation response respectively. Ilavarasan *et al.* (2005) reported the main constituents responsible for antiinflammatory activity of *Cassia fistula* are flavnoids and bioflavonoids.

2.8. Plant samples

1) *Camellia sinensis* (L.) Kuntze var.

Camellia sinensis (L.) Kuntze var. assamica (J.Masters) Kitam. is belonged to Theaceae family. Common name in Thailand is Miang pa, Miang, Miang Doi or Tea. It has been provided tea drinking. It is particularly rich in flavonoids, which are strong antioxidant and protecting the individual for oxidative stress diseases (Coimbra *et al.*, 2006). Cai *et al.* (2003) reported about antioxidant activity, phenolic compounds and anticancer of *C. sinensis* by using young leaf. It was found that, methanolic extract showed higher total phenolic content than aqueous extract which were 5268.6 and 3610.9 g/100g dry weight respectively. Similar resulted in TEAC assay, methanolic extract showed higher antioxidant activity than aqueous extract which were 17.4 and 13.6 μmol trolox/100g dry weight respectively.

Major types of phenolic compounds contained flavonols (catechin, epicatechin), flavonols (kaempferol and quercetin glycosides) and condensed tannins. Furthermore, *C. sinensis* known as green tea associated with anticancer might be a potential source of powerful natural

antioxidants and beneficial chemopreventive agents. Catechins contained in green tea exhibited significance in cancer prevention due to their structure similarity with chaperones and their interactions with target molecules. These properties suggest that green tea can prevent cancer due to their chaperone like activity (Kuzuhara *et al.*, 2008). Tea extract exhibits beneficial properties such as antimutagenic, antitumour, antioxidant, anticoagulant, antiviral, blood pressure and cholesterol lowering activity (Jarald and Jarald, 2006).

Figure 2.4 ***Camellia sinensis***

(Source : https://www.dr.hauschka.com/en_GB/knowledge-base/medicinal-plant-facts/tea/)

2) ***Careya sphaerica*** **Roxb.**

Careya sphaerica Roxb. is belonged to the Lecythidaceae family. Thai local name is Kradon or Kradonbok. Phomkaivon (2009) found that *C. sphaerica* showed high antioxidant activities and could be potential as rich sources of natural antioxidants and total phenolic content. Sriket (2014) found that the *Careya sphaerica* Roxb had the highest TPC and DPPH radical scavenging activity among other extracts were (32 mg GAE/g DW and 85.58% respectively. It was noted that extracts have high inhibition activities. This result suggested that both extracts were a very potent radical scavenger.

Maisuthisakul and Pongsawatmanit (2004) studied the effect of different sample preparations, and extraction time on antioxidant activity of *Careya sphaerica* Sample preparation were (1) hot air drying by tray dryer at 40 °C for 18 h, (2) vacuum drying by vacuum dryer at 40 °C (3) air drying at room temperature 25 °C for 12, (4) slowing freezing by freezing fresh leaves in freezer at -30 °C after packing in HDPE bag (5) fast freezing by contacting with dry ice and further freezing at -30 °C. The results revealed that sample preparation methods affected on total phenolic content and antioxidant activity. On the other hand, it does not effect on yield of the extract. In case of fast freezing, it could retard shelf life of the leaves before analysis time. It also

gave a higher yield of the extract, total phenolic content and antioxidant activity when compare with those obtained other drying method. After that, they were extracted with alcoholic solvent. It was found that yield of the extract and total phenolic content were mostly constant after 3 h of extraction time at room temperature. Extraction time from 4.5 to 6 h gave the lowest EC_{50} value when compared with those from other extraction times. These results interpreted that keeping plant leaf using fast freezing before analyzing. It was the appropriate preparation method and extraction time from 4.5-6 h gave the highest yield of the extract and antioxidant activity.

Figure 2.5 ***Careya sphaerica***

(Source: https://toptropicals.com/catalog/uid/Careya_arborea.htm)

3) ***Castarnopsis inermis*** **(Lind. Ex Wall) Benth. & Hook f**

Casternopsis inermis (Lind. Ex Wall) Benth. & Hook f is belonged to the Fagaceae family. Ko khao or Ko is Thai local name. Phomkaivon (2009) reported the major compound including alkaloid, steroid and terpene, flavonoild saponin and essential oil. Also, it had high antioxidant activities. *C inermis* extract showed antitrypanosomal with IC_{50} values which is more than12.5 µg/ml whereas it was no affected testing for their cytotoxic activities on a mamalian cell line (Vero) (Norhayati *et al*., 2013). Kaewdana (2013) found that *C. inermis* showed high potential for anti-rancidity in soybean oil.

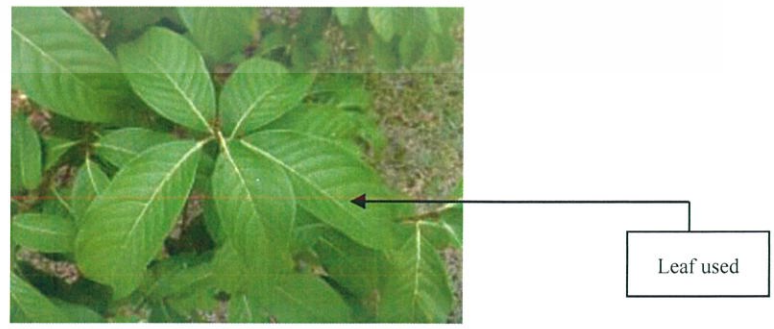

Figure 2.6 ***Castarnopsis inermis***

(Source: https://www.flickr.com/photos/yeochowkhoon/8039067256/in/set-72157631657346345/)

4) *Cratoxylum formosum* (JACK) Dyer spp.

Cratoxylum formosum (JACK) Dyer spp. is belonged to the Clusiaceae family. Tio khao or Tio is the Thai local name. In Thailand, it is consumed either raw with spicy minced pork salad or cooked in Tomyum. Maisuthisakul *et al.* (2007a) reported the major phenolic compound is chlorogenic acid. *C. formosum* extract was more effective than alpha-tocopherol in inhibiting lipid oxidation in bulk oil but were less effective in an oil-in-water emulsion in accordance with the polar paradox. Maisuthisakul *et al.* (2007b) reported that *C. formosum* has total flavonoids 25.5 mg RE/g dry basis and total phenolics 63.4 mg GAE/g dry basis. In term of anticancer, *C. formosum* extract selectively induced HepG2 cell death compared with normal Vero cells. It showed about a 1.5 times higher apoptotic effect compared with melphalan was induced by 120 µg/ml of the 50% ethanol mixed water of *C. formosum* extract. Both type of apoptotic cell death are extrinsic and intrinsic caspase-dependent pathways in dose- and time-dependent manners were found in HepG2 cells by significantly increasing the activities of caspase 3/7, 8, and 9, decreasing the mitochondrial membrane potential, and causing apoptotic body formation and DNA fragmentation (Nonpunya *et al.*, 2014).

Figure 2.7 *Cratoxylum formosum*

(Source: http://bpp24udon.com/data/plant/cratoxylum_formosum)

5) *Eleutherococcus trifoliatus* (L.) S.Y. Hu

Eleutherococcus trifoliatus (L.) S.Y. Hu is belonged the Araliaceae family. Phak- pam or pam is a common name. It is a common shrub in montane forest and thickets, about 1,100-1,400 m above sea level. It is occasionally grown by the natives in the north of Thailand for young shoots. Generally, it is used as folk medicine in Vietnam, Taiwan, and China for bruising, neuralgia, impotence, and gout because it has ginseng-like properties (Yook *et al.* 1999). Many authors have been reported the activities of secondary metabolic compounds from the Araliaceae.

Triterpenes such as ginsenoside and ciwujianosides isolated from Panax species and *E. senticosus*, respectively. Some compounds of this family have been found to be important on biological activities, including anti-inflammatory, anti-cancer, anti-amnestic, and anti-aging effect, which led to greater interest in this group of plants (Jung *et al.* 2003; Cheng *et al.* 2005; Seo *et al.* 2005).

Figure 2.8 ***Eleutherococcus trifoliatus***

(Source: https://www.shutterstock.com/image-photo/eleutherococcus-trifoliatus-l-sy-635478857)

6) *Ficus auriculata* Lour

Ficus auriculata Lour. is belonged the Moraceae family. Thai local name is Duea wa. A large group of phytochemicals are sterols and/or terpenes in genus Ficus (Kuete *et al.*, 2008; Djemgou *et al.*, 2009), flavonoids and coumarins (Chen *et al.*, 2010), in addition to minor alkaloids (Subramaniam *et al.*, 2009).

El-Fishawy *et al.* (2011) revealed eight known compounds contained in alcoholic extracts of the leaves and fruits of *F. auriculata* Lour were isolated by petroleum ether, chloroform and ethyl acetate fractions including: betulinic acid, lupeol, stigmasterol, bergapten, scopoletin, β-sitosterol-3-O-β-D-glucopyranoside, myricetin, and quercetin-3-O-β-D glucopyranoside. *F. auriculata* had a potential to inhibit gram positive bacteria (*Staphylococcus aureus*, *Bacillus aureus* and *Bacillus subtilis*) and gram negative bacteria (*Esherichia coli* and *Pseudomonas aeruginosa*) by agar well diffusion method. Whereas, the alcoholic extract of leaves at the concentration of 500mg/kg showed anti-inflammatory effect by using carrageenan-induced rat hind paw oedema model antioxidant effect. The results suggested that increasing the concentrations of the extracts increasing the antioxidant effect in a dose dependent manner. Moreover, the fruits extract showed higher potential than the leaves. Also, the extracts exhibited slight hepatoprotective and antidiabetic activities.

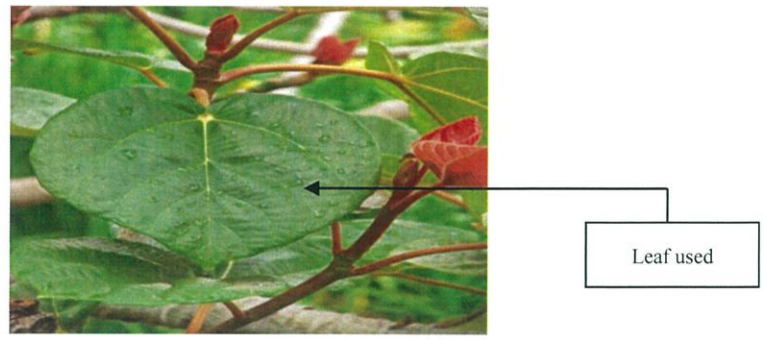

Figure 2.9 ***Ficus auriculata***

(Source: https://www.feedipedia.org/content/roxburgh-fig-ficus-auriculata-leaf)

7) *Glochidion sphaerogynum* (MULL.Arg) Kurz

Glochidion sphaerogymum (MULL.Arg) Kurz is belonged to the Euphorbiaceae family. Some researches have been reported about efficacy in treating cancer and reducing inflammation that stems from its constituents such as flavonoids, triterpenoid saponins, triterpenoid glycosides, and alkaloids (Ilango and Chitra., 2009; Takeda *et al*.,1998). In Thailand, *Glochidion sphaerogynum* is a local plant in the north of Thailand. The local name is Man Pla or Khai Man Pla. It is traditional consumed either cooked or raw. Jaivisen (2011) reported high total phenolic content and antioxidant activity were found in *G. sphaerogynum* extract. Phomkaivon (2009) revealed that *G. sphaerogymum* extract showed highest antioxidant activities among 49 alcoholic plant extracts in DPPH radical scavenging, trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) except thiobarbutiric acid reactive substances (TBARS). Putthawan *et al*. (2017) reported that *G. sphaerogymum* extract had the highest potential against lipid oxidation in 30% creamy salad dressing among other extracts.

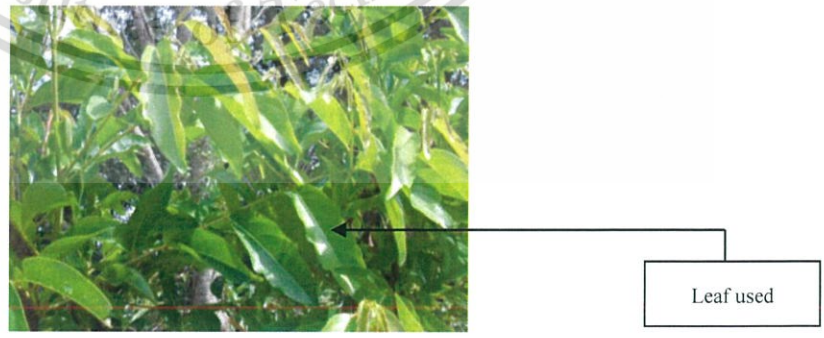

Figure 2.10 ***Glochidion sphaerogynum***

(Source: http://pgcp.crru.ac.th/frm_show_data_plants_user.php?plants_id=1293)

8) *Persicaria odorata* (Lour.) Soja'k

Persicaria odorata (Lour.) Soja'k is belonged to the Polygonaceae family. *P. odorata* is a common plant and well known in Malaysia as Daun kesum that is commonly used in cuisines and has various medicinal properties (Rasha *et al.*, 2014). Rau ram, phak phai, or phak praew as known in Thailand, is an evergreen perennial with tiny white flowers, that is native to South East Asia. The leaves of this plant have a similar, yet milder flavor than cilantro. The more mature plant leaves also have a hint of lemon flavor. It is used as a condiment and is a basic ingredient in the Vietnamese soup known as pho (Bilton, 2011).

Sasongko *et al.*, (2011) studied using hydrodistillation to extract the essential oil from the fresh and dry leaves of *P.odorata* for antibacterial activity. The main volatile compounds of *P.odorata* were dodecanal, caryophyllene, alpha-caryophyllene, drimenol and decanal. The antibacterial testing was examined by disc diffusion method. The essential oils from both of fresh and dry leaves of *P. odorata* showed antibacterial activity against both of gram positive and negative bacteria such as *Staphylococcus aureus* and *Escherichia coli* respectively. Moreover, essential oil from dry leaves exhibited more antimicrobial activity than fresh leaves, *S. aureus* showed an inhibition zone at 26 mm for 100 μl/ml concentration.

Figure 2.11 *Persicaria odorata*

(Source: http://tdaily.us/15700-20190303.html)

9) *Schima wallichii* (D.C.) Korth

Schima wallichii (D.C) Korth. (Theaceae family) is widely grown in Southeast Asia, and its astringent corollas are used for treatment of uterine disorders and hysteria, and also as an ointment to treat smallpox, in Indonesia and Malasia (Perry,1980; Burkill, 1966). In Thailand, Talo or Mung Tan is a local name. This plant is known to be rich in saponin and tannins (Burkill, 1966). In dried flowers of *S. wallichii* contained ten polyphenolic including camellin B (8), a

dimeric hydrolzable tannin, and two new hydrolysable tannins named schimawalins A and B (Yoshida *et al.*, 1991). Diantini *et al.* (2012) found that Kaempferol-3-*O*-rhamnoside as an active compound was isolated from the leaves of *Schima wallichii* inhibits cell proliferation in a dose-dependent manner and promotes apoptosis in MCF-7 breast cancer cells via the activation of the caspase signaling cascade, which includes caspase-9, caspase-3 and PARP.

Figure 2.12 ***Schima wallichii***

(Source: https://calphotos.berkeley.edu/cgi/img_query?enlarge=0000+0000+0110+0076)

10) ***Vaccinium sprengelii*** (G.Don) Sleumer

Vaccinium sprengelii (G.Don) Sleumer, Thai local name is sompi or maohin, maohuawaen, sompae, somsad. Phomkaivon and Areekul (2009) reported the phenolic content and antioxidant activities of *V. sprengelii* extract in TPC, DPPH concentration (EC_{50}), TEAC and FRAP found to be 95.42±6.26 mg GAE/g dry basis, 0.53±0.01 mg dry basis, 56.06±0.13 mM TE/g dry basis and 63.67±0.27 mg AscAE/g dry basis respectively. Utilization for anticancer and antimicrobial also nutrition of this plant is not yet reported.

Figure 2.13 ***Vaccinium sprengelii***

(Source: https://www.shutterstock.com/search/sprengelii)

2.9 Cell lines

Cell lines are produced from proliferating cells in culture derived from various organ and sources. They are commonly used in basic research and early step drug development studies. The use of cell line depends on the organism of study, the disease investigated, and the tissue or organ of interest.

1) HT-29

HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology. These cells are sensitive to the chemotherapeutic drugs 5-fluorouracil and oxaliplatin, which are standard reference treatment choices for colorectal cancer. In addition to being a growth of tumor model for colorectal cancer, the HT-29 cell line is also used as an *in-vitro* model to study absorption, transport, and secretion by intestinal cells. Under standard culture conditions, these cells grow as a nonpolarized, undifferentiated multilayer (Memorial Sloan Kettering Cancer Center, 2019).

Figure 2.14 HT-29 Cell line

2) HepG2

HepG2 is a human liver carcinoma cells, derived from the liver tissue of a 15-year-old Caucasian male who had a well-differentiated hepatocellular carcinoma. Hepatocellular carcinoma is the fifth most-common cancer worldwide. The morphology of HepG2 cells is epithelial and contains 55 chromosome pairs. HepG2 cells can be grown successfully at a large scale, and secrete many plasma proteins, such as transferrin, fibrinogen, plasminogen and albumin. They can be activated with human growth hormone. HepG2 cells are adherent, epithelial-like cells growing as monolayers and in small aggregates (HepG2: Cell Culture and Transfection Protocol, 2019).

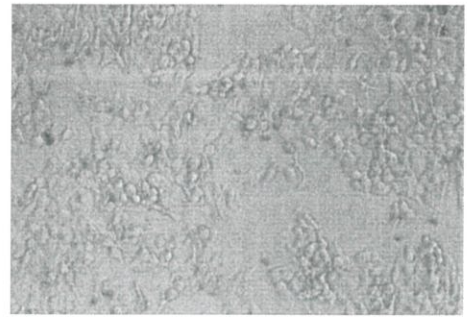

Figure 2.15 HepG2 Cell line

3) KB

Another most common malignancy in the world is oral cancer. Smoking and alcohol-considered major risk factors are present in 90% of cases (Dissanayaka *et al*, 2012). It is characterized by a high degree of local invasiveness and a high rate of metastases to cervical lymph nodes. The migration of oral squamous cell carcinoma (OSCC) into maxillary and mandibular bones is a common clinical problem.

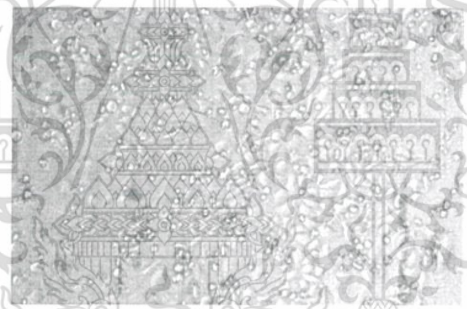

Figure 2.16 KB Cell line

4) Vero cell

African green monkey kidney cells (Vero cells) are species of cells used in cell cultures. Vero cells are derived from normal kidney cells. They are used for many objectives, including to screen the toxin of *Escherichia coli* (Evan *et al*., 2000) , to measure replication in the presence or absence of a research pharmaceutical, to test the presence of rabies virus, or the growth of viral stocks for research purposes as host cells for eukaryotic parasites (Kistne *et al*., 2007)

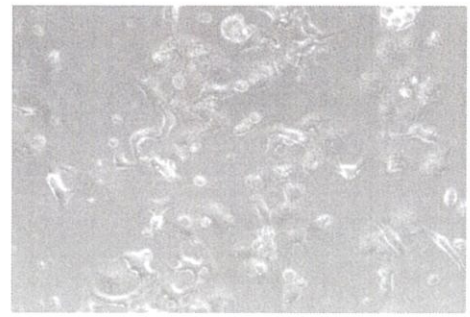

Figure 2.17 Vero Cell line

5) TK6

TK6 is a lymphoblastoid cell line heterozygous at the thymidine kinase locus (Skopek *et al.,* 1978). TK-6 cell line has been used to study the *in vitro* genotoxic effects of *trans*- and *cis*-2-ethylhexyl 4-methoxycinnamate (EHMC). It has also been used to study the interaction of silver nanomaterials with the cells.

Figure 2.18 TK6 Cell line

The numbers of cancer patients have increased every year and cancer is the second cause of death worldwide. To reduce these side effects from conventional therapeutic methods, novel or alternative measures have been sought for instead drugs as treatments for cancer patients. The therapy is based on plants or plant extracts, it usaully has less side effects, and lower costs for treatment. Recent studies indicated that some medicinal plants may have anticancer effects. Hajiaghaalipour *et al.* (2015) found that *Camellia sinensis* extract exhibited anti-proliferative effects on HT-29 cells. Devika and Mohandas (2014) reported that extracts of *Foeniculum vulgare* induced apoptosis in cervical cancer cells and had anti-proliferative effects through DNA fragmentation. Manapradit *et al.* (2015) showed that butanolic leaf extracts from *Barleria strigosa* exhibited the highest cytotoxicity against the P-388 cell line with a CC50 of 127.42 µg/ml. Momtazi-Borojeni *et al.* (2013) reported the *Avicennia marina* was extracted with methanol

had potentially antiproliferation of MDA-MB 231 cell (human breast cancer cell) with CC_{50} values of 250 µg/ml.

Chapter 3

Research Methodology

3.1 Raw materials

3.1.1 Plant samples *C. sphaerica* and *C. formosum* were collected from Yasothon province and the other eight plants from Chiang Mai province. All plant materials were harvested from May to August in 2014 as shown in Table 3.1

Table 3.1 Scientific name of 10 edible Thai local plants and parts used

Scientific name	Family	Thai local name	Part used	Harvested
Camellia sinensis (L.) Kuntze var. assamica (J.Masters) Kitam.	Theaceae	Miang pa, Miang, Miang Doi, Tea	Leaf	June
Careya sphaerica Roxb.	Lecythidaceae	Kradon, Kradonbok	Leaf	May
Castarnopsis inermis (Lind. Ex Wall) Benth. & Hook f	Fagaceae	Ko khao, ko	Leaf	May
Cratoxylum formosum (JACK) Dyer spp.	Clusiaceae	Tio khao ,Tio	Leaf	May
Eleutherococcus trifoliatus (L.) S.Y. Hu	Araliaceae	Phak paem, Paem	Stem and Leaf	May
Ficus auriculata Lour	Moraceae	Duea wa	Leaf	June
Glochidion sphaerogymum (MULL.Arg) Kurz	Euphobiaceae	Man Pla, Khai Man Pla	Leaf	June
Persicaria odorata (Lour.) Soja'k	Polygonaceae	Phak phai, Phak praew	Stem and Leaf	August
Schima wallichii (DC.) Korth	Theaceae	Talo, Mung Tan	Leaf	August
Vaccinium sprengelii (G.Don) Sleumer	Ericaceae	Som Pi, Maohin, maohuawaen,sompae,somsad	Leaf	June

3.1.2 Cell line

The cell lines, Human colon adenocarcinoma (HT29), Human hepatocellular carcinoma (HepG2), Human epidermoid carcinoma (KB) Vero Cell line and Human lymphoblastoid cell line (TK6) are obtained from the National Cancer Institute (Thailand).

3.2 Chemicals and Reagents

1) ABTS+ (2,2 azinobis (3-ethylbenzothiazoline-6-sulfonate)) (Sigma Aldrich, USA)
2) Absolute ethanol (Merck, Germany)
3) Acetate buffer (Merck, Germany)
4) Acetic acid (Lab scan, Ireland)
5) Agarose gel (Vivantis, USA)
6) Aluminium chloride ($AlCl_3$) (Sigma Aldrich, USA)
7) Anixine v (Vivantis, USA)
8) Annexin-binding buffer (Vivantis, USA)
9) Bovine Serum Albumin (BSA) (Sigma Aldrich, USA)
10) Catechin (Sigma Aldrich, USA)
11) Chloroform ($CHCl_3$) (Lab scan, Ireland)
12) Compound 48/80 (Sigma Aldrich, USA)
13) di-Potassium hydrogen phosphate 3 hydrate (K_2HPO_4 (Merck, Germany)
14) Dimethyl Sulfoxide (DMSO) (Sigma Aldrich, USA)
15) 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphynyltetraaolium bromide (MTT) (Gibco, USA)
16) Diosgenin (Sigma Aldrich, USA)
17) DPPH (2,2 –diphenyl-1-picrylhydrazyl) (Sigma Aldrich, USA)
18. DNA Marker 1kb (Vivantis, USA)
19) Dye solution Annexin X and Propidium iodide (Vivantis, USA)
20) Ethyl alcohol (Liquor Distillery Organization,Thailand)
21) Ferric chloride ($FeCl_3$) (Lab chem, USA)
22) Fetal Bovine Serum (FBS) (Gibco, USA)
23) Folin-Ciocalteu (Carlo, Italy)

24) GF-1 Blood DNA Extraction Kit (Vivantis, USA)

25) Hyaluronidic acid (Nacalai Tesque, Inc., Tokyo Japan)

26) Hyaluronidase from bovine testis type IV-S (Sigma Aldrich, USA)

27) Hydrochloric acid (Merck, Germany)

28) Iron (III) chloride hydrate (Carlo, Italy)

29) Iso-octane (2,2,4- trimethylpentane) (Lab scan, Ireland)

30) Lecithin (MP Biomedicals, USA)

31) Linoleic acid (Sigma Aldrich, USA)

32) Lipoxygenase type I-B (Sigma Aldrich, USA)

33) Malonaldehyde (1,1,3,3- tetraethoxypropane) (Merck, Germany)

34) Methyeneblue trihydrate (Sigma Aldrich, USA)

35) Mitomycin C (Kyowa Hakko Kirin, Japan

36) Nordihydroguaiaretic acid (NDGA) (Sigma Aldrich, USA)

37) Propidium Iodide (PI working) (Vivantis,USA)

38) Potassium dihydrogen phosphate (KH_2PO_4) (Merck, Germany)

39) Potassium iodie (KI) (Merck, Germany)

40) Perchloric acid (Merck, Germany)

41) *p*-Anisidine (Sigma Aldrich, USA)

42) *p*-dimethyaminobenzaldehyde (*p*-ADB) (Nacalai Tesque, Inc., Tokyo Japan)

43) Roswell Park Memorial Institute (RPMI 1640) (Gibco, USA)

44) Rutin (Sigma Aldrich, USA)

45) Sodium azide (Lab chem, USA)

46) Sodium borate (Sigma Aldrich, USA)

47) Sodium carbonate (Carlo, Italy)

48) Sodium dodecyl sulfate (SDS) (Merck, Germany)

49) Sodium hydroxide (NaOH) (Merck,Germany)

50) Sodiumthiosulphate ($Na_2S_2O_3$ (Merck, Germany)

51) Starch (Merck, Germany)

52) Tannic acid (Nacalai Tesque, Inc., Tokyo Japan)

53) Thiobarbituric acid (TBA) (Sigma, USA)

54) TPTZ(2,4,6-tripyridyl-s-triazine) (Sigma, USA)

55) Triethanolamine (Merck, Germany)

56) Trypsin 0.25% (Gibco, USA)

57) Tween 20 (Merck, Germany)

58) Tween 80 (Merck, Germany)

59) Vanilin (Merck, Germany)

60) Xanthan gum (Nutrition Sci, USA)

3.3 Equipment

1) Autopipette (Eppendrop, Germany)

2) Centrifuge (5,500 rpm) (Hettich Zentrifugen EBA 20, Germany)

3) Centrifuge (9,000 rpm) (Thermo Scientific Legend Mach 1.6R, England)

4) Fluorescence microscope (BX51, Olympus, USA).

5) Gas Chromatography (GC) (7890A, Agilent Technologies, Wilmington DEL)

6) Hot air oven (Memmert UFB 400, Germany)

7) Hot plate magnetic stirrer (IKA® C-MAG HS7, Japan)

8) Homogenizer (IKA® T25 digital- Ultra-Turrax, Germany®)

9) Mass spectrometer (5975C Network Mass Selective Detector, Agilent Technologies)

10) Microplate reader (Beckman couterl Multimode Detector DTX 880, USA)

11) Microplate reader (SH-1000Lab, Corona Electric, Ibaraki, Japan).

12) Rotary evaporator (Buchi R215, Canada)

13) pH meter (Mettler Toledo, USA)

14) UV-VIS Spectrophotometer model Evolution 201 (Becthai, Bangkok Equipment & Chemical)

15) Water bath (Memmert WB 29, Germany)

3.4 Methodology

3.4.1 Preparation of plant extracts

All plant samples were washed two times then blanched at 80 °C for 30 s and drained. Next, they were dried at 50°C in a tray dryer for 20-30 h to reduce their moisture content to below 10%. After that, the dried samples were finely ground and mixed with 80% ethanol (50°C) at a ratio of 1:5 (w/v) or weighed 25 g sample and extracted with 125 ml of ethanol and shaken at 200 rpm by shaking machine for 8 h at room temperature. The extracts were filtered by filter pater and the residues were then re-extracted at a ratio of 1:3 and shaken continuously for another 8 h. The first and second extracts after filtered were then combined and concentrated by rotary evaporator at 40°C. When preparing for use, the crude extracts were diluted with ethanol to a final concentration of 400 mg/ ml (0.4 g of extract in 10 ml of ethanol) and kept at -20°C after flushing with nitrogen until analyses.

3.4.2 Effect of plant extract concentrations on oxidative stability of 30% O/W emulsion

3.4.2.1 Preparation of o/w emulsion

Thirty percent o/w emulsion was prepared according to Di Mattia *et al.* (2009) with slightly modifications. The emulsion was prepared by dispersing 30% wt soybean oil in 70% phosphate buffer (0.1 M, pH=5.4) containing Tween 80 (1%), lecithin (0.3%), xanthan gum (0.5%) and sodium azide (0.02%).

The extracts were added to obtain the final concentration of 200 and 500 µg/ml in the mixture and then were homogenized at 11,000 rpm by homogenizer for 15 min. A commercial antioxidant, 100 ppm tertiary butylhydroquinone (TBHQ), was dissolved in ethanol and used as a positive control for comparison. A sample without addition of antioxidant was used as a control. 25 g of emulsions were packed into glass bottle and incubated in the oven at 35°C for 1 month. The samples were randomly taken for lipid oxidation analysis every week through out.

3.4.2.2 Measurements of lipid oxidation

Four measurement methods were used for lipid oxidation including thiobarbituric acid reactive substances (TBARS), peroxide value (PV), anisidine value (AV), and oil stability index (OSI). For TBARS measurement, emulsion samples were analyzed

immediately without being frozen. On the other hand, samples analyzed by the other three methods were kept frozen at -20°C overnight at first then were thawed to room temperature. Oil layers were separated from their buffer layer when centrifuged at 9000 rpm for 15 min. After that, the oil layers were analyzed by the three methods.

1) Thiobarbituric acid value (TBARS)

TBARS values are measured of the malonaldehyde content which is the secondary products of lipid oxidation according to the method described by McDonald and Hutin (1987) with slightly modifications. Briefly, the TBARS reagent was prepared by mixing 15% w/v trichloroacetic acid and 0.0375% w/v 2 –thiobarbituric acid in 0.25 N hydrochloric acid. The emulsion sample (0.1g) was mixed with distilled water (0.9 ml) and then the TBARS reagent (2 ml) was added in test tubes. Then, the sample was placed in a boiling water bath for 15 min and cooled down to room temperature for 10 min, then was centrifuged at 5,500 rpm for 10 min. The supernatant was collected and absorbance was measured at 532 nm. Concentrations of TBARS was calculated from standard curve using 1,1,3,3-tetramethoxypropane.

2) Peroxide value (PV)

Peroxide value monitors primary products of lipid oxidation according to AOCS official method Cd 8-53 (1997a). Weight 0.50±0.05 g of oil samples was taken into a 250-ml erlenmeyer flask with glass stopper and added 3ml of the ratio 3:2 acetic-chloroform solution. Flask was swirled to dissolve the sample. After that, 0.05 ml of saturated KI solution was added with occasional shaking for 1 min and then immediately added 3mL of distilled water. It is titrated with 0.01 N sodium thiosulfate using starch as indicator and a blank determination of the reagent daily must not exceed 0.1 ml of 0.01 N sodium thiosulfate solution. PV was calculated and expressed as milliequivalents peroxide per kg of oil sample;

$$\text{PV (mequiv/kg)} = \frac{(S-B) \times N \times 1000}{W} \qquad (3.1)$$

Where S is the volume of titrant (ml) of sample, B is the volume of titrant (ml) of blank, and N is a normality of sodium thiosulfate solution, W is weight of sample (g)

3) *p*-Anisidine value (*p*-AV)

Anisidine value is measured of aldehyde production (secondary oxidation product) during oxidation reaction were carried out following AOCS official method Cd 18-90 (1997b). The oil 0.5-4.0 g was added into a 25 ml volumetric flask and made up to the mark with isooctane. The absorbance (A_1) of the samples was measured while a pure isooctane is used as blank. 5 ml of oil sample solution or 5 ml isooctane (as blank) was then transferred to 10 ml test tubes. Then, 1 ml of *p*-ansidine solution (0.25% w/v solution in glacial acetic acid) was added. After that, it was mixed together for 10 second and allowed for 10 min. The absorbance (A_2) was read at 350 nm while the isooctane containing *p*-ansidine was used as blank. The *p*-AV values were calculated as following;

$$p\text{-AV} = \frac{25 \times (1.2 \times A_1 - A_2)}{W} \quad (3.2)$$

Where A_1 is the absorbance of sample added isooctane

Where A_1 is the absorbance of sample added isooctane and *p*-ansidine solution

Where W is weight of sample (g)

4) Oil stability index (OSI)

An automated Metrohm Rancimat apparatus was used to determine induction periods of the oils in order to monitor the volatile compounds of oxidation reaction accelerated by heated under constant aeration determined according to a slightly modified version of the AOCS official method Cd 12b-92 (1997c). Briefly, the oil was carefully weigh about 3.01-3.0200 g into each of the eight reaction vessels under condition was 120°C and air flow of 20l/h and analyzed simultaneously. Induction periods of the samples was recorded automatically and corresponded to the break point in the plotted curves and reported in the unit of hour.

3.4.3 Effect of various pHs and storage temperatures on plant extracts

Phosphate buffers of pH 3, 5 and 7 were used to prepare emulsion samples in the same way as the preparation in Section 3.4.2.1 and then two extracts were chosen from previous experiment added to achieve the chosen concentration of 500 µg/ml. Then, the samples were

transferred into screw-capped 120 ml glass bottles. All samples were incubated in an oven at 25, 35, and 45 °C for 63, 49, 35 days respectively and randomly taken for analysis every week.

Lipid oxidation including PV, TBARS, *p*-AV and oil stability index were monitored in the same way as the method in Section 3.4.2.2 and reported as reaction rate constant (k), it was calculated by the slope of variation of PV, TBARS and *p*-AV during storage.

Shelf life prediction was based on not allowing a PV value over 30 meq/kg (a TISI standard for acceptable emulsion). Shelf life (days) was calculated according to the order of reaction rate constant. In addition, secondary oxidation products were studied by solid phase microextraction (SPME) using GC-MS.

The pH of emulsion showing the lowest rancidity for each temperature was selected from section 3.4.3 After that, emulsion containing plant extracts at this pH were prepared again, then stored at 25, 35, and 45°C for 62, 49 and 35 days, respectively. The samples were prepared according to the method described by Whiton and Zoecklein (2000) with slightly modifications. Five grams of emulsion was mixed with 2.5 g of sodium chloride in vial. The sample was heated at 40°C along with agitated samples for 30 min. Extraction of headspace volatiles was done by using solid-phase micro-extraction (SPME) (50/30μm DVB/CAR/PDMS) (Divinylbenzene/Carbowax/ Polydimethylsiloxane). This fiber is suitable for analyzing polar and non-polar volatile compounds. After completion of sampling, the fiber was removed from the sample vial and inserted into the injection port of the GC. The volatiles were analyzed on a Hewlett-Packard model HP 6890 GC (Hewlett-Packard, Palo Alto, CA, USA) equipment with a mass selective detector model HP 5973. Capillary Polar column, HP-5 (60 m length, 0.25 mm ID and 0.25 lm film thickness) and Polar column DB-WAX (60 m length, 0.25 mm ID and 0.25 lm film thickness) is used. Helium was used as the carrier gas. The oven program starts the initial temperature at 50°C for 5 min, then increases with 5°C/min until 200°C. The temperature was set steady for 5 min. The data was calculated from internal standard curve of hexanal. The samples were analyzed at The National Nanotechnology Center (Nanotech) of Thailand.

3.4.4 Evaluation of biological activities of 10 edible Thai local plant extracts

3.4.4.1 Preparation of Plant Extracts

Plant extracts were prepared in the same way as the preparation in section 3.4.1, ten plant extracts at 2,000 μg/ml were tested for their biological activities as following:

3.4.4.2 Phytochemical contents

1) Flavonoid

The flavonoids content was determined using a method as described by Kumaran and Karunakaran (2007) with a little modification. 1ml of extracts was mixed with 1ml of aluminium trichloride in ethanol (10 g/l) and then, diluted with ethanol to 25 ml and incubated at 20°C for 40 min. Blank was prepared from 1 ml of each plant extracts and a drop of acetic acid and then diluted to 25 ml with ethanol. Rutin was used as a standard compound. The absorbance was examined at 415 nm. The data was reported as the milligrams rutin equivalence/ ml of extracts.

2) Saponin

The total saponins content of different extracts using a method as described by Chen *et al.* (1996) adopting few modification, diosgenin as a reference compound. 1ml of extracts was taken into volumetric flask (50 ml) and followed; 0.8 ml perchloric acid and vanilic acetic acid (5%). After completely mixed, the flask was transferred in a water bath for 15 min at 60°C. Afterward, the samples were cooled in an ice water bath. 5 ml of glacial acetic acid was added and allowed for 10 min. Each flask was diluted with 70% ethanol to 50 ml. The absorbance of each flask was measured at 554 nm with 1 ml blank solution as contrast. The data was expressed as the milligrams diosgenin equivalence/ml of extracts.

3) Tannin

Tannin content of different extracts using a method as described by Hagerman and Butler (1978) using catechin as a reference compound. Each extract was mixed with bovin serum albumin (0.2 mol/l acetate buffer pH5) at the ratio 2:1 into centrifuge tubes and stood for 15 min with slowly agitation. Then, the solution was centrifuged for 10 minutes at 6000 rpm. The supernatant was carefully poured off (1), retaining the pellet was in the centrifuge tube. Then, 250 µl of the buffer A (washing buffer) was slowly added to the pellet sample (2). After that, it was centrifuged for 1 minute at 6000 rpm (3). Step 1, 2 and 3 was repeated to wash the pellet a second time. The supernatant was carefully pour off and then added 875 µl of buffer C (5%triethanolamine (v/v and 1% SDS (w/v) and incubated for 10 min. Tube was mixed until the pellet completely dissolved. After dissolving the pellet was stood for 10 min and then read the absorbance at 510 nm before adding the ferric chloride reagent (10mM). The data was recorded.

Then, 125 µl of the ferric chloride reagent was added, mixed together, stood for 10 min. The absorbance at 510 nm was re-read. The data was calculated and reported as the milligrams catechin equivalence/ ml of extracts.

3.4.4.3 Antioxidant capacities

1) Total phenolic content (TPC)

TPC was determined using Folin -Ciocalteu method with some modification of Singleton *et al.* (1999). Briefly, each extract (250 µl) was added in 96 well plates. Afterward, 12.5 µl Folin–Ciocalteu reagent was added followed by 50 µl of 10% sodium carbonate and mixed together. The extract was kept in the dark for 10 min. The absorbance was measured at 695 nm. The results were reported as milligrams of gallic acid equivalence (GAE)/ml of extracts.

2) Ferric-reducing antioxidant power assay (FRAP)

The procedure described by Benzie and Strain (1996) was used with minor modification. Briefly, each extract (10 µl) was mixed with 300 µl of FRAP solution in 96 well plates. The extract was kept in the dark for 8 min. The absorbance was read at 595 nm by microplate reader. The results were expressed as milligrams Trolox equivalence /ml of extracts.

3) DPPH free radical-scavenging assay

The method of Brand- Williams *et al.* (1995) was adopted for evaluating the free radical scavenging with little modification. Briefly, 70µl of each extract was mixed with 210 µl of 0.2 mM DPPH in 96 well plates and the extracts was kept in the dark for 30 min. The absorbance was measured at 520 nm by microplate reader. The reduction in DPPH radical of each plant extract was calculated DPPH radical scavenging activity which was reported as milligrams Trolox equivalence/ml of extracts.

4) Trolox equivalent antioxidant capacity (TEAC)

The procedure described by Zhou and Yu (2004) was used with minor modification. Briefly, each extract (30 µl) was added in 96 well plates. Afterwards, 300 µl (5mM) of ABTS+ was added. The solution was stood at room temperature for 6 min. The absorbance

was measured at 734 nm by microplate reader. The scavenging activity of each plant extracts was calculated and reported as milligrams Trolox equivalence/ ml of extracts.

5) Thiobarbutyric acid reactive substances (TBARS)

TBARS described by McDonald and Hultin (1987) was used with some modification. Briefly, each extract (200 µl) was added into tube. 800 µl of linoleic emulsion (1%) was added. Each tube was moved in water bath at 50°C for 18 hrs. Then, 200 µl of TBARS reagent was added and boiled for 15 min. After that, the sample was cooled down with cold water and then 300 µl of samples was measured at 532 nm by microplate reader. The inhibition of each plant extracts were calculated and reported as milligrams Trolox equivalence/ ml of extracts.

3.4.4.4 Cytotoxicity Assay

10 plant extracts were prepared for analysis as following 0.2 g of each 80% alcoholic crude extracts was diluted with 1ml of DMSO, followed by 9 ml of phosphate buffer saline (PBS). Then, they were centrifuged at 1,500 rpm, passed through the 0.45 µm filter.

1) Cell culture

Cells lines including Human colon adenocarcinoma (HT29), Human hepatocellular carcinoma (HepG2), Human epidermoid carcinoma (KB), African green monkey kidney cells (Vero cells) and Lymphoblastoid cell (TK6) were maintained in Roswell Park Memorial Institute (RPMI 1640 medium) supplemented with inactivated fetal bovine serum (FBS 8%), gentamycin 50 µg/ml. The cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

2) 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphynyltetraaolium bromide (MTT) assay

MTT assay was described by Mosmann (1983) with a slight modification. The cell line, Human colon adenocarcinoma (HT29), Human hepatocellular carcinoma (HepG2) and Human epidermoid carcinoma (KB) was in a 96-well-plate with 1x 10^5 cells/well of concentration. Cell lines were incubated at 37°C and 5%CO_2 for 24 h. Ten plant extracts were added in each well at the final concentration 2000 µg/ml, the cells treated with Mitomycin C (cytotoxic drug) at 50 µg/ ml was used as a positive control while cells treated with 0.2% DMSO was used as a negative control. Untreated cell cultures (control) and blank wells without cells

contained 100 µL of medium and incubated for 21 h. Next, MTT solution (2 mg/ml) was added to each well and incubated for 3-4 hrs. Afterwards, 100 µl of mixing DMSO and absolute ethanol (ratio 1:1) was added to the wells followed by shaking to solubilize the formazan crystal. Absorbance was measured at 540 nm. Surviving cell was calculated as percentage of cytotoxicity. After that, four plant extracts show high antiproliferation were chosen for test cytotoxic concentration (CC_{50}) at the final concentration 250, 500, 1000, 2000, and 4,000 µg/ml. Four plant extracts were tested toxic with a normal cell line (Vero) at the concentration of 4000 µg/ml).

3) DNA fragmentation

Cell lines were prepared according to Mat-Akhir *et al.* (2011) method with a little modification. The HT29 and HepG2 cell lines with 1x 10^5 cells/well of concentration were treated with four plant extracts (500 and 1000 µg/ml) and incubated for 48 h. DNA extraction was performed using blood DNA extraction kit (Vivantis, USA) according to the manual instructions. The cells were pelleted and washed with binding buffer (BB). Then, 20 µl of proteinase K was added. The cells were mixed and incubated at 65°C for 10 min. After that, 20µl of RNase was added and re-incubated at 37°C for 5 min. Absolute ethanol (200µl) was added and mixed immediately. Samples were transferred to column and centrifuged at 10000 rpm for 1 min. The supernatant was discarded and washed with buffer 1. Then, it was centrifuged and washed with buffer 2 (2 times). The supernatant was discarded. Pre-incubated elution buffer (70 µl) was added and stood at room temperature for 10 min. Afterward, it was centrifuged at 14000 rpm for 1 min. The DNA pellets was re-suspended in TE buffer prior to loading (10 µl) onto a 2% agarose gel. Then, gel was ran at 100 V for 30 min. DNA fragments was visualized under UV illumination and the picture was taken with a gel documentation system. DNA marker (1kb) was used to estimate the size of DNA fragment.

4) Apoptosis observation

The Human lymphoblastoid cell line (TK6) was prepared according to Mosmann (1983) method with some modifications. The cells were taken in a 24-well-plate with 1X10^5 cells/well of concentration. The morphology of cells was monitored during cell growth after treatment with the two extracts (chosen from DNA fragment), control (no adding extract) and positive control (Mitomycin C 100 µg/ml). The plates were incubated at 37°C for 24h. Then,

cell was harvested by centrifuged at 1500 rpm for 5 min. After that, supernatant was discarded. 500 µl of cold phosphate buffer saline (PBS) was added and re-centrifuged. Apoptosis analysis were performed according to the manual instructions (Apoptosis Kit). 2 µl of Annexin v was mixed into tube followed by 1 µl of PI working solution (100 µg/ml). Then, the solution was mixed and incubated at 37°C for 15min. Afterward, it was centrifuged at 1500 rpm for 5 min. The supernatant was discarded. After that, 1x annexin-binding buffer is added, mixed together. The cells were taken on clean slides before checking under the fluorescence microscope (BX51, Olympus, USA).

3.4.4.5. Anti-inflammatory assay

Inflammation is a response of humans and other mammals to the presence of pathogens and chemical or mechanical injury. In inflammatory responses, several inflammation-related enzymes are known to be involved in generating inflammatory mediators or enhancing tissue damage. So, ten plant extracts were used to evaluate. Crude extracts were diluted with DMSO to the final concentration of 400mg/ml for evaluating lipoxygenase and hyaluronidase inhibition.

1) Lypoxygenase inhibition

The lipoxygenase assay was performed based on the change of methylene blue color of during the enzymatic reaction as described by Anthon and Barrett, (2003); Suda *et al.*, (1995). Linoleic acid solution at a 50 mM stock in a volume of 1.0 ml was first prepared, including Linoleic acid (56 mg), Tween 20 (112 mg), 1N NaOH (24 µl), and water (811.2 µl), and stored at -20°C under N_2 gas condition. Lipoxygenase was prepared in 0.1 M phosphate buffer (pH 6.0). The standard mixture is NDGA. It was prepared at varying concentration. For the samples were prepared in a final volume of 350 µl is contained 17.5 µl of the sample solution, 140 µl of 0.2 M phosphate buffer (pH 6.0), 5.6 µl of 25 mM linoleic acid solution, 14 µl of 250 µM methylene blue, 102.9 µl of distilled water and 70 µl of 212 kU/ml lipoxygenase. The reaction was started after adding the enzyme, allowed to proceed for 0 min and 30 min at room temperature in a 96-well plate. The mixture was measured using a grating microplate reader at 660 nm. The lipoxygenase inhibitory activity will be calculated as the percentage inhibition as following equation;

$$\text{Lipoxygenase inhibition (\%)} = [1-\{(OD_{Sample}- OD_{Blank})/(OD_{Control}-OD_{Blank})\}]x100 \qquad (3.3)$$

OD_{sample} is optical density of plant extracts containing reagents and enzyme

OD_{blank} is optical density of all chemical reagents except plant extracts and enzyme

$OD_{control}$ is optical density of all chemical reagents and enzyme except plant extracts

2) Hyaluronidase inhibition

The hyaluronidase assay was carried out using hyaluronic acid as the substrate depending on the detection of liberated *N*-acetyl glucosamine end group was described by Aronson and Davidson,1967; Kakegawa *et al.*,1985. First, hyaluronidase, compound 48/80, and hyaluronic acid potassium salt was prepared in 0.1 M acetate buffer (pH 4.0). The standard assay mixture was tannic acid and was prepared at varying concentrations. In the samples were prepared in a final volume of 30 µl, contained 5µl the sample solution, 15 µl of 0.1 M acetate buffer (pH 4.0), and 10 µl. 2kU/ml hyaluronidase. After a pre-incubation for 20 min at 37°C, 20 µl of 1mg/ml compound 48/80 solution was added to activate the enzyme and incubated for 20 min. The reaction was started by the addition of 50 µl 0.8 mg/ml hyaluronic acid allowed to proceed for 40 min at 37°C, terminated by adding 20 µl of 0.4 N NaOH solution and kept on ice. Next, 20 µl of sodium borate buffer (pH 9.1) was added into the assay mixture, heated for 3 min at 100°C and cooled down on ice. 600 µl of *p*-dimethyl aminobenzaldehyde (*p*-ADB) reagent (5 g of *p*-ADB was dissolved in 43.75 ml of glacial acetic acid and 6.25 ml of 10 N HCl) was added. After that, the solution was kept at 4°C as 10x stock solution, and diluted with acetic acid before use. Then, the solution was centrifuged, the supernatant (350 µl) was measured at 585 nm using a microplate reader. The hyaluronidase inhibitory activity was expressed as the percentage inhibition;

$$\text{Hyaluronidaseinhibition}(\%)=[1-\{(OD_{Sample}-OD_{Blank})/(OD_{Control}-OD_{Blank})\}]x100 \quad (3.4)$$

OD_{sample} is optical density of plant extracts containing reagents and enzyme

OD_{blank} is optical density of all chemical reagents except plant extracts and enzyme

$OD_{control}$ is optical density of all chemical reagents and enzyme except plant extracts

3.4.4.6 Statistical analysis

All analyses were done in duplicate. The results were reported as the mean ± SD. A completely randomized experimental design used to analyze. Analysis of variance was performed by ANOVA tests and significant differences were $p < 0.05$ using IBM SPSS software, version 24 (IBM Singapore Pte. Ltd., Changi, Singapore).

Chapter 4

Results and Discussion

4.1 Effect of plant extract concentrations on oxidative stability of o/w emulsion

1) Peroxide values

Changes in the peroxide values of o/w emulsion added plant extracts at 200 µg/ml during storage were investigated (Figure 4.1). Each plant extract showed different anti-oxidative activities. The rates of peroxide formation in emulsion added plant extracts were divided into 2 groups. First, the peroxide values of three plant extracts; *P. odorata*, *E. trifoliatum* and *S. wallichii*, sharply increased during storage indicating their pro-oxidant properties which speeds the oxidation process. Another group showed the rates of peroxide formation closed to control and TBHQ. However, the rates of peroxide formation in the second group were indifferent during the first 14 days of storage ($p>0.05$). When comparing with the control and the sample added TBHQ, the lower rates of peroxide formation were found in *G. sphaerogynum*, *C. sphaerica* and *V. sprengelii* extracts but the higher rate of those in *C. inermis and F. auriculata* extracts were observed ($p\leq0.05$). These results indicate that 4 plant extracts can delay peroxide formation in the initiation step of lipid oxidation reaction by donating H-atom and convert to more stable or non-radicals.

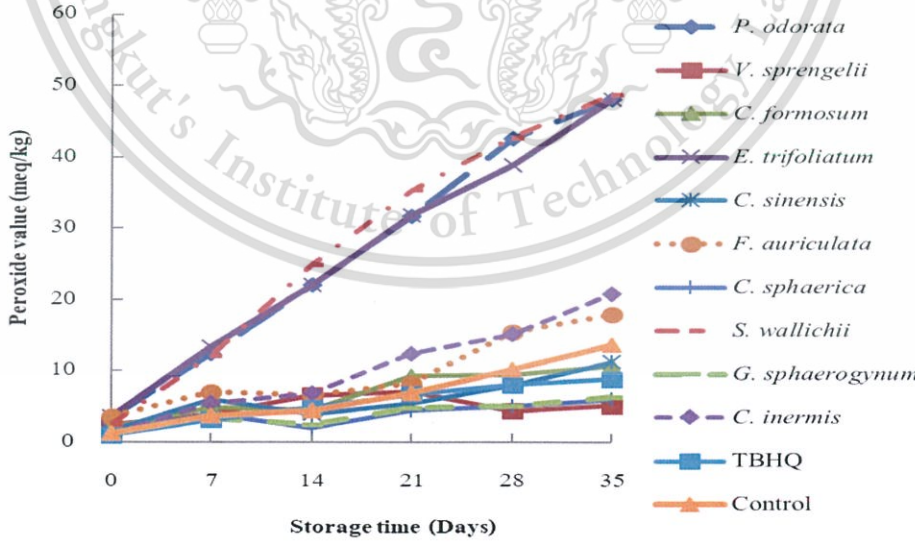

Figure 4.1 Peroxide values of O/W emulsion added plant extracts at 200 µg/ml during storage at 35 °C

PV values of each emulsion with 500 µg/ml of plant extract were shown in Figure 4.2. Only, two plant extracts; *P. odorata* and *E. trifoliatum* extracts were statistically significantly higher PV values than the control ($p \leq 0.05$). Unlike the 200 µg/ml concentration (Figure 4.1), there was three plant extract had higher PV than the control including *P. odorata*, *E. trifoliatum* and *S. walliichii* extracts. This may be due to the increase in concentration of *S. walliichii* extract, resulting in the increase efficiency of the extract. In addition, it was found that five extracts including *V. sprengelii, C. sphaerica, G. sphaerogynum, S. wallichii* and *C. formosum* had effectively inhibit the formation of hydroperoxide better than TBHQ. Moreover, it was indicated that most plant extracts had hydroperoxide generation rates lower than those with 200 µg/ml plant extracts. It was shown that the increasing concentration of the extract could enhance the inhibition of hydroperoxide formation. It may cause from chemical compositions in those plants donating hydrogen atom to reactive peroxyl (ROO) and alkoxyl (RO) in propagation step of lipid oxidation reaction resulted in reduce peroxide formation. The result was in accordance with Di Mattia *et al.* (2009) found that phenolic compounds exhibited improved antioxidant capacity when added to concentrations from 250 to 500 µM. Similar result was noted in 6 plant extracts at high concentration extracts resulting in enhancement of their potential anti-rancidity in sunflower oil emulsion by Abdalla and Roozen (1999).

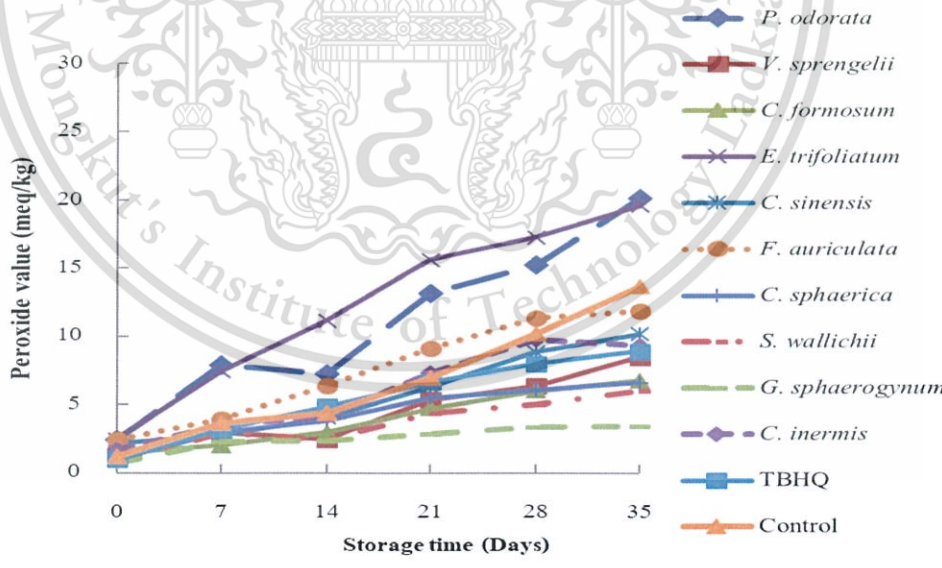

Figure 4.2 Peroxide values of O/W emulsion added plant extracts at 500 µg/ml during storage at 35 °C

2) TBARS

TBARS is a method for determining the secondary oxidation compounds production. The plant extracts at 200 µg/ml influenced on TBARS values during storage (Figure 4.3). Similar result to PV, plants were divided into 2 groups. First, TBARS of the emulsions containing three plant extracts; *P. odorata*, *E. trifoliatum* and *S. wallichii*, rapidly increased in TBARS value until the end of storage. Whereas, the other seven plant extracts were indifferent value in the first week of storage ($p>0.05$). After that, only *F. auriculata* and *C. inermis* extracts slightly increased value close to control while other five extracts remained constant. In addition, *G. sphaerogynum*, *C. sphaerica* and *C. formosum* extracts showed lower TBARS values compared with control at the end of storage ($p\leq0.05$). Ramful *et al.* (2011) reported that plant extracts at high levels of total phenolic, and a comparable amount of total flavonoids and proanthocyanidins inhibited lipid oxidation of TBARS assay in 30% soybean oil emulsion. *C. formosum* containing chlorogenic acid showed an effectiveness for inhibiting lipid oxidation of TBARS in soy bean oil emulsion (Maisuthisakul *et al.*, 2006) it was similar trend of *C. formosum* extract in this experiment. From the results, it can be interpreted that phytochemicals of plants can delay hydroperoxide decomposed resulted in reduce secondary oxidation products for TBARS.

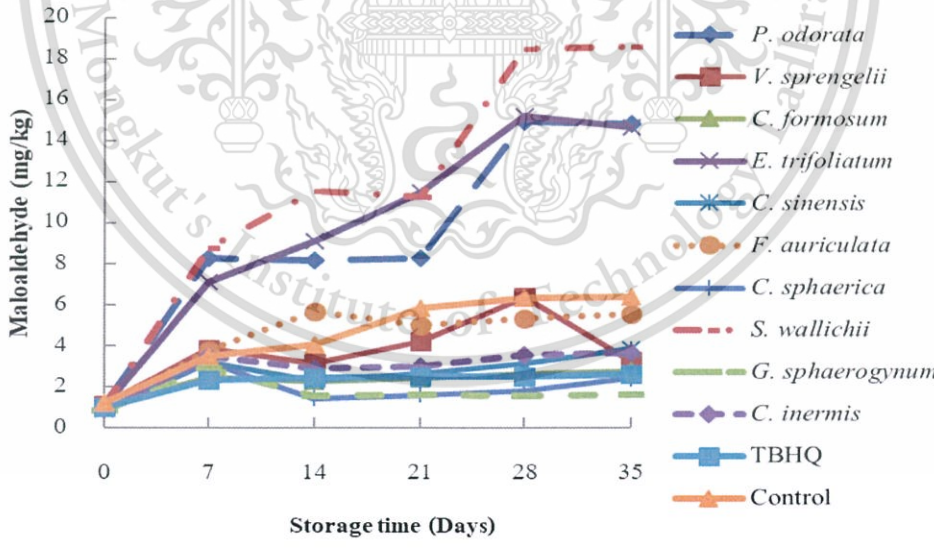

Figure 4.3. TBARS values of o/w emulsion added plant extracts at 200 µg/ml during storage at 35 °C

After emulsion storage with 500 µg/ml plant extracts, most plant extracts had a lower TBARS than emulsions with 200 µg/ml extracts and lower than that control (Figure 4.4). Especially, increasing the extract of *S. wallichii* from 200 to 500 µg/ml resulted in increased ability of aldehyde inhibition than control. According to Zhou and Elias (2013), the concentration of EGCG increased resulting in the TBARS values in the in flaxseed oil-in-water (o/w) emulsions decreased. Interestingly, *G. sphaerogynum* extract at both concentrations had the lowest TBARS values. This result was similar to the PV, and *p*-AV. Therefore, *G. sphaerogynum* could inhibit malonaldehyde formation from lipid oxidation reaction. The results indicated that increasing plant extracts to 500 µg/ml would increase the antioxidative. Interestingly, *G. sphaerogynum* showed the high antioxidation than TBHQ. The high polyphenol content which contained in *G. sphaerogynum* extract may be an important factor to delay lipid oxidation reaction.

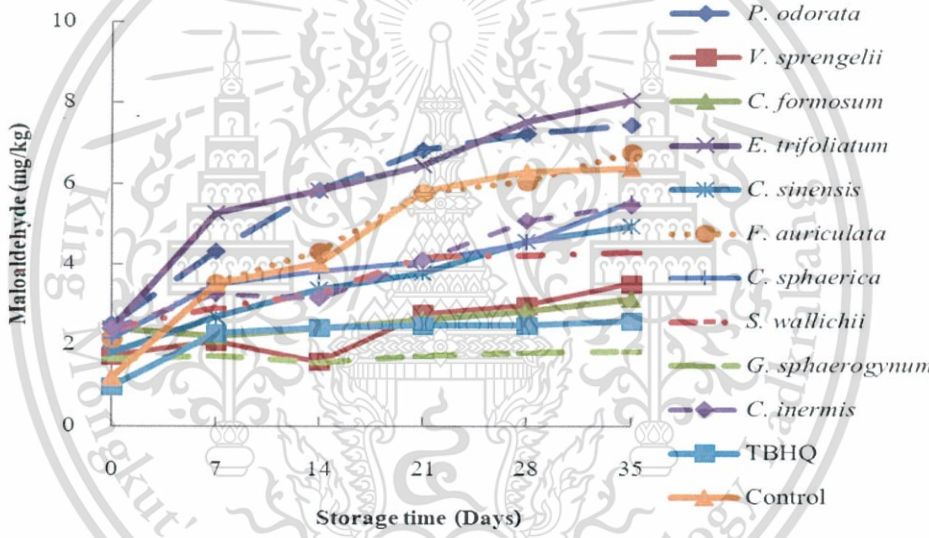

Figure 4.4 TBARS values of o/w emulsion added plant extracts at 500 µg/ml during storage at 35 °C

3) Anisidine values (*p*-AV)

The aldehydes, secondary oxidation products, are measured using the *p*-AV method. The results are presented in Figure 4.5. It was found that *p*-AV of all samples were gradually increasing during storage. The four plants; *G. sphaerogynum*, *C. sinensis*, *C. sphaerica and C. formosum* showed significantly lower *p*-AV values compared to control ($p \leq 0.05$). The results indicated that those plant extracts had high potential anti-rancidity. However, the rates of *p*-AV formation were lower compared to those of PV and TBARS formation. It may be caused by

decomposition to secondary product occurred slowly. A similar result was observed when plant extracts increased to 500 µg/ml in emulsion (Figure 4.6). However, when comparing in the same plant extract, the *p*-AV value of emulsion added 500 µg/ml extracts showed lower value compared with that added 200 µg/ml. The results also confirmed the potential of plant extracts for retarding lipid oxidation in emulsion.

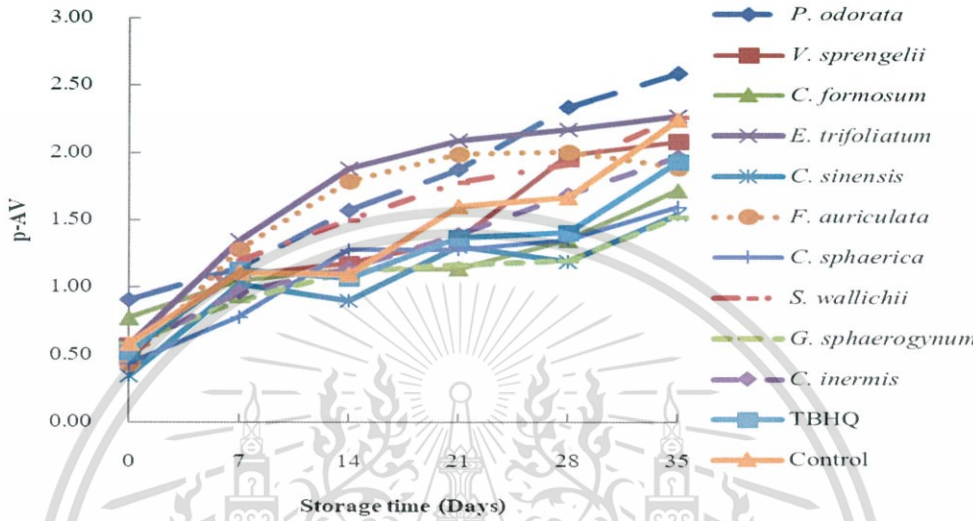

Figure 4.5 Anisidine values (*p*-AV) of o/w emulsion added plant extracts at 200 µg/ml during storage at 35 °C

After emulsion storage with 500 µg/ml plant extracts (Figure 4.6) the emulsions added with plant extracts had similar *p*-AV values throughout the storage period of 35 days. Only two plant extracts *E. trifoliatum* and *P. odorata* with *p*-AV values were significantly higher than control throughout the storage period, the same trend with PV and TBARS values.

It was found that there was different in plant extracts at 200 µg/ml. There were four species of plants, namely, *P. odorata*, *E. trifoliatum*, *S. wallichii* and *F. auriculata* were higher *p*-AV than control. Therefore, increasing of plant extracts concentration resulted in *S. wallichii* and *F. auriculata* extracts had lower *p*-AV values than control. It was indicated that, increasing concentration of plant extracts resulted in lower *p*-AV than emulsions with 200 µg/ml extracts. The increase in extract concentration did not effect on *E. trifoliatum* and *P. odorata* extracts. They still showed as the prooxidant effect (a substance that accelerates the oxidation). It is reported that *P. odorata* had the iron (Fe) in the structure. It may cause from some plants contained the metals in the molecules resulted in accelerates the oxidation in emulsion.

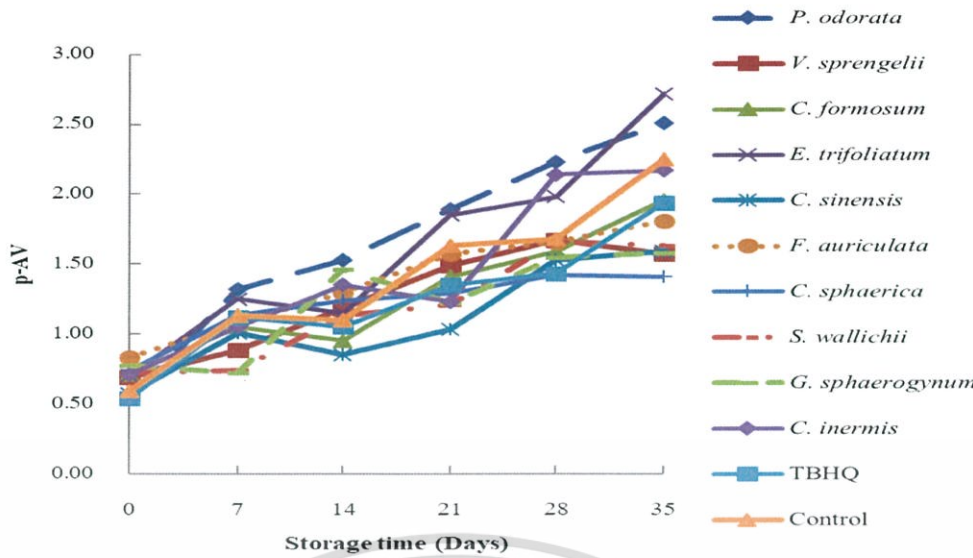

Figure 4.6 Anisidine values (*p*-AV) values of o/w emulsion added plant extracts at 500 µg/ml during storage at 35 °C

4) Oil stability index

Oxidation stability was measured by Rancimat presenting in induction time (h). At concentration of 200 µg/ml, each extract showed different oxidation stability at the end of storage varying from 2.23 – 2.98 h while TBHQ and control were equal to 3.14 and 2.84 h, respectively (Table 4.1). The induction time of *P. odorata*, *E. trifoliatum* and *S. wallichii* had lower value than that of control ($p \leq 0.05$) indicating their less effective oxidation stability. Whereas, the induction times of *V. sprengelii, C. formosum, F. auriculata* and *C. sphaerica* extracts were not significant difference compared to control ($p > 0.05$). Only plant extract, *G. sphaerogynum* showed the highest induction as well as TBHQ ($p > 0.05$). At 500 µg/ml of the extracts in emulsion, increasing concentration of plant extracts lead to increased induction time. Most extracts; *C. formosum, C. sphaerica, V. sprengelii, S. wallichii* and *C. sinensis* had higher induction time than those of 200 µg/ml of each extracts. Moreover, they had the potential as TBHQ. Obviously, only extract *G. sphaerogynum* had higher induction time than TBHQ ($p \leq 0.05$). It was suggested that, *G. sphaerogynum* extract could retard lipid oxidation in o/w emulsion.

Table 4.1 Induction time of o/w emulsion added extracts at 200 and 500 μg/ml at 35 days

Extracts	Induction time (h)	
	200 μg/ml	500 μg/ml
P. odorata	2.23±0.18^{a}	2.53±0.11^{a}
V. sprengelii	2.75±0.01^{bcd}	3.13±0.09^{d}
C. formosum	2.72±0.02^{bcd}	3.05±0.09^{d}
E. trifoliatum	2.25±0.18^{a}	2.67±0.07^{ab}
C. sinensis	2.60±0.01^{b}	3.06±0.07^{d}
F. auriculata	2.73±0.16^{bcd}	3.01±0.09^{cd}
C. sphaerica	2.87±0.06^{cd}	3.07±0.09^{d}
S. wallichii	2.35±0.16^{a}	3.13±0.11^{d}
G.sphaerogynum	2.98±0.08^{de}	3.39±0.10^{e}
C. inermis	2.61±0.01^{bc}	2.83±0.06^{bc}
TBHQ	3.14±0.16^{e}	3.14±0.08^{d}
Control	2.84±0.02^{bcd}	2.84±0.02^{bc}

Data within a column followed by different letters are significant different ($p \leq 0.05$)

4.2 Effect of plant extracts at various pHs and storage temperatures

1) Peroxide values

PV indicated the amounts of primary products of lipid oxidation. The changes in PV were shown in Figure 4.7. During storage, PV of all emulsions tended to increase. The pH highly affected on oxidative stability of emulsions. At pH 3, PV were higher than those at pH 5 and 7 ($p \leq 0.05$). Moreover, it was higher than that control at the same pH. This result indicated the poor oxidative stability of emulsion at pH 3 whereas emulsions at pH 5 and 7 were more effective in slowing down hydroperoxide formation. The oxidative stability of emulsions prepared at neutral pH was better than emulsions at acidic pH (Hu and Jacobsen, 2016). Heş *et al.* (2017) found that the antioxidant efficiency of these extracts seemed to depend on pH conditions. The protective properties of antioxidants towards methionine were higher in a pH of isoelectric point whereas towards lysine in pH below this point.

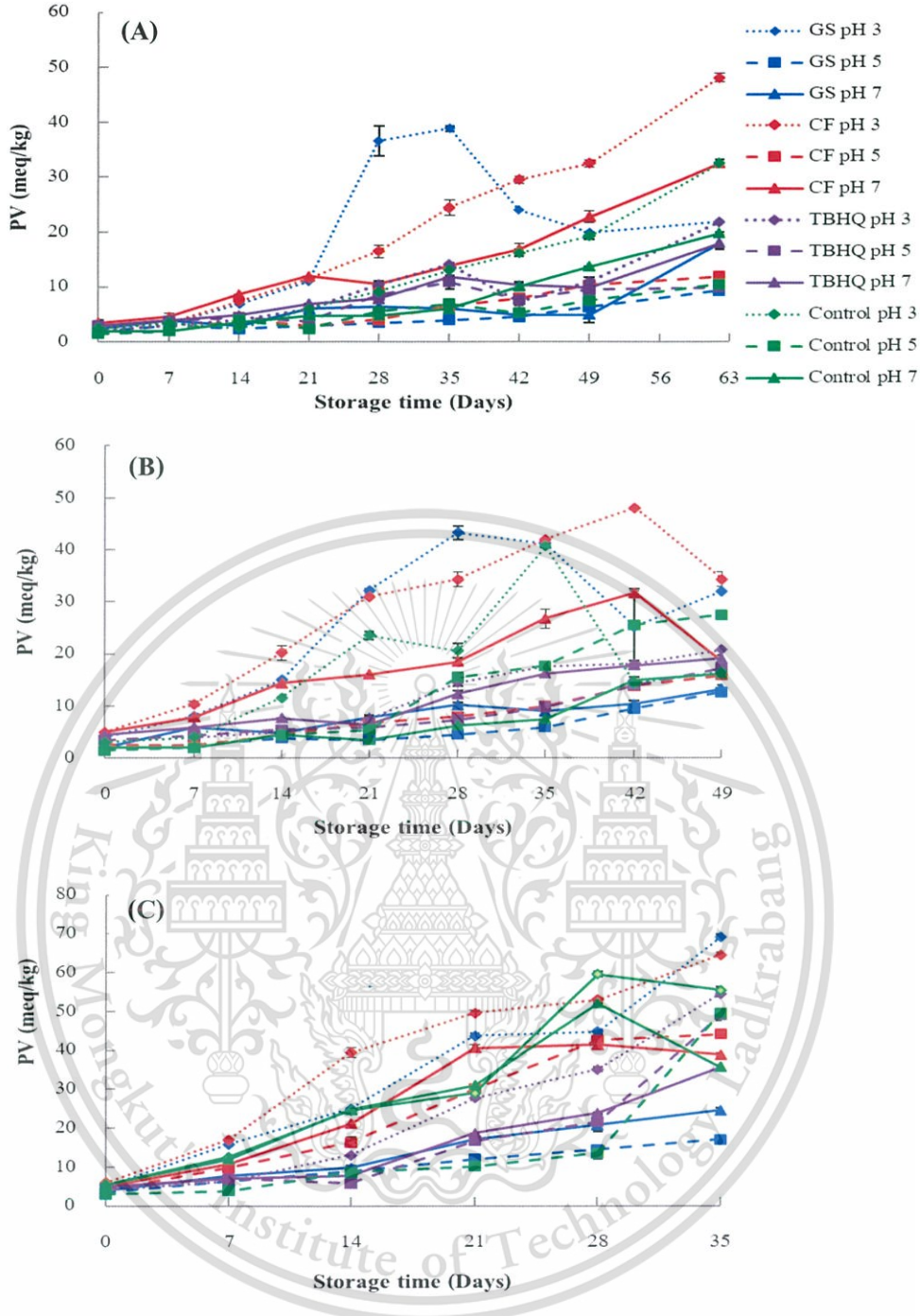

Figure 4.7. Changes in the peroxide value (meq/kg) of emulsion during storage at 25°C (A), 35°C (B), and 45°C (C). (GS; *G. sphaerogynum* extract, CF; *C. formosum* extract, TBHQ 100 µg/ml, Control; No adding any antioxidants)

Storage temperatures also affected on PV. The high temperature caused higher PV during storage. It may be caused by an instabilization of emulsion such as creaming or coalescence phenomena followed by separation between oil and liquid phase. Oil move up the top of emulsion, it was easy to contact the air leading to oxidation. Mohsin *et al.* (2016) studied

formulation and stability of topical water in oil emulsion containing corn silk extract found that at higher temperatures slight phase separation might be because of lowering of viscosity of oil phase that resulted in the sedimentation of the heavier phase under centrifugal force. It did not show significant ($p \leq 0.05$) changes at normal storage conditions (8 and 25 ^{o}C). From experiment, it was noted that emulsion at low pH and high storage temperatures resulted in the separation of water and leading to hydrolysis reaction. This phenomenon may cause high lipid oxidation reaction.

At 25°C, the PV of emulsion added *G. sphaerogynum* extract at pH 3 decreased on Day 42 whereas emulsion at 35°C decreased on Day 35 after the constant increase since Day 0. Emulsion added *C. formosum* extract pH 3 at all storage temperatures were higher than that of all samples. There was only pH 5 at 35°C had the lower than that of control at the same pH. However, it was found that, emulsion added *G. sphaerogynum* extract of all pHs and storage temperatures had the higher potential for inhibiting PV than *C. formosum* extract. Mohsin *et al.* (2016) studied formulation and stability of topical water in oil emulsion containing corn silk extract found that at higher temperatures slight phase separation might be because of lowering of viscosity of oil phase that resulted in the sedimentation of the heavier phase under centrifugal force.

Shahidi and Zhong (2010) reported that the falling in the PV values was due to the unstable primary oxidation products are decomposition, forming carbonyl compounds. The PV of emulsion stored at high temperature more rapidly increased than at low temperature. The *G. sphaerogynum* extract at pH 5 and 7 every storage temperature exhibited a greatest ability to retard the PV of emulsion. They showed significantly lower than that control and TBHQ at the same pH ($p \leq 0.05$). Ramful *et al.* (2011) found that *Eugenia pollicina* leaf extract at a concentration of 0.02% was also effective in retarding hydroperoxide formation in soybean oil emulsion during 13 days of storage at 40°C. Wang and Wang (2008) reported that PV of low pH 3 emulsion quickly increased to above 40 meq/kg after 10 to 15 days, and PV value of neutral pH emulsion were less than 20 meq/kg at the same time. The pH influences the charge of extract. Emulsion with acidic pH has positively-charged droplets and pro-oxidants in its continuous phase causing accelerated oxidation.

2) TBARS

TBARS are based on the amounts of secondary oxidation products. The changes in TBARS values are shown in Figure 4.8. Increasing of TBARS value occurs simultaneously with a decrease of PV (Nawar, 1996). Emulsion oxidation was affected by the pHs and temperatures ($p \leq 0.05$). It was the same trend with PV. TBARS values tended to increase till the end of storage. TBARS values of emulsion containing *G. sphaerogynum* extract at pH 5 and 7, and TBHQ were lower than that of the control in all of storage temperatures ($p \leq 0.05$). While, only emulsion added *C. formosum* extract pH 5 at 35 °C was lower than that of the control. TBARS values of emulsion containing *G. sphaerogynum* extract and *C. formosum* extracts at pH 3 rapidly increased higher than control of all storage temperatures probably due to effect of compound from extracts decomposition at low pH. Wang and Wang (2008) suggested that the effect of pH may be because of its catalytic activity in breaking the primary products to secondary products. McClements and Decker (2000) explained that emulsion with acidic pH has positively-charged droplets and pro-oxidants in its continuous phase causing accelerated oxidation.

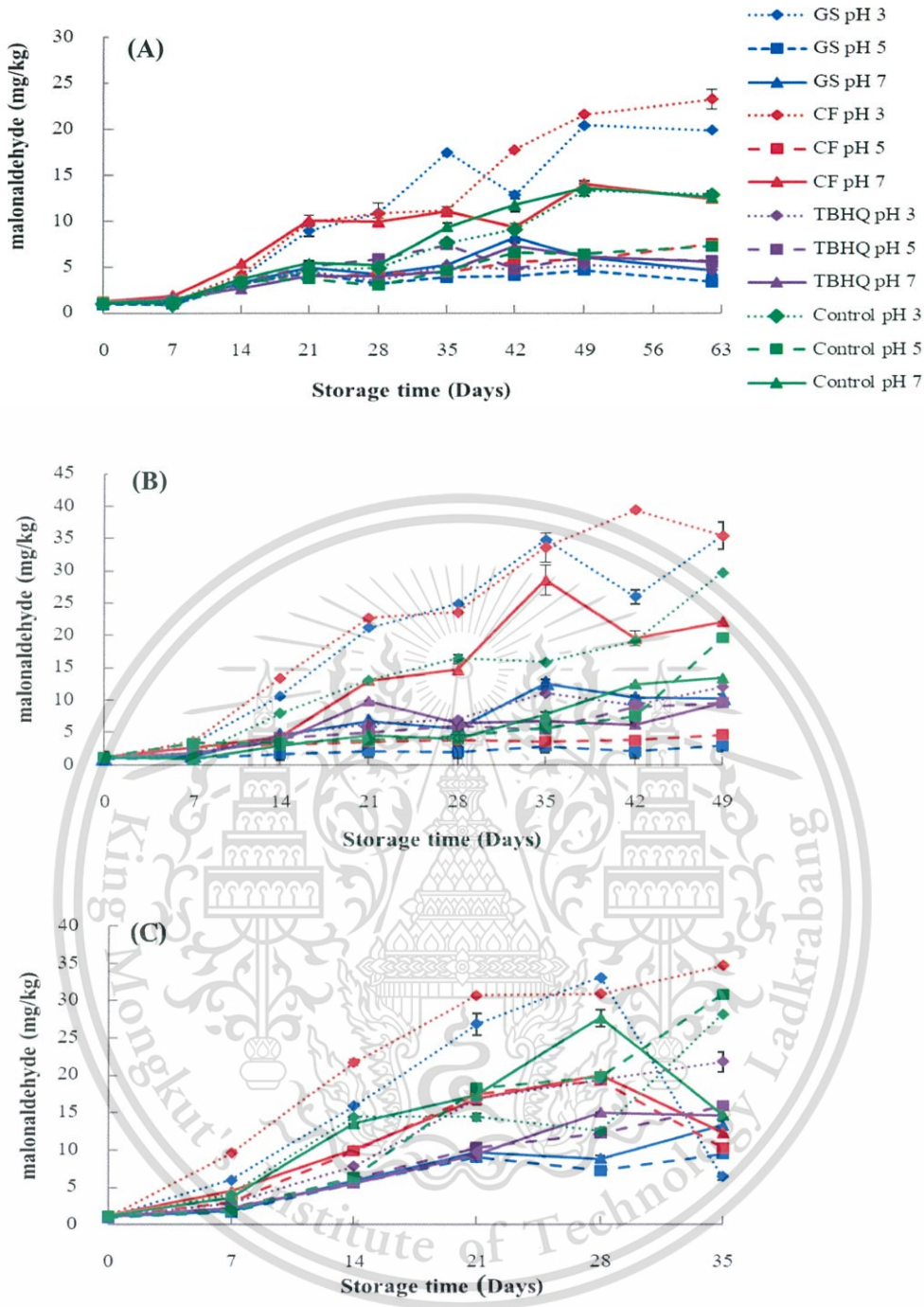

Figure 4.8 Change in the malonaldehyde (mg/kg) of emulsion during storage at 25°C (A), 35°C (B), and 45°C (C). (GS; *G. sphaerogymum* extract , CF; *C. formosum* extract, TBHQ 100 µg/ml, Control; No adding any antioxidants)

3) Anisidine values (*p*-AV)

Anisidine value (*p*-AV) is one of the methods for determining secondary oxidation products, especially aldehydes (2-alkenals). The changes in TBARS values are shown in Figure 4.9. At 25 °C, *p*-AV of all emulsion had a little change from the first day. Whereas, at 35 °C and 45 °C, emulsion added *G. sphaerogynum* , *C. formosum* extracts and the control at pH 3 were sharply increased in the last weeks of storage time ($p \leq 0.05$). However, emulsion added *G. sphaerogynum* extract at pH 5 was lower than that of all samples of storage temperatures. Moreover, at 45 °C of storage temperature, emulsion added *G. sphaerogynum* extract at pH 5 and 7 had potential than that of TBHQ at the same pHs. It was indicated that *G. sphaerogynum* extract pH 5 and kept at 25 °C had the high potential for inhibiting lipid oxidation.

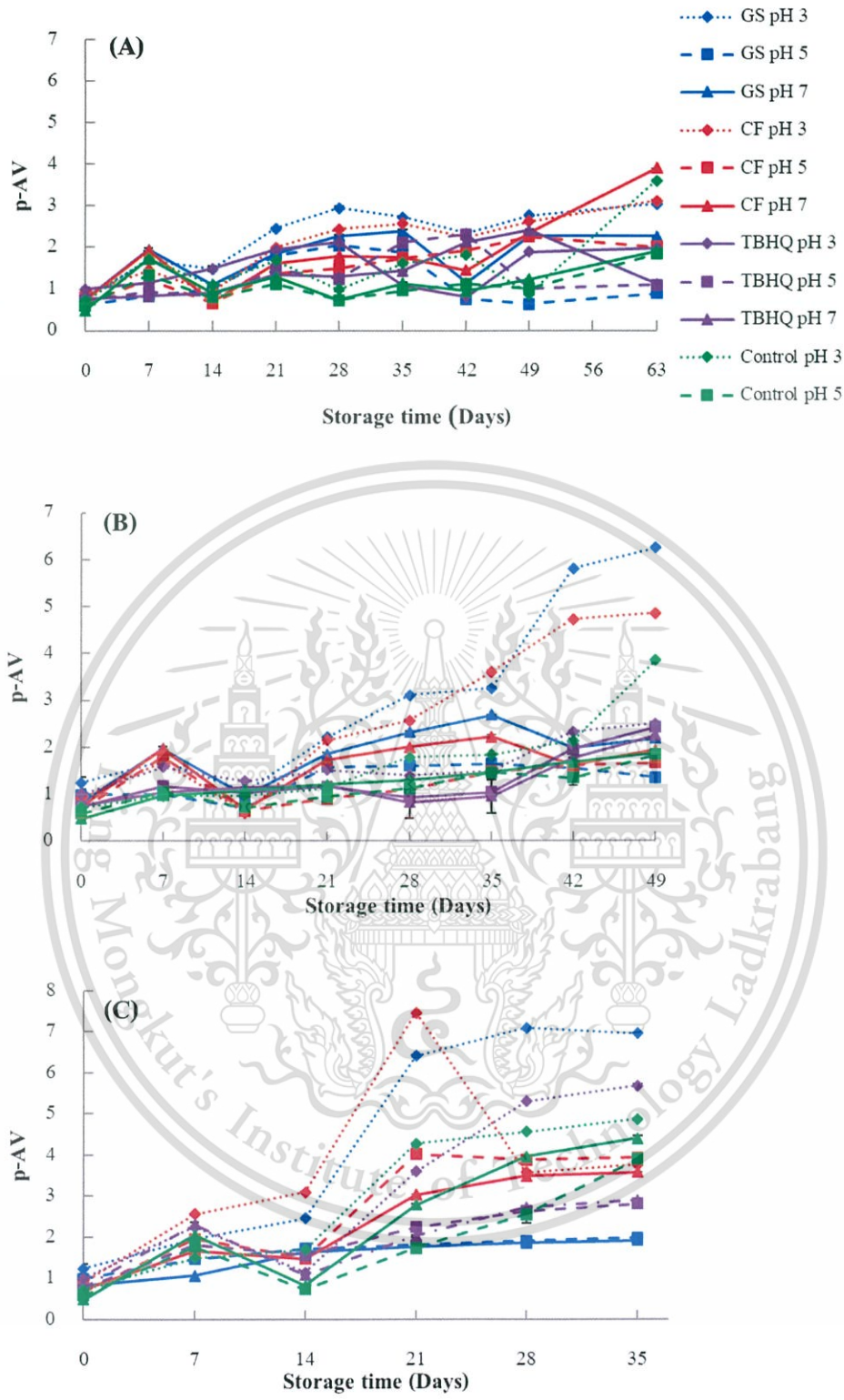

Figure 4.9. Change in the *p*-AV of emulsion during storage at 25°C (A), 35°C (B), and 45°C (C). (GS; *G. sphaerogynum* extract, CF; *C. formosum* extract, TBHQ 100 µg/ml, Control; No adding any antioxidants)

4) Oil Stability Index (OSI) analysis

OSI analysis is measured with a Rancimat machine. It is calculated as induction time, higher induction time indicating the higher oxidative stability. OSI in emulsions were measured at 0 day and at the end of storage. The effects of *G. sphaerogynum*, *C. formosum* extracts and TBHQ on the oxidative stability of emulsion are shown in Figure 4.10. The induction time of emulsion with *G. sphaerogynum* extract and TBHQ were found to be significantly longer than that of the emulsion added *C. formosum* extract and without antioxidant. Initially, the emulsions with TBHQ of all pHs showed higher induction time values (7.3-8.3 h) than emulsions with *G. sphaerogynum* extract (3.5-3.8 h) and control (3.7-3.8 h). Hamed *et al.* (2012) reported that TBHQ revealed the highest protection oxidation in sunflower oil due to the long induction periods. The induction time of all emulsion samples at the end of storage decreased. The emulsion with low pH had a short induction time. TBHQ showed stronger activity of antioxidant than emulsion added extracts of all pHs at 25 and 35°C. On the other hand, at highest storage temperature (45°C), the emulsion added *G. sphaerogynum* extract at pH 5 and 7 showed higher induction time than TBHQ. This phenomenon was probably due to a partial oxidation of polyphenols which may have even higher antioxidant potential than their non-oxidized counterpart (Cheigh *et al.* 1995; Manzocco *et al.* 1998; Nicoli *et al.* 2000).

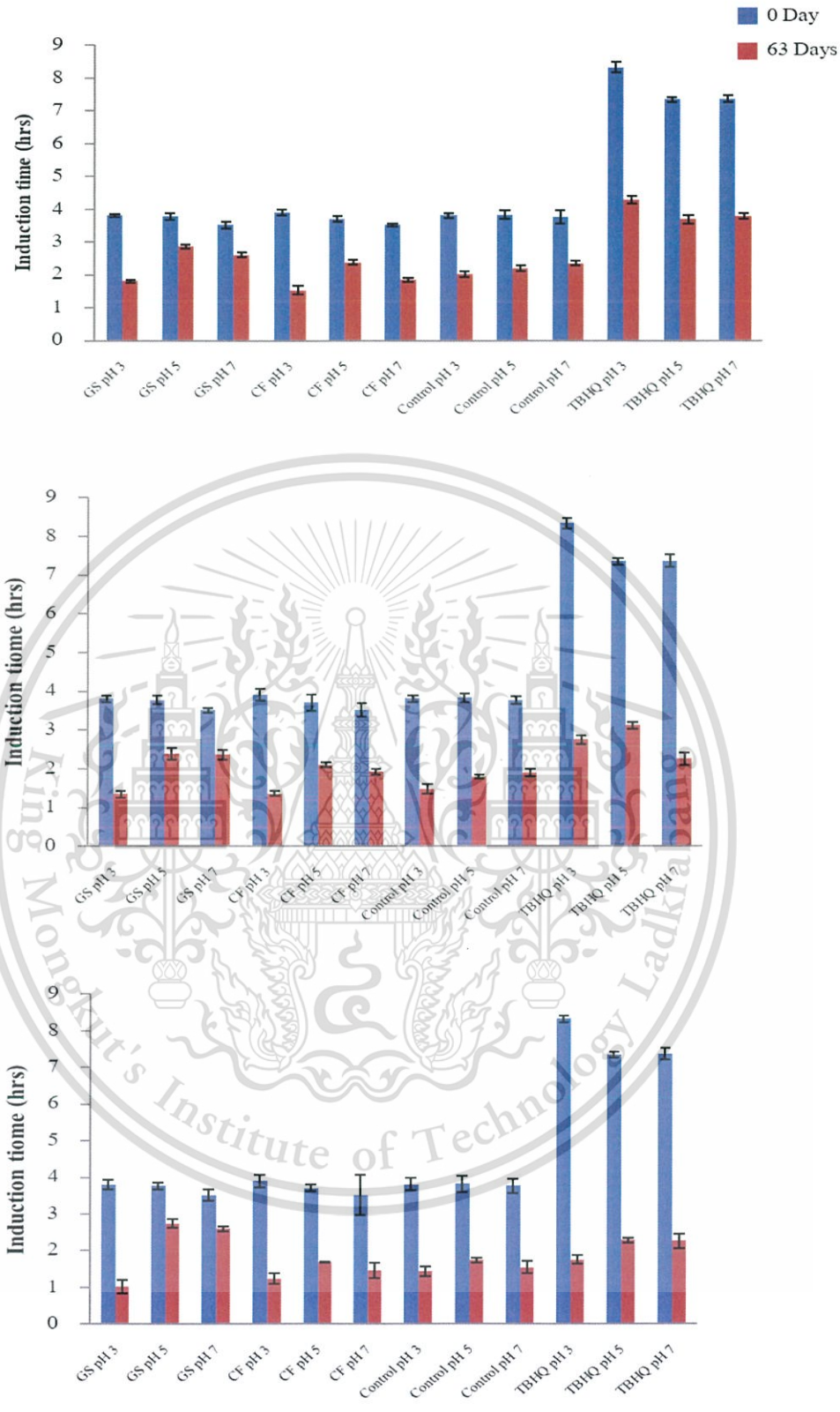

Figure 4.10 Induction period of emulsion during storage at 25°C (A), 35°C (B), and 45°C (C) (GS; *G. sphaerogynum* extract, CF; *C. formosum* extract, TBHQ 100 ppm, Control; No adding any antioxidants)

5) SPME GC-MS

The secondary oxidation products were analyzed by using the SPME to absorb rancid odor. After that, they were identified using the GC-MS. The samples for analysis were selected from section 2.2 which was emulsion at pH 5 due to plant extracts showed the great resistance to lipid oxidation reaction. The peak area from graphs (Retention time about 4 min) of each sample was calculated as hexanal content (mmol/g emulsion) by using hexanal standard curve (Figure 4.11). The results are shown in Table 4.2.

Figure 4.11 Peak area of emulsion added *G. sphaerogynum* (A), *C. formosum* (B) extracts, and control (C) kept at 25 °C by GC-MS

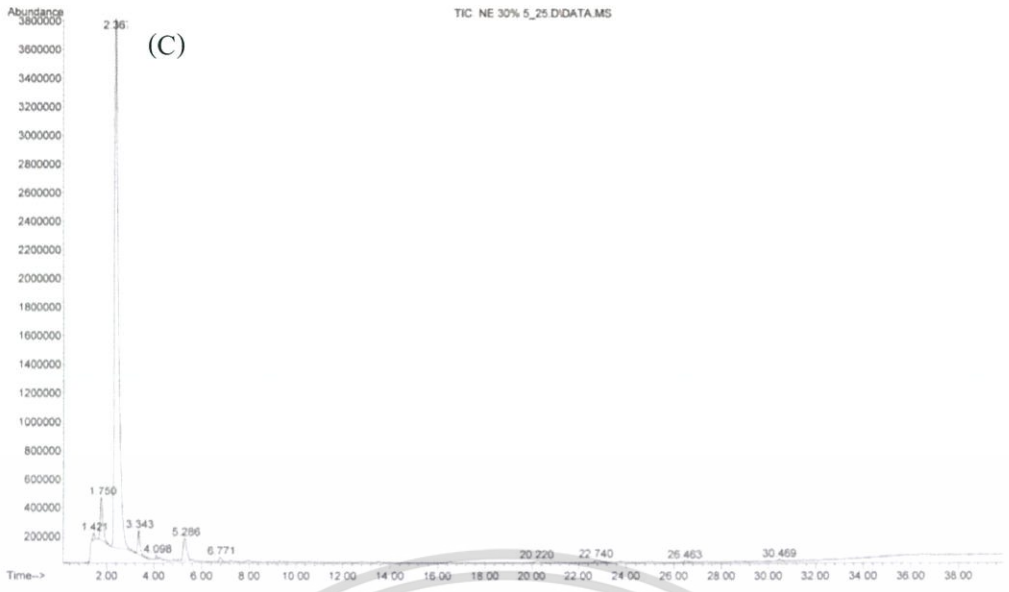

Figure 4.11 (Cont.)

Table 4.2 Hexanal content of different extracts added in emulsions and the control after kept at 25 35 and 45 °C for 62 49 and 35 days, respectively

Samples	Temp (°C)	Hexanal (mmol/g emulsion)	
		First day	Last day
G. sphaerogynum	25	0.050	0.066
	35		0.723
	45		0.463
C. formosum	25	0.073	0.100
	35		0.140
	45		0.945
Control	25	0.043	0.052
	35		4.490
	45		0.455

The concentration of volatile secondary oxidation products increases during oxidation, and hexanal is generally the main volatile produced from soybean oil emulsion. Initially in the first day, the concentration of hexanal was low in all O/W emulsion samples kept in 3 storage temperatures, and it increased gradually during the storage period. Emulsions containing extracts,

especially *G. sphaerogynum* extract showed lower concentrations of hexanal than *C. formosum* extract at 25 and 45 °C of storage temperatures.

Considering a storage temperature at 25 ° C, most samples had very little change from day 0, possibly due to the relatively low storage temperature resulted in retarding the oxidation reaction. Moreover, both plant extracts did not affect the oxidation reaction and secondary oxidation products formation because they are not different from control samples ($p>0.05$). Considering the storage temperature of 35 ° C, all samples of emulsions increased from day 0. However, it was found that both types of plant extracts had the ability as antioxidation. It could be seen that the emulsion added plant extracts showed less hexane than control (without extract). It was consistent with Gallego *et al.* (2017) found that emulsions containing extracts, especially those with added *Caesalpinia decapetala* extract, showed lower concentrations of hexanal than the negative control (without antioxidant) during the experiment. Storage at maximum temperature of 45 ° C was observed. All samples showed hexanal content increase from day 0 to the last day of storage time. Both extracts could not inhibit hexanal formation due to it gave the higher hexanal values than the control. May be due to excessive temperature may affect the structure of the substance relatively with inhibit oxidation reaction resulting in the resistance oxidation ability decreased. Moreover, it also supported the action of oxidation (prooxidant).

4. Reaction rate constants (k) of PV, TBARS and *p*-AV

Values of the oxidation rate constant for PV TBARS and *p*-AV of emulsions at different pHs and storage temperatures are reported in Table 4.3. Higher k values indicate lower stability of emulsion. The values of k_{PV} k_{TBARS} and $k_{p\text{-AV}}$ of all samples increased with the temperature, which confirmed that both factors considerably influenced the rate of the autoxidation reaction. Tan (2001) reported the reaction rate constant as a function of temperature. The reaction rate constant of emulsion without antioxidants (control) can be attributed to higher than emulsion with antioxidant. The pH of emulsion affected on reaction rate constant at lower pH than higher pHs. The overall results found that, emulsion added *G. sphaerogynum* extract at pH 5 showed the lower of k_{PV} k_{TBARS} and $k_{p\text{-AV}}$ than *C. formosum* extract at all storage temperatures. When compared with TBHQ, it had the lowest in reaction rate constant at pH 5. Moreover, among all samples showed high k at pH 3 but TBHQ was quite stable every storage temperatures.

Table 4.3 Reaction rate constants (k) of PV, TBARS and *p*-AV in emulsions at different pHs and storage temperatures

Samples	pH	k_{PV} (Day^{-1})			k_{TBARS} (Day^{-1})			$k_{p\text{-AV}}$ (Day^{-1})		
		25°C	35°C	45°C	25°C	35°C	45°C	25 °C	35°C	45°C
G. sphaerogynum	3	0.41	0.64	1.74	0.35	0.73	0.49	0.03	0.11	0.20
	5	0.10	0.20	0.39	0.05	0.04	0.26	0.001	0.01	0.02
	7	0.17	0.19	0.61	0.08	0.22	0.35	0.02	0.03	0.03
C. formosum	3	0.74	0.79	1.68	0.40	0.81	0.98	0.04	0.09	0.09
	5	0.17	0.27	1.27	0.1	0.05	0.42	0.01	0.02	0.1
	7	0.43	0.43	1.15	0.2	0.51	0.44	0.02	0.02	0.09
TBHQ	3	0.27	0.41	1.44	0.06	0.23	0.66	0.01	0.02	0.14
	5	0.13	0.27	1.13	0.08	0.17	0.44	0.01	0.03	0.05
	7	0.21	0.33	0.88	0.08	0.15	0.44	0.02	0.02	0.05
Control	3	0.46	0.39	1.61	0.22	0.54	0.66	0.02	0.05	0.13
	5	0.14	0.59	1.07	0.11	0.27	0.87	0.01	0.02	0.08
	7	0.28	0.30	1.13	0.23	0.27	0.58	0.01	0.02	0.11

5. Shelf life evaluation

Shelf life (days) was calculated according to the order of reaction rate constant. This study, the lipid oxidation reaction in the oil-in-water emulsion was apparently zero-order degradation reaction with a high R^2 value, where the reaction rate did not depend on the concentration of the oxidizing substrate. Therefore, zero order equation was used to calculated;

$$C_A - C_{AO} = -kt \quad (4.1)$$

where C_A is the limited concentration of hydroperoxide in the emulsion, C_{AO} is the initial concentration of hydroperoxide, k is the rate constant of the reaction.

In this study, shelf life was defined as the time for PV to reach 30 meq kg^{-1} of emulsion. It was the limit specified in the TISI quality index. The results were shown Table 4. 4. It was found that both extracts added emulsion at pH 3 had a shorter shelf life than those at the other pHs at every storage temperature. As for the emulsion samples at pH 5 and 7, the one at pH 7 had a shorter shelf life than the one at pH 5 when they were kept at 25 °C and 35°C, but at 45°C, the one at pH 7 of TBHQ had a longer shelf life than the one at pH 5. Out of all tested conditions, the *G. sphaerogynum* extract added emulsion had the high effective than *C. formosum* at all conditions. Moreover, *G. sphaerogynum* extract adjusted to pH 5 and stored at 25°C were shown to be the best condition more than TBHQ. It was extended shelf life for 280 days. The emulsion was stable at pH 5. This is an appropriate condition to donate H atom from plant extracts to free radicals in emulsion. The overall results indicated that the *G. sphaerogynum* extract had a good potential for usage as a new natural antioxidant for 30% oil-in-water emulsion.

Table 4.4 Shelf life evaluation of emulsion added plant extracts compared with TBHQ and control at different pHs and storage temperatures

Samples	pH	Shelf life (Day)		
		25°C	35°C	45°C
G. sphaerogynum	3	67	41	14
	5	**280**	**140**	**68**
	7	161	148	43
C. formosum	3	37	32	14
	5	165	101	21
	7	39	58	18
TBHQ	3	104	67	18
	5	208	98	23
	7	129	78	29
Control	3	60	69	15
	5	204	48	25
	7	100	93	22

Plots of the natural logarithm (ln) of the rate constant versus the inverse of temperatures (1/T) of emulsion added *G. sphaerogynum* at pH5 showed a good linear relationship (r^2=0.9671). This kinetics behavior followed the Arrhenius equation: ln k= ln A-Ea/RT. The reaction rate constant depends on temperature changing, higher temperature resulted in higher the rate of reaction.

This higher ln k results in a higher kinetic energy, which has an effect on the activation energy of the reaction. From the relationship between ln k and 1/T (K), the slope of graph was 4,741.9. The activation energy calculated from slope = Ea/R. Therefore, the values of the activation energy of emulsion added *G. sphaerogynum* at pH5 was 37.93 kJ/mol.

For prediction the shelf life at other storage temperatures of emulsion added *G. sphaerogynum* at pH5 can be calculated from the equation between ØS (take ln in the shelf life in Table 4.4) and 1/T as shown in Figure 4.12

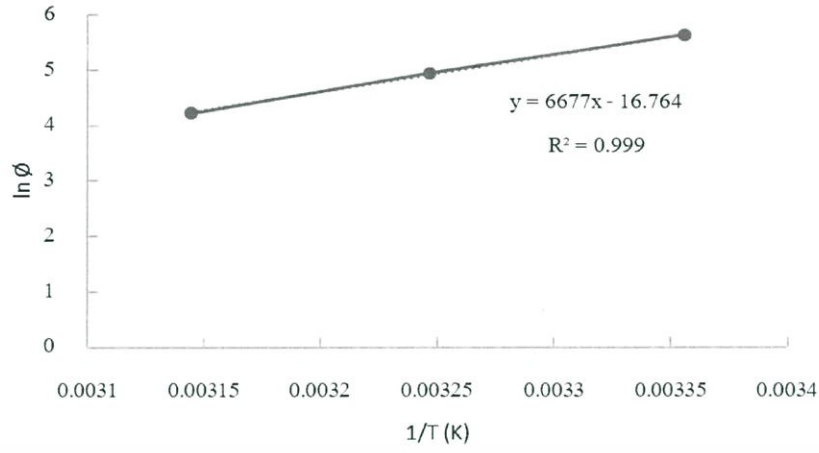

Figure 4.12 The relationship of ln ØS versus 1/T (K)

From the Figure 4.12, the shelf life (ØS) of emulsion added *G. sphaerogynum* at pH5 and stored at 15 °C can be calculated as the equation;

$$ØS = Exp\ (6{,}677)\ x\ (1/T(K) - 16.764 \quad (4.2)$$

Therefore, the shelf life of emulsion added *G. sphaerogynum* at pH5 and stored at 15 °C was 376 days or about 1 year. From experiment, it was found that decrease in storage temperature resulted in extend the shelf life of emulsions.

4.3. Evaluation of biological activities of edible Thai local plant extracts

4.3.1 Phytochemical content

The phytochemical composition (defined by the total polyphenol content, total flavonoid, total tannin, and total saponin contents of *C. sinensis, C. sphaerica, C. inermis, C. formosum, E. trifoliatum, F. auriculata, G. sphaerogynum, P. odorata, S. wallichii, and V. sprengelii* extracts) were estimated (Table 4.5). The findings in the present study showed that the spices were relatively high but not very high in polyphenols. The composition and content of the phenolic acids varied depending on the type of plants. Total phenolic contents of the ten spices decreased in the following order: *G. sphaerogynum* > *C. inermis* > *S. wallichii* > *C. sphaerica* > *V. sprengelii* > *C. formosum* > *P. odorata* > *C. sinensis* > *F. auriculate* > *E. trifoliatum*.

Table 4.5 Phytochemical contents of 10 edible Thai local plant extracts at 2000 µg/ml

Extracts	Phytochemical contents			
	TPC (µgGAE/ml)	Tannin (µgCE/ml)	Saponin (µgDE/ml)	Flavonoild (µgRE/ml)
C. sinensis	2,188^{a}±25.0	134.0^{c}±20.5	195.8^{e}±2.12	34.13^{c}±0.35
C. sphaerica	3,267^{e}±35.0	**784.5^{f}±2.83**	106.5^{b}±4.24	78.13^{d}±1.06
C. inermis	3,698^{e}±66.2	80.63^{b}±0.35	46.50^{a}±1.06	17.75^{a}±1.41
C. formosum	2,411^{b}±19.1	18.38^{a}±1.06	149.4^{d}±1.77	31.31^{b}±0.53
E. trifoliatum	1,249^{a}±13.3	14.63^{a}±0.35	83.88^{a}±0.35	23.19^{a}±0.53
F. auriculata	2,018^{c}±75.6	163.8^{d}±0.71	**426.5^{g}±2.83**	33.25^{c}±0.71
G. sphaerogynum	**4,346^{f}±52.5**	526^{e}±1.41	156.0^{d}±2.12	90.13^{e}±1.41
P. odorata	2,378^{b}±28.4	104.3^{b}±0.07	348.3^{f}±3.54	**144.0^{g}±0.18**
S. wallichii	3,310^{e}±36.1	695.5^{e}±29.7	136.9^{c}±0.35	99.63^{f}±0.35
V. sprengelii	3,146^{d}±71.3	8.380^{a}±1.06	135.5^{e}±1.41	95.25^{e}±1.06

Data within a column followed by different letters are significant different ($p \leq 0.05$)

The tannin content of the plant extracts varied from 8.38 to 784.5 µgCE/ml. The *C. sphaerica* extract found to be rich in tannin content (784.50 µgCE/ml) followed by *S. wallichii* extract (695.5µgCE/ml). Saponin content ranged from 83.88 to 426.5 µgRE/ml. The highest saponin content was found in the *F. auriculata* extract (426.5µgDE/ml) followed by *P. odorata* and *C. sinensis* extracts (348.3µgDE/ml and 195.75µgDE/ml respectively). The highest flavonoid content, was found in the *P. odorata* extract (144.0µgRE/ml) followed by *S. wallichii* (99.63µgRE/ml) and *V. sprengelii* extracts (95.25µgRE/ml), respectively. As a result, different plant species demonstrated various types of bioactive compounds, which could be responsible for their antioxidant activities. Moreover, the phytochemical content in plant foods is influenced by various factors, such as growth, season, geographic location, climate, weather, soil conditions, degree of ripeness, processing and storage. Phytochemicals are biologically active compounds that occur naturally in plants. The phytochemicals investigated, as shown in this study, have been reported to possess strong antioxidant activities due to their ability to adsorb, quench free radicals and decompose peroxides generated in the system (Adedapo *et al.*, 2008). Phenolic compounds were known to exhibit strong antioxidant activities, which have direct antioxidant properties due to the presence of hydroxyl groups, which act as hydrogen donor (Ozgen *et al.*, 2010). In this study, *G.*

sphaerogynum extract had the highest phenolic compound also showed the highest effective against lipid oxidation in o/w emulsion. Flavonoids were hydroxylated phenolics and are potent water-soluble antioxidants which help in radical scavenging and prevention of oxidative cell damage. They had been reported to possess strong antioxidant activities (Loots *et al.*, 2007). Tannins are known to be useful for the prevention of cancer as well as treatment of inflamed or ulcerated tissues (Okwu and Emenike, 2006). This study, *P. odorata*, *S. wallichii* extracts was rich in flavonoid and tannin resulted in the antiproliferation in cancer cell lines.

Moreover, the ability of a substance to act as an antioxidant depends on its strength to reduce ROS by donating hydrogen atom (Mondal *et al.*, 2005). The reducing power of the extracts was the concentration dependent and the antioxidant activities. There were many reports that support the use of antioxidants supplementation in the reducing level of the oxidative stress and in slowing or preventing the development of complication associated with disease. Bhattacharyya *et al.* (2014) reported that natural antioxidant pathways can limit the adverse effects of ROS, their levels could be stimulated by many oxidative stressors and maintained such that they contribute to tissue damage.

4.3.2 Antioxidant activities

The degree of color change (either an increase or decrease of absorbance) was correlated to the concentration of antioxidants in the sample. TEAC, DPPH and TBARS were decolourisation assays indicated the high antioxidant activity, whereas increasing in the color of FRAP indicated the higher antioxidant. Antioxidant activities were shown in Table 4.6. Different plant extracts showed different antioxidant activities. The TEAC assay ranged from 662.9 to 3,194 µg TE/ml. *G. sphaerogynum* extract had the most radical scavenging activity (3,194 µg TE/ml). The DPPH assay varied from 82.60 to 2,791 µg TE/ml and FRAP assay varied from 4,316-9,655 µg TE/ml. The highest DPPH radical scavenging activity and ferric reducing power was found in *C. inermis* (2,791 and 9,655 µg TE/ml respectively). The TBARS assay range from 56.58 - 511.0 µg TE/ml. Three plant extracts showed the highest inhibiting lipid oxidation including *C. sinensis, C. inermis*, and *F. auriculata* (511,509 and 439.7 µg TE/ml respectively).

Most plant extracts had a high potential in FRAP assay while TBARS assay had the lowest. The similar results of many researches were found that TBARS had a little relationship with polyphenol content and other antioxidant activities. Each method of antioxidant activities had different mechanisms for indicating potential of plant extracts and also depending on the compounds contained in each plant material. In addition, it also depends on the basis of mode of action, antioxidants can be classified into two main groups, namely, hydrogen atom transfer (HAT) and single electron transfer (SET) assay. DPPH, FRAP, and HAT are HAT while TBARS are SET (Karadag *et al.*, 2009)

.

Table 4.6 Antioxidant activities of 10 edible Thai local plant extracts at 2000 µg/ml

Samples	ABTS (µg TE/ml)	DPPH (µg TE/ml)	FRAP (µg TE/ml)	TBARS (µg TE/ml)
C. sinensis	1,543^{c}±45.1	227.1^{b}±22.6	7,822^{f}±116	**511.0^{h} ±0.57**
C. sphaerica	2,112^{f}±101	1,994^{e}±146	5,759^{b}±259	56.58^{d} ±0.47
C. inermis	2,697^{g}±104	**2,791^{e}±7.41**	**9,655^{h} ±286**	**509.0^{h}±0.33**
C. formosum	1,034^{d}±64.8	290.1^{b}±12.1	6,319^{a} ±151	124.7^{c} ±2.06
E. trifoliatum	829.8^{bc} ±31.6	279.3^{b}±16.8	6,064^{d} ±401	414.7^{f}±12.6
F. auriculata	662.9^{a}±19.4	82.60^{a}±2.62	4,316^{a} ±66.3	**493.7^{h} ±7.43**
G. sphaerogynum	**3,194^{h}±128**	2,245^{d}±115	8,567^{g} ±127	80.76^{b} ±23.1
P. odorata	927.6^{cd}±32.4	258.4^{b}±21.6	5,468^{b} ±232	469.2^{g} ±24.0
S. wallichii	2,057^{f}±135	2,044^{e}±67.9	7,131^{e} ±377	345.5^{f} ±15.6
V. sprengelii	793.8^{b}±49.1	261.3^{b}±11.2	5,410^{b} ±103	270.7^{d} ±3.24

Data within a column followed by different letters are significant different ($p \leq 0.05$)

The current study also revealed that TPC of samples had significantly positive correlation with their DPPH, ABTS and FRAPS methods. It meant antioxidant activities of all samples were linearly correlated in DPPH, ABTS and FRAP methods. It could be concluded that phenolic compounds are the main contributor in most antioxidant activities, increasing TPC increasing antioxidant activities by DPPH, ABTS and FRAP assay. The previous research studied the correlation between two antioxidant testing methods by determining linear regression of calibration curve. Antioxidant activities of nineteen sweet potato with varying flesh colors by DPPH method were linearly correlated to ABTS method.

Whereas, TPC in samples had a negative correlation with their TBARS. It can be predicted that there were no relationship between antioxidant activities by TBARS method (Fidrianny *et al.*,2018).

In this study, the correlation coefficients (*r*) between studied parameter TPC and ABTS DPPH TBARS and FRAP in 10 edible Thai local plants (Table 4.7). The results indicated that phenolic are major components responsible for the antioxidant activities of extracts high amount of TPC tended to increase ABTS DPPH and FRAP content except TBARS. The correlation coefficient between the DPPH free radical scavenging activity and ABTS was the highest of 0.920 followed by the relation between two methods for determination of antioxidant activity, FRAP and ABTS, with a correlation coefficient equal to 0.818. Similar results with Phomkaivon and Areekul (2009) reported that a strong correlation was found in FRAP and TEAC (ABTS•+). On the other hand, there was no correlation between total phenolic content and TBARS. It may be due to different types of phenolic compounds. The antioxidant activity of plant extracts is not limited to phenolics (Javanmardi *et al.*, 2003). The antioxidant activity may also contribute from the other antioxidant secondary metabolites such as volatile oils, carotenoids and vitamins (Wongsa *et al.* 2012). Antioxidant activity of substances may not be solely characterized by the total phenolic components and their particular structural characteristics (Shahidi *et al.*, 1992).

Table 4.7 Pearson correlation of the different methods of extracts

	TPC	ABTS	DPPH	FRAP	TBARS
TPC	1	0.837	0.813	0.575	-0.475
ABTS		1	0.920	0.818	-0.337
DPPH			1	0.703	-0.288
FRAP				1	0.026
TBARS					1

4.3.3 Cytotoxic activities

1) MTT Assay

All the plant extracts exhibited antiproliferative activity against HT-29, HepG2, KB cells with different levels (Table 4.8). Against HT-29, nine plant extracts showed higher

cytotoxicity than the positive control or the cytotoxic drug Mitomycin C at 50 µg/ml except *G. sphaerogynum* extract. Three plant extracts showed high cytotoxic activity including *P. odorata*, *S. wallichii* and *C. sinensis*. Interestingly, only have both extracts; *P. odorata* and *S. wallichii* extracts exhibited strong anticancer activity in against HT-29 cells with cytotoxic concentration levels of more than 50% cell death there were 66.86 and 65.42% respectively. In case of HepG2 cells, no plant extracts showed stronger anticancer activity than positive control. However, the extracts from three species: *P. odorata*, *V. sprengelii* and *S. wallichii* inhibited HepG2 cell growth more than 50% there were 68.94, 62.20 and 54.94% respectively. Against KB cells, *P. odorata* extract showed the highest antiproliferative activity was 69.57% followed by *V. sprengelii*, and *S. wallichii* extracts were 58.98 and 52.84 respectively.

Table 4.8 Cytotoxicity activity on HT-29, HepG2 and KB cell lines of 10 edible Thai local plant extracts at 2000 µg/ml and Mitomycin C at 50 µg/ml (Positive control)

Extracts	Cytotoxic activity (%)		
	HT-29	HepG2	KB
C. sinensis	45.15^{e}±13.20	44.38^{bcde}±15.50	45.72^{cd}±2.76
C. sphaerica	37.52^{c} ±3.83	31.24^{bcd}±15.40	48.16^{cd}±2.31
C. formosum	35.25^{c}±5.95	17.13^{b} ±0.58	46.82^{cd}±3.94
E. trifoliatum	35.51^{c} ±2.08	45.06^{bcde}±15.70	32.16^{b}±9.20
F. auriculata	31.02^{bc} ±2.11	30.56^{bc}±7.04	31.73^{b}±7.57
P. odorata	**66.86^{d}±12.90**	**68.94^{e}±17.70**	**69.57^{e}±1.04**
S. wallichii	**65.42^{d}±2.64**	54.95^{cde}±12.00	52.84^{cd}±1.59
V. sprengelii	42.29^{c}±12.50	62.20^{de}±7.76	58.98^{de}±3.39
C. inermis	20.08^{b}±6.38	11.38^{b}±3.21	14.58^{a}±4.90
G. sphaerogynum	5.620^{a}±1.01	6.785^{a}±0.55	10.94^{a}± 1.62
Mitomycin C	17.32^{b}±3.75	81.35^{f}±10.20	82.37^{g}±8.73

Data within a column followed by different letters are significant different ($p \leq 0.05$)

The potent biological activities of plant extracts are associated with their phytochemical constituents. *P. odorata*, *S. wallichii* *V. sprengelii* and *C. sinensis* have high polyphenol content. Polyphenols have been shown to induce pro-apoptotic effects. Ramos

(2007) described the pro-apoptotic properties of dietary polyphenols on a variety of human cancer cell lines derived from colon, prostate, lung and breast cancer and leukemia.

As a result, flavonoids found rich in *P. odorata* extract, similar result to Wojdylo *et al.* (2007) flavonoids were found to be the major phytochemical that important role for anticancer activity in *P. odorata* The biological activity of flavonoids exhibit anti-inflammatory, anti-spasmodic and anti-allergic activities, as well as protective effects against hepatic and vascular disorders (Okuda *et al.*,1992). Flavanoids also show inhibiting cell growth in several cancer cells, of which (+)-catechin and (-)-epicatechin were shown to protect liver cancer cells against N-nitrosodimethylamine, N-nitrosopyrrolidine and benzo (a) pyrene induced DNA damage (Delgado *et al.*, 2008).

Tannins found rich in *S. wallichii* extract was consistent with the study of Burkil, (1966). It was found that major phytochemical of *S. wallichii* are saponins and tannins. Saponins are steroid and triterpenoid glycosides that display several biological activities and have the potential for pharmaceutical application (Faizal and Geelen, 2013). They were reported about preventing the proliferation of cancer cells (Yildirim and Kutlu, 2015). Hu *et al.* (2014) found that the main component of triterpene saponins extracted from *Nigella glandulifera* (Nepenthaceae) is Nigella A. It could inhibit the growth of human lung carcinoma A-549 cell line. Saponin fractions were isolated from the leaves of *Panax notoginseng* (Araliaceae) showed cytotoxic effects against 4 cell line including KP4 cells (human pancreatic cancer), NCI-H727 cells (human lung cancer), HepG2 cells (human hepatocellular cancer) and SGC-7901 cells (human gastric adenocarcinoma) (Qian *et al.*, 2014). Tannins also exhibit biological activities such as anticancer activity. For example, hydrolysable tannin isolated from *Cuphea hyssopifolia* (Lythraceae) showed antitumor activity against to HL-60 cells (Wang *et al.*, 2000). Gallotannin inhibited the proliferation and to induce apoptosis in a human colon cancer cell line (T-84) (Gali-Muhtasib *et al.*, 2001).

As a result, MTT assay indicated that the *P. odorata, S. wallichii*, *V. sprengelii* and *C. sinensis* extracts had the strong antiproliferative activity on three cell lines. Therefore, they were selected for further study to determine 50% cytotoxic concentration (CC_{50}). The level of cytotoxicity tended to gradually increase with the increasing of the concentration of extracts (Figure 4.13-4.15). The *S. wallichii* and *C. sinensis* extracts had higher potential than the *P.*

odorata and *V. sprengelii* extracts in HepG2 cell line tested. The CC_{50} of *S. wallichii* extract on HepG2 was 347 µg/ml and 360 µg/ml in *C. sinensis* extract while *P. odorata* and *V. sprengelii* extracts were 1,666 and 1,514 µg/ml respectively (Figure 4.13). Buranrat *et al.* (2017) found that 50% alcoholic extract of C*ratoxylum formosum* was strongly inhibited in a dose- and time-dependent manner with half maximal inhibitory concentration (IC_{50}) values of 219.03±9.96 µg/ml at 24 h and 124.90±6.86 µg/ml at 48 h. Hajiaghaalipour *et al.* (2015) found that *Camellia sinensis* extract exhibited anti-proliferative effects on HT-29 cells. The choloroform extract of henna (*Lawsonia inermis*) displayed the strongest cytotoxic effect on human liver cancer cell line (HepG2) with an IC_{50} value of 0.3 µg/ml (Endrini *et al.*, 2007).

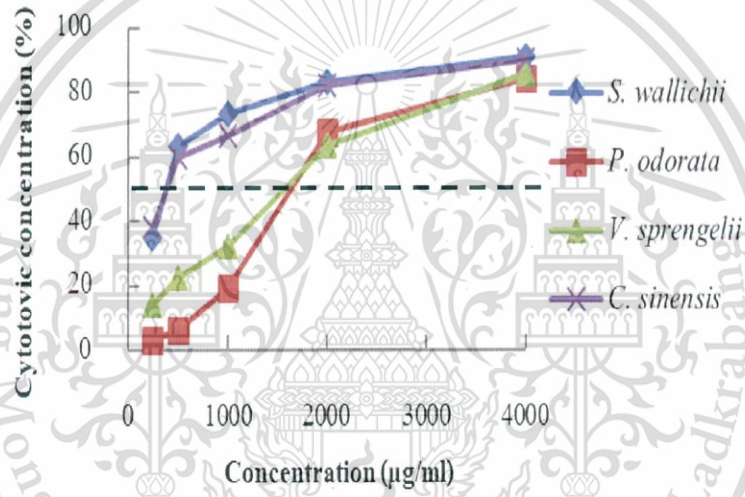

Figure 4.13 Cytotoxic concentration (CC_{50}) of 4 plant extracts on HepG2 cell line

S. wallichii extract had the highest potential on HT-29 cell. The CC_{50} of *S. wallichii* extract on HT-29 was 453 µg/ml, while *V. sprengelii*, *P. odorata* and *C. sinensis* extracts were 494, 775, and 1,214 µg/ml respectively (Figure 4.17-4.20). Figure 4.14 showed that *S. wallichii* extract higher toxic than *P. odorat*a extract on HT-29 cell.

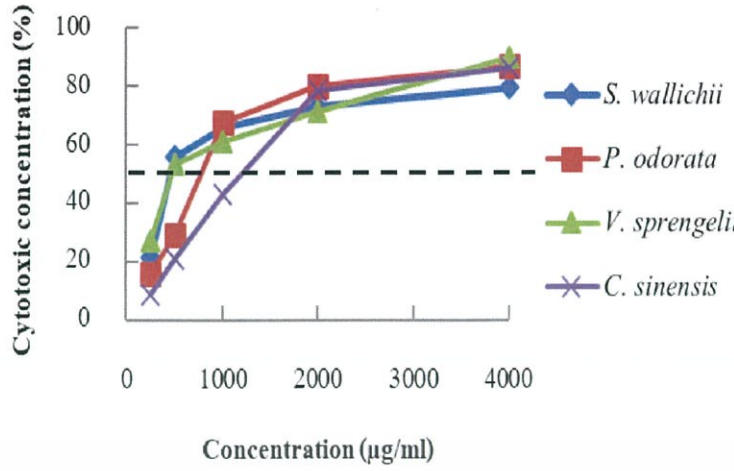

Figure 4.14 Cytotoxic concentration (CC_{50}) of 4 plant extracts on HT-29 cell line

In case of KB cell (Figure 4.15), *S. wallichii* extract had the highest antiproliferative was 287 µg/ml followed by *P. odorata* and *V. sprengelii* extracts were 496, 530 µg/ml respectively while *C. sinensis* extract had the lowest antiproliferative activity was 889 µg/ml.

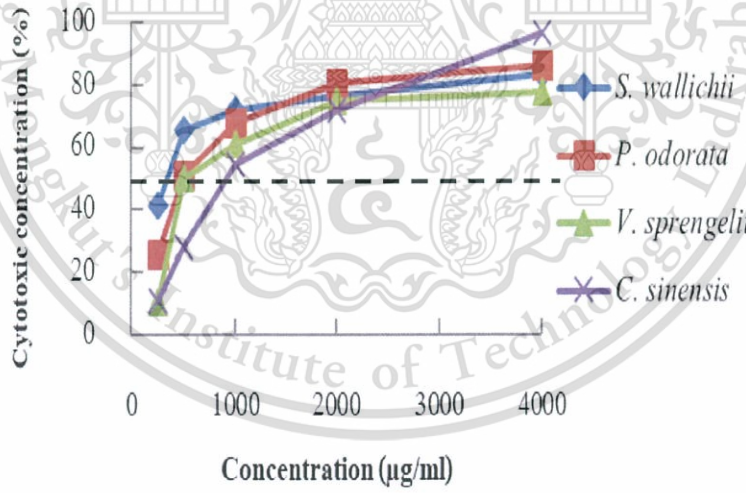

Figure 4.15 Cytotoxic concentration (CC_{50}) of 4 plant extracts on KB cell line

S. wallichii extract was more effective against on KB cells considering with CC_{50} less than HT-29 and HepG2 cells, which indicated that phytochemical in *S. wallichii* extract had more specific on KB than HT-29 and HepG2 cell lines. In addition, anti-cancer activity

of phytochemicals mainly depends on their multi-target mechanism of action, including antimutagenic, antioxidant and antiproliferative activities (Catalano, 2016).

The results indicated that the *S. wallichii* extract had a significantly higher cytotoxicity than the *P. odorata*, *V. sprengelii* and *C. sinensis* extracts against all three cell lines. However, three plant extract can destroy the Vero cell line (normal cells) except *P. odorata* extract had a little activity (Figure 4.16). The CC_{50} of *P. odorata, S. wallichii, C. sinensis* and *V. sprengelii* and extracts which were 1,140, 488, 388 and 218 respectively. The result of of *P. odorata* extract was the same as Adebayo *et al.* (2015) the extracts of *Zanthoxylum capense* were the least cytotoxic (IC_{50} > 1000 µg/mL) when the extract toxicity was determined against Vero (African green Monkey) kidney cell lines. The safety of herbal medicines remains a concern because few reports exist on the safe use of these products. Many extracts have been shown to contain potentially harmful substances that could impact adversely on human health when consumed (Watt *et al.*, 1962). Although, the study suggests that extracts of *Z. capense* had low toxicity on Vero cell lines (≥1000 µg/ml). Another research, *Cotinus coggygria* exhibit greater activity on the HeLa cell line and little activity on the Vero cell line which were IC_{50} of 293 mg/ml and >1,000 mg/ml respectively (Artun *et al.*,2016).

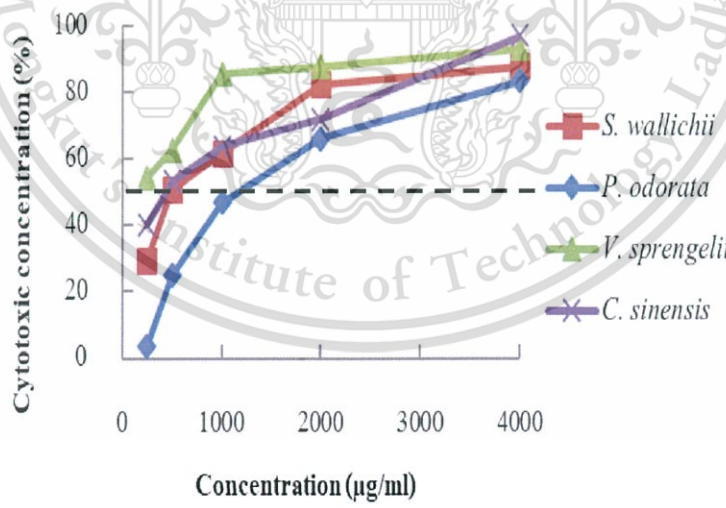

Figure 4.16 Cytotoxic concentration (CC_{50}) of 4 plant extracts on Vero cell line

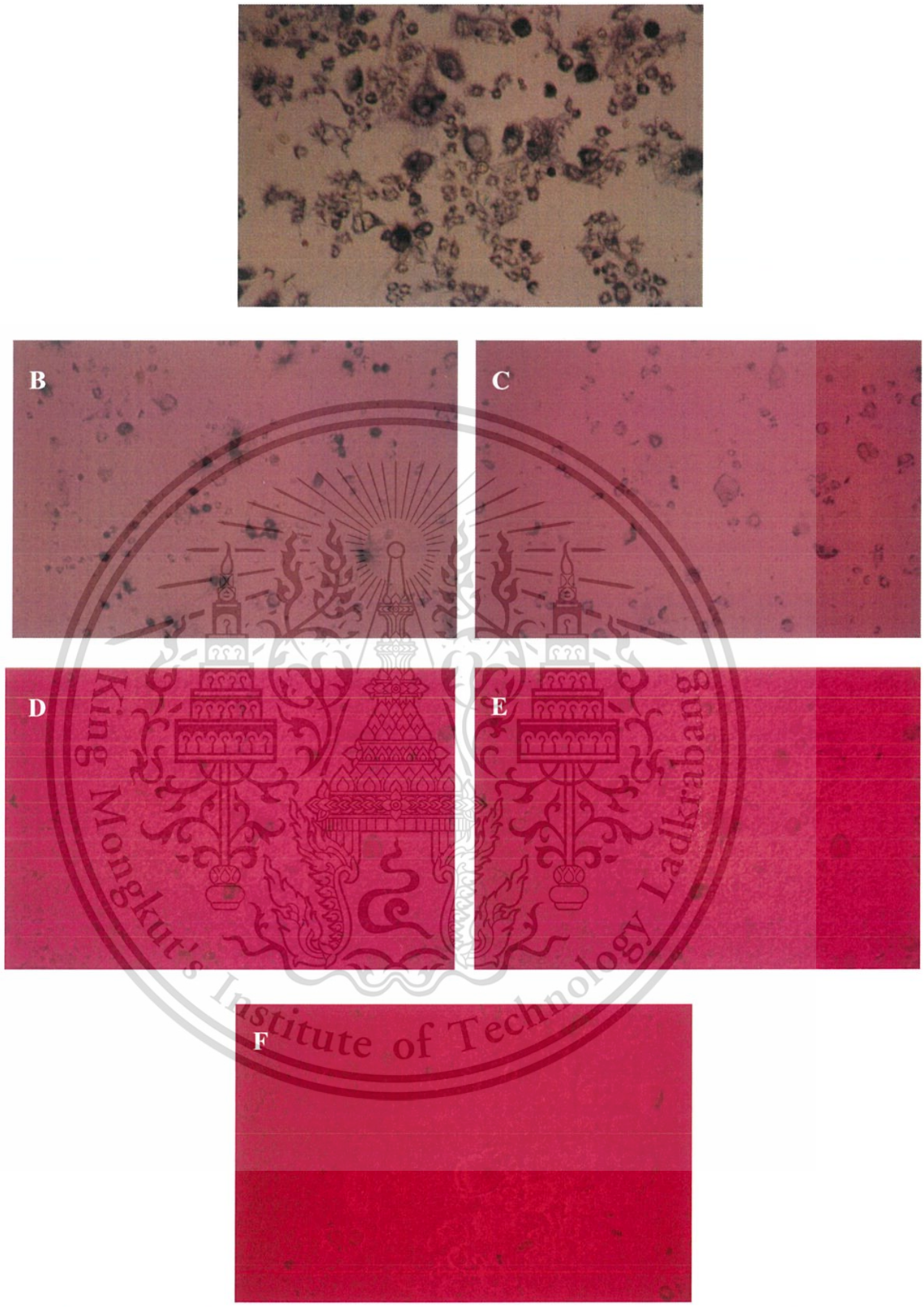

Figure 4.17 Anticancer effect (CC_{50}) of *S. wallichii* extract on HT-29 cell line; A: control, B: 250 µg/ml, C: 500 µg/ml, D: 1000 µg/ml, E: 2000 µg/ml, F: 4000 µg/ml

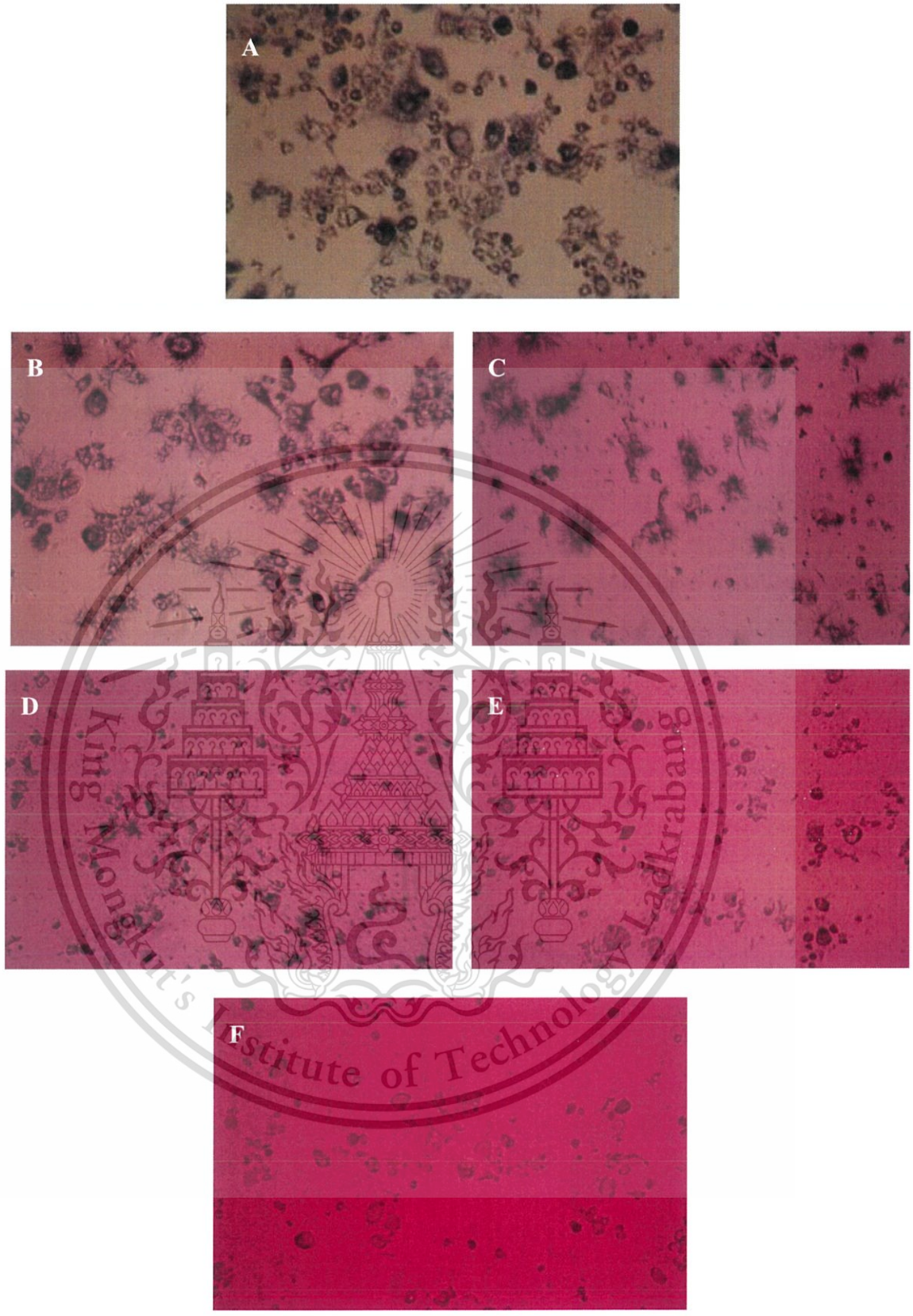

Figure 4.18 Anticancer effect (CC_{50}) of *V. sprengelii* extract on HT-29 cell line; A: control, B: 250 µg/ml, C: 500 µg/ml, D: 1000 µg/ml, E: 2000 µg/ml, F: 4000 µg/ml

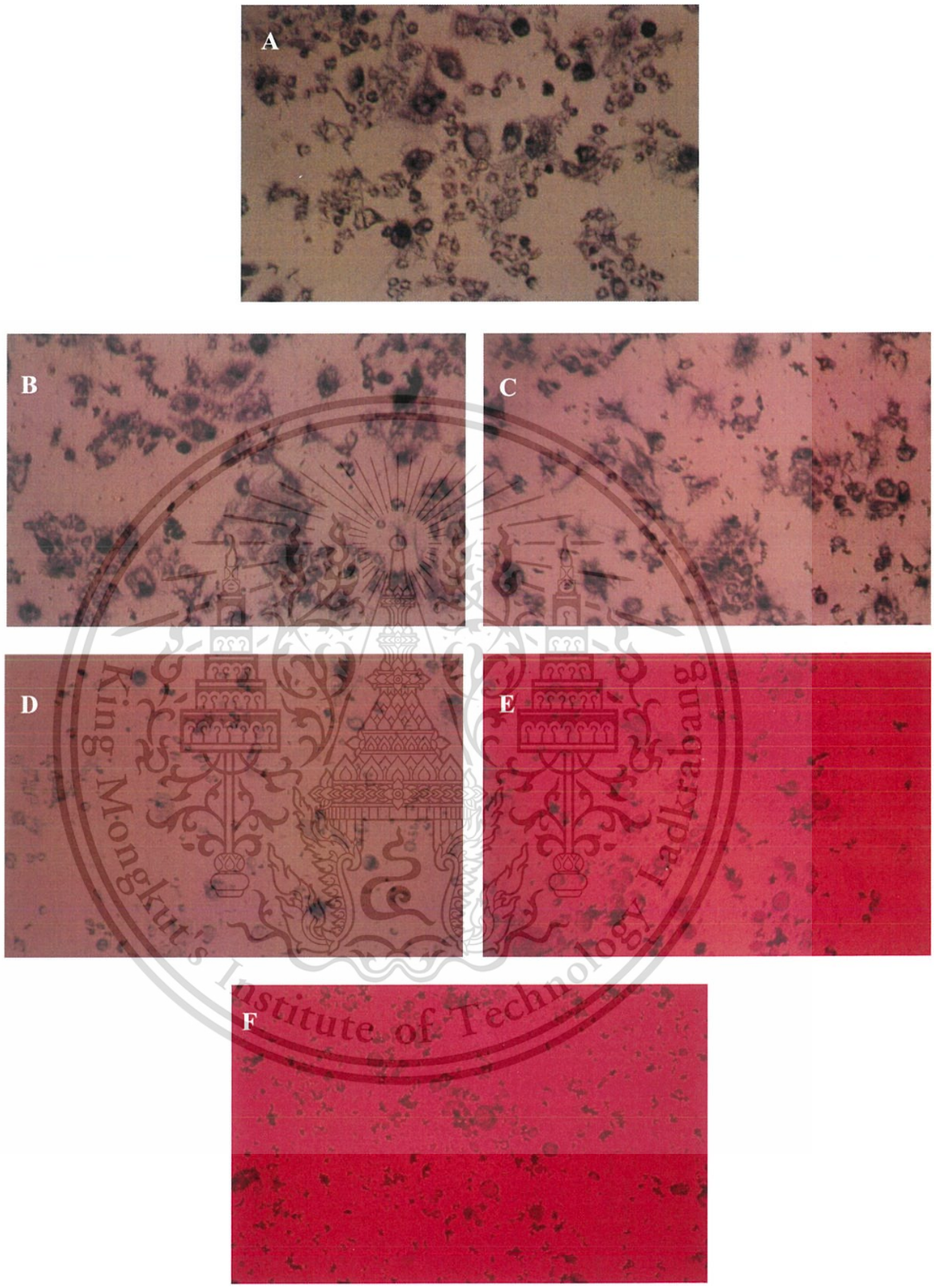

Figure 4.19 Anticancer effect (CC_{50}) of *P. odorata* extract on HT-29 cell line; A: control, B: 250 µg/ml, C: 500 µg/ml, D: 1000 µg/ml, E: 2000 µg/ml, F: 4000 µg/ml

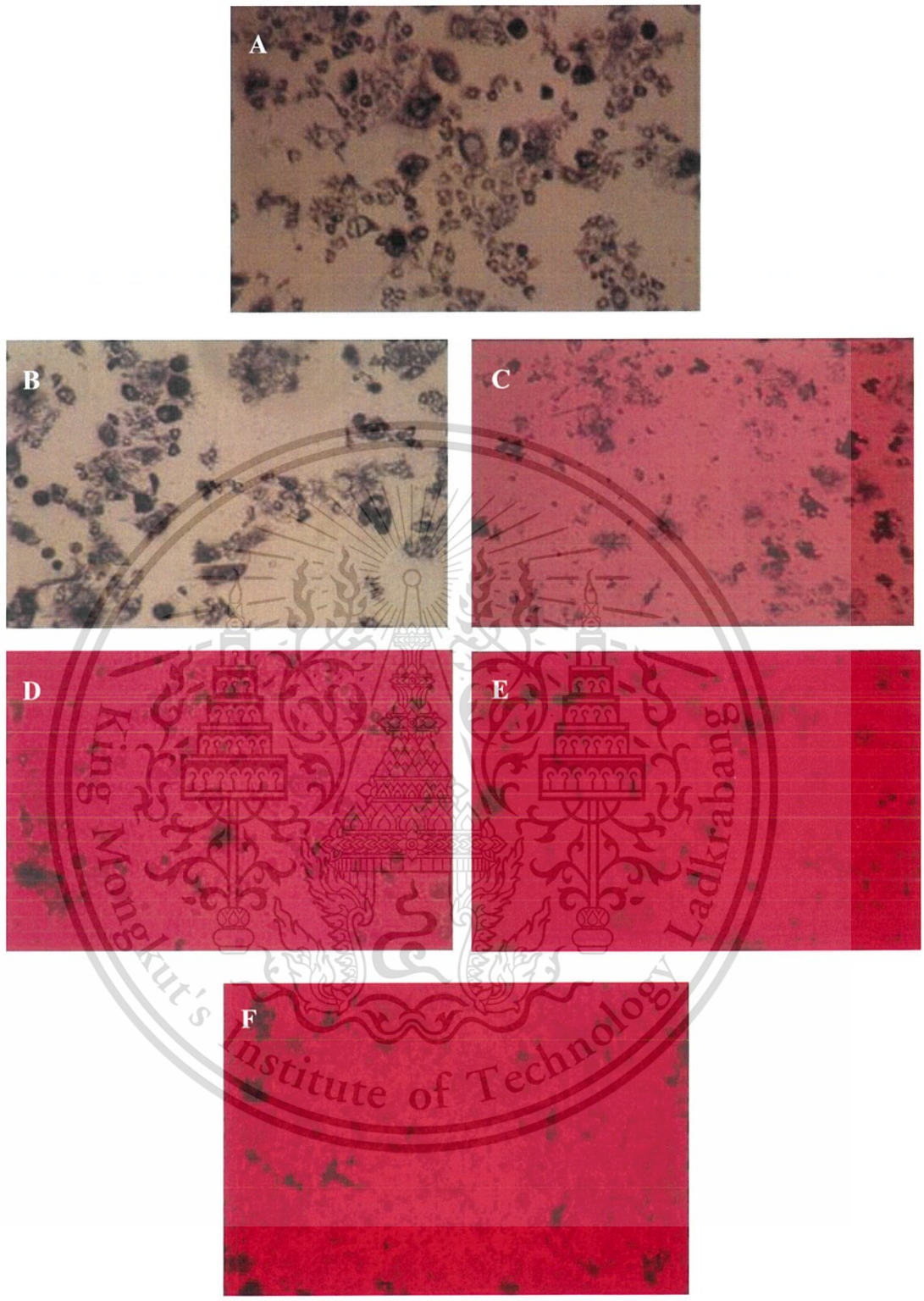

Figure 4.20 Anticancer effect (CC_{50}) of *C. sinensis* extract on HT-29 cell line; A: control, B: 250 µg/ml, C: 500 µg/ml, D: 1000 µg/ml, E: 2000 µg/ml, F: 4000 µg/ml

2) DNA Fragmentation

Figure 4.21-4.24 showed the effectiveness of the extracts that induced a very high fragmentation in HT-29, HepG2 and KB cell lines. Cells treated with *S. wallichii*, *P. odorata, V. sprengelii* and *C. sinensis* extracts induced a smear pattern when compared with the DNA ladder; meanwhile, smear pattern of damaged DNA was not observed in case of control (no adding extract). It was indicated that apoptosis in HT-29, HepG2 and KB cell lines was induced by the extracts.

Four plant extracts showed high potential in DNA fragmentation. In case of HT-29 cells, *S. wallichii* extract was more effective than *P. odorata* extract (Figure 4.21). Whereas, *P. odorata* extract was more effective than *S. wallichii* extract and other extracts in HepG2 cells (Figure 4.22). It was clearly that, *P. odorata* extract more effectively to destroy DNA than other extracts at 500 and 1000 µg/ml concentrations. It may occur from phytochemicals contained in *P. odorata* extract activate caspase family of proteases better than other extracts, and eventually lead to the degradation of chromosomal DNA of HepG2 cells. Yang *et al.* (2006) reviewed tea and cancer prevention on molecular mechanisms, molecular targets and human relevance of tea constituents. Various natural phytochemicals and dietary compounds such as phytochemicals including curcumin, resveratrol, lycopene, folates and tea polyphenols possess chemopreventive properties, and in-vitro and animal studies support that these compounds may modulate signaling pathways involved in cell proliferation and apoptosis in transformed cells (Kotecha *et al.*, 2016). When the results of both extracts was compared with positive control, Mitomycin C at 100 µg/ml, positive control much more destroyed DNA in HepG2 compared to those in HT-29 cell lines.

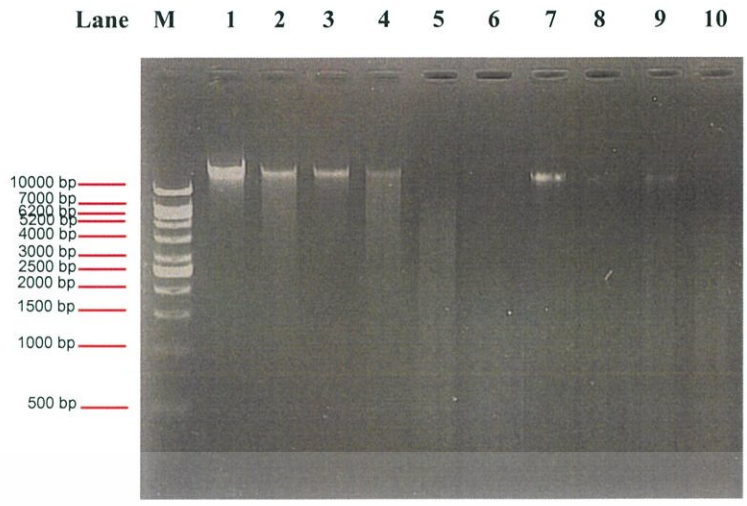

Figure 4.21 Agarose gel electrophoresis of DNA fragmentation on HT-29 cell line and cells treated with *P. odorata* (PO), *S. wallichii* (SW), *V. sprengelii.* (VS), and *C. sinensis* (CS) extracts for48 h; M: marker 1kb, 1: control, 2: Positive control (100 µg/ml), 3-10: cell treated with PO (500 µg/ml), PO (1000 µg/ml), SW (500 µg/ml), SW (1000 µg/ml), VS (500 µg/ml), VS (1000 µg/ml) and CS (500 µg/ml), CS (1000 µg/ml) respectively.

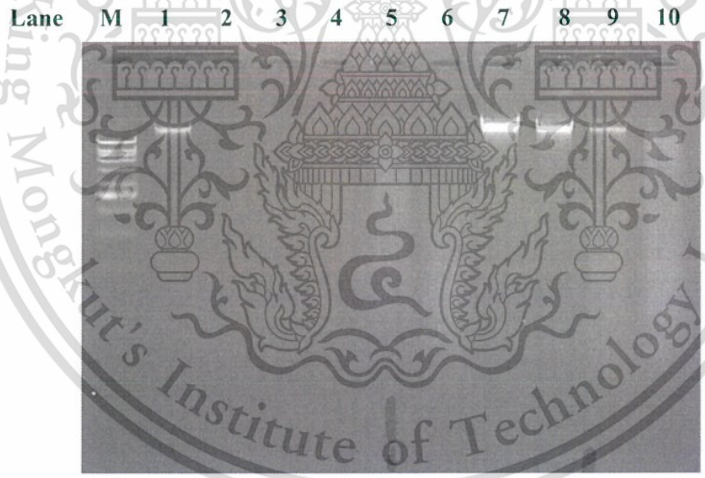

Figure 4.22 Agarose gel electrophoresis of DNA fragmentation on HepG2 cell line and cells treated with extracts for48 h; M: marker 1kb, 1: control, 2: Positive control (100 µg/ml), 3-10: cell treated with PO (500 µg/ml), PO (1000 µg/ml), SW (500 µg/ml), SW (1000 µg/ml) , VS (500 µg/ml), VS (1000 µg/ml) and CS (500 µg/ml), CS (1000 µg/ml) respectively.

In case of KB cells, four extracts showed smear pattern of DNA fragmentation. It was found that *S. wallichii* extract was highest destroy DNA among other extracts (Figure 4.23)

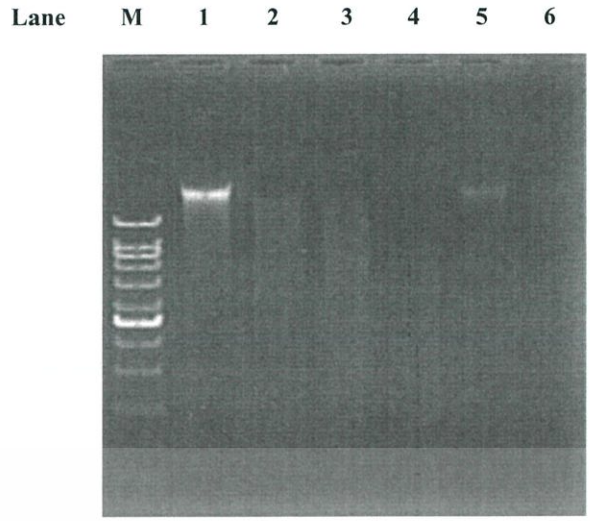

Figure 4.23 Agarose gel electrophoresis of DNA fragmentation on KB cell line and cells treated with extracts for48 h at 1000 µg/ml; M: marker 1kb, 1: control, 2: Positive control (100 µg/ml), 3-6: cell treated with PO, SW, VS and CS respectively.

DNA fragmentation on TK6 cell line (normal cell line) treated with extracts were investigated (Figure 4.24). The results showed that, all plant extracts affected on normal cell line resulting in DNA fragment. *P. odorata* extract had the highest effect followed by *S. wallichii* , *V. sprengelii* and *C. sinensis* extracts.

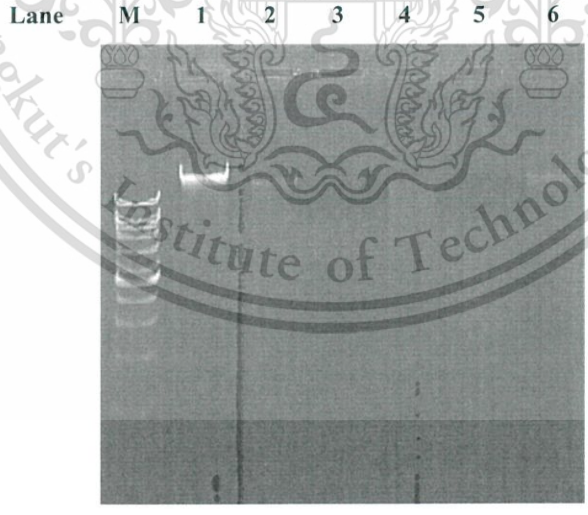

Figure 4.24 Agarose gel electrophoresis of DNA fragmentation on TK6 cell line and cells treated with extracts for48 h at 1000 µg/ml; M: marker 1kb, 1: control, 2: Positive control (100 µg/ml), 3-6: cell treated with PO, SW, VS and CS respectively.

The result of *P. odorata* extract for destruction of DNA on HepG2 cells was opposite to CC_{50} of the MTT assay. In MTT assay, it was found that *S. wallichii* extract was more effective than the *P. odorata* extract on HepG2 cells. Due to the fact that, MTT assay, it is indirect measurement, plant extracts inhibited succinase dehydrogenase in mitochondria, result in the MTT reagent change to formazan crystals whereas DNA degradation associated with caspase-3-mediated cleavage. It releases caspase-activ, DNA degradation associates with caspase-3-mediated cleavage. It releases caspase-activated DNase (CAD) lead to degrading DNA into oligonucleosomal fragments (Enari *et al.*, 1998).

It was concluded that *P. odorata* extract had a higher effective for DNA fragmentation than *S. wallichii* extract on HepG2. Phytochemical in *P. odorata* extract may activate caspase 3-mediated cleavage leading to DNA fragments. Increasing the concentration of *P. odorata* and *S. wallichii* extracts had the typical DNA laddering in agarose gels which were observed in both cell lines. It may occur from some phytochemicals in *P. odorata* extract activate CAD resulted in DNA fragmentation. The result of *S. wallichii* extract was similar to those of Halimah *et al.* (2015) who reported that kaempferol-3-*O*-rhamnoside, the major compound found in the ethyl acetate fractions of the *S. wallichii*, inhibited the proliferation of MCF-7 cells through the activation of caspase-9 and caspase-3 proteins inducing apoptosis.

3) Apoptosis observation

Two plant extracts (*P. odorata* and *S. wallichii*) were investigated on normal cell line (TK6) as shown in Table 4.9. The effect of plant extracts, *P. odorata* and *S. wallichii*, were stained with fluorescence dyes. AnnexinV and PI were used to detect apoptotic cell. The morphological change of TK6 cells treated with 500 and 1000 µg/ml of both extracts were compared to untreated control after fluorescence staining. AnnexinV exhibits viable cells and early apoptotic cells with green nuclei and PI stained with late apoptotis. As shown in Figure 4.25.

The results showed that the cells were treated with extracts occur membrane blebbing, cell shrinkage and chromatin condensation. Both extracts induced apoptotic cells in TK6 cell line of both concentrations of extracts after 24 h. It was also those observed in the control cells However, most cells treated with *P.odorata* extract showed orange-stained cells

under fluorescent microscope indicating necrosis effect while cells treated with *S. wallichii* extract showed red-strain indicating that apoptotic effect. The apoptosis mechanism involves several signal transduction pathways. Apoptotic proteins may form membrane pores and cause mitochondrial swelling and increase the permeability of the mitochondrial membrane and leak out the apoptotic effectors. Caspases, which carry out the cell degradation and are normally suppressed by IAPs, proceed for cell apoptosis process (Fesik and Shi, 2001)

The present study demonstrates that *P.odorata* and *S. wallichii* extracts showed high antiproliferative activity against HT-29 , HepG2 and KB cell lines. However, they have toxic to normal cell line (TK6). Dunkem *et al.* (2003) studied cytotoxicity and apoptosis induced by MNNG in a pair of human lymphoblastoid cells expressing wild-type p53 (TK6) and mutant p53 (WTK1) and show that TK6 cells are more sensitive than WTK1 cells to cell killing (determined by a metabolic assay) and apoptosis. Furthermore, it was similarly results with Bhouri *et al.* (2012) studied investigation of the apoptotic way induced by digallic acid (DGA) that purified from *Pistacia lentiscus* in human lymphoblastoid TK6 cells and found that TK6 occurs by apoptosis as shown by oligonucleosomal DNA cleavage ("DNA ladder"). It was indicated that TK6 cells sensitive with reagents or plant extracts.

Table 4.9. Percentage of apoptosis of two plant extracts on normal cell line (TK6)

Samples	**% Apoptosis**
P.odorata (500μg/ml)	82.75
P. odorata (1000 μg/ml)	89.60
S. wallichii (500 μg/ml)	85.67
S. wallichii (1000 μg/ml)	88.46
Control	30.87

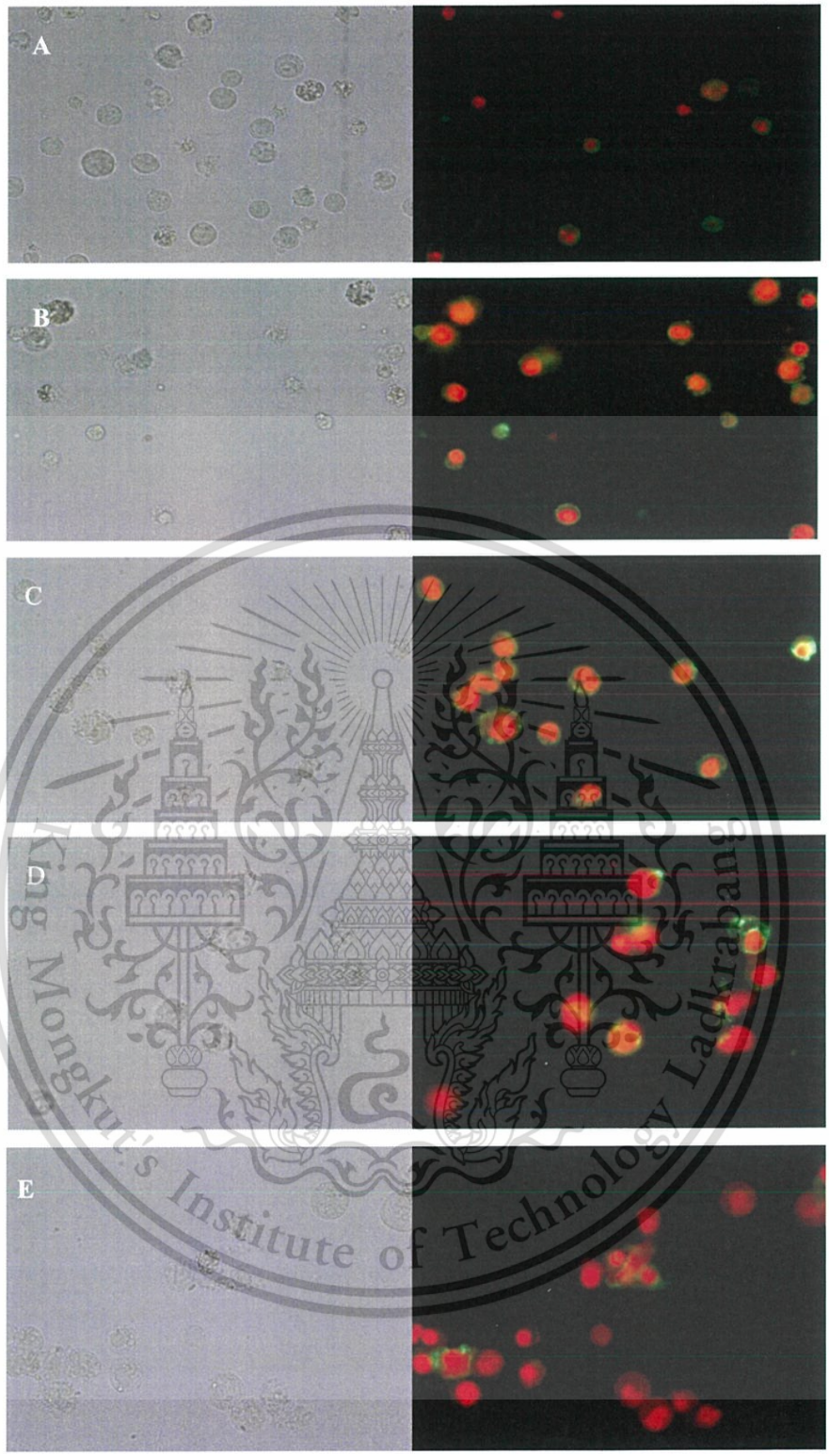

Figure 4.25 Photomicrographs of *P.odorata* (PO) and *S.wallichii* (SW) extracts on TK6 cell line (left) before and (right) after under the fluorescence microscope; A: cells without treatment (control), B-C: PO 500 and 1000 µg/ml, D-E: SW 500 and 1000 µg/ml respectively.

4.3.4 Anti-inflammatory activities

1) Lypoxygenese assay

Lipoxygenases are a group of oxidative enzymes which are involved in the regulation of inflammatory responses by generation of pro-inflammatory mediators. Antioxidant can exert inflammatory effect. Therefore, antioxidant of ten plant extracts for inhibit lipoxygenase were investigated at 100 and 500 µg/ml (Figure 4.26).

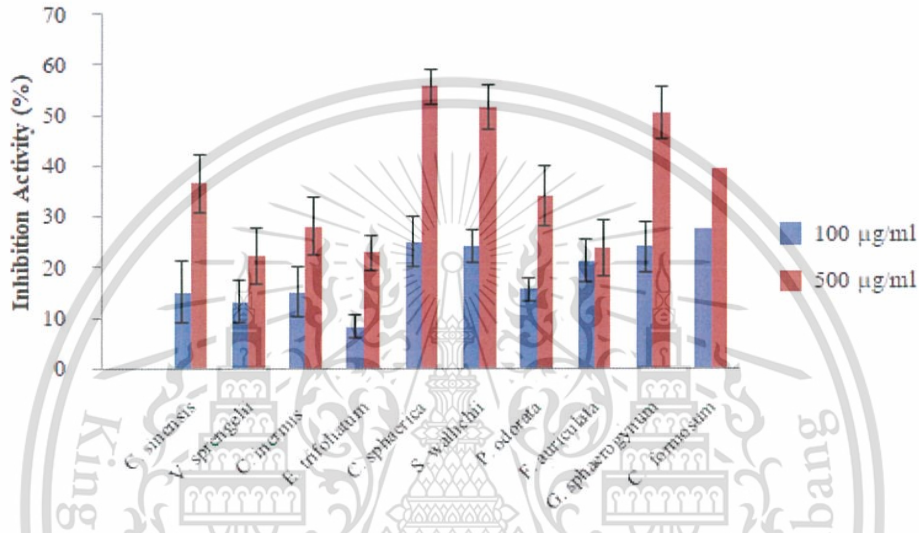

Figure 4.26 Inhibition activity of 10 plant extracts at 100 and 500 µg/ml on lypoxygenase

For lipoxygenase assay, ten plant extracts at the concentration of 100 µg/ml showed lower activity than 50%. When the concentration of extracts was fixed at 500 µg/ml, two plant extracts (*C. sphaerica* and *S. wallichii*) exhibited moderate inhibition on lipoxygenase inhibition (55.67 and 51.59% respectively). The calculated half –maximal inhibitory concentration (IC_{50}) of these plant extracts were 339.5 and 470.2 µg/ml, respectively, whereas the standard sample, NDGA provided 4.05 µg/ml (Figure 4.27). Nishida *et al.* (2014) reported that the *Scilla scilloides* extracted by ethyl acetate exhibited inhibitory effects on lipoxygenase and hyaluronidase activities with IC_{50} values 31.5 and 169 mg/ml, respectively.

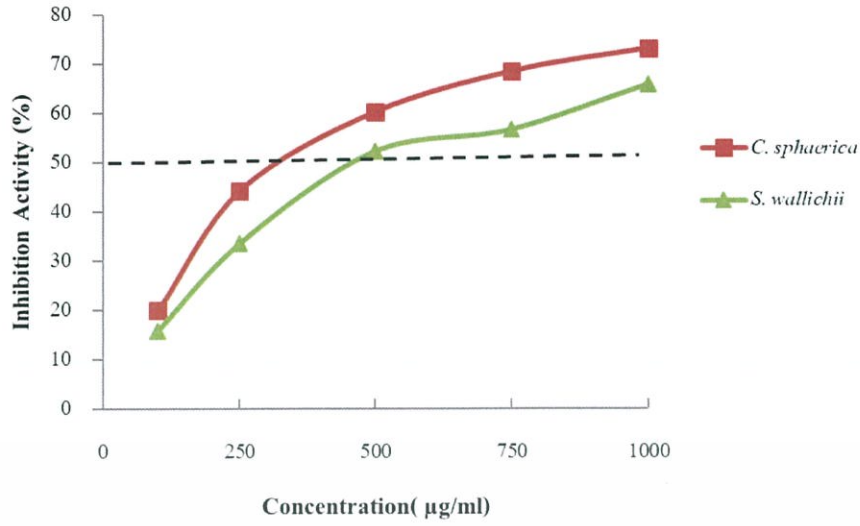

Figure 4.27 IC_{50} of *C. sphaerica* and *S. wallichii* extracts on lypoxygenase

C. sphaerica and *S. wallichii* extracts contained the rich of tannins and flavonoids. They showed the potential for inhibiting lipoxygenase. The possibility of flavonoids, they could contribute in inhibiting enzyme activity. Similar result to Paguigan *et al.* (2014) found that *C. diffusa* and *E. hirta* extract were both positive for the presence of flavonoids and terpenoids. It is possible that the compounds contained the extract are responsible for the observed bioactivity especially inhibits lipoxygenase. In the literature, natural compounds reported as 15-Lypoxygenase inhibitors comprise of flavonoids. The same results with Guardia *et al.* (2011) three plant flavonoids; rutin, quercetin and hesperidin were found to have anti-inflammatory effects. Onions (*Allium cepa*) contain a high concentration of quercetin and studies confirmed the anti-inflammatory activities of onion juice and extracts Oliveira *et al.* (2015). *Abutilon indicum* extract also contains high amounts of quercetin and has significant anti-inflammatory activity.

2) Hyaluronidase assay

Hyaluronidase is known to be a family of key enzymes promotes the spread of inflammatory mediators throughout tissues during allergy and other inflammatory responses (Guerra, 1946; Horton *et al.*, 1998; Kakegawa, 1988; Mio and Stern, 2002). In the hyaluronidase inhibitory assay, the extracts showed moderate to low anti-hyaluronidase activities at 500 and 1,000 µg/ml concentrations in comparison to the reference standard tannic acid. All samples had lower activity than 50%, while the standard sample, tannic acid provided IC_{50} of 97.16 µg/ml (Figure 4.28). There are only two plant extracts *C. sphaerica* and *G. sphaerogynum* at 1000 µg/ml showed moderate activities were 30% and 38% respectively exhibited the highest anti-hyaluronidase activities compared to the other extracts.

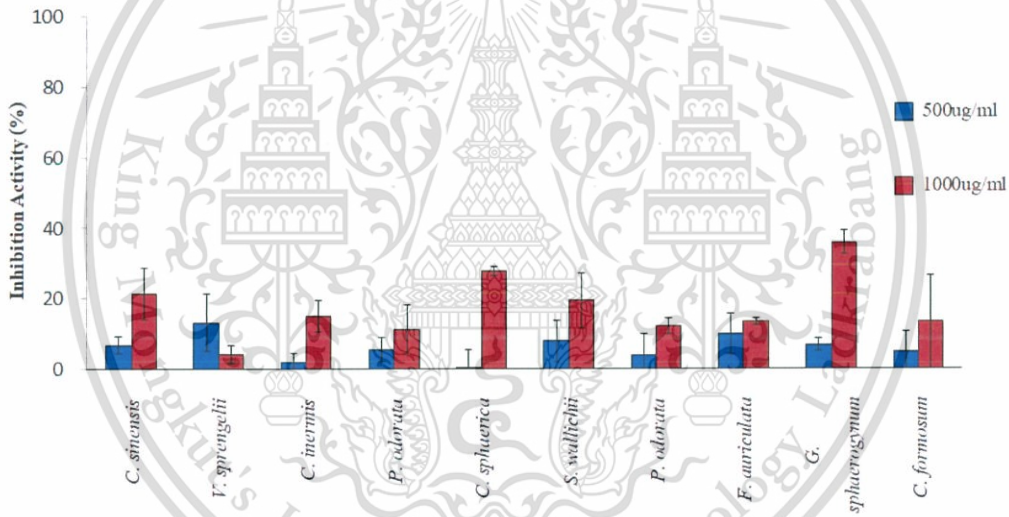

Figure 4.28 Inhibition activity of ten plant extracts at 500 and 1000 µg/ml on hyaluronidase

Melshandi-Perera *et al.* (2018) found that the hyaluronidase inhibitory activities ranged from 16.27 ± 1.00 to 42.31 ± 2.00% for the ethanol bark extracts at 500 µg/ml. The ethanol extract of bark of *Flacourtia indica* has exhibited the highest bio-activities irrespective of possessing moderate to low contents of polyphenols and flavonoids. It may be attributed to the strong aroma of the ethanol bark extract of *F. indica* such as Linoleic acid ethylester is another bio-active compound detected in the extract of bark of *F. indica* which is known to possess anti-inflammatory properties. In addition, garlic contains large amounts

of allicin (essential oil) which exerts potent anti-inflammatory effects (Oliveira *et al.* 2015). This study suggested that the polyphenolic, flavonoid and other secondary metabolite compounds of the extracts may not be solely responsible for the hyaluronidase inhibitory. It may be caused by other substance such as volatile compounds contained those extracts.

Chapter 5

Conclusions

Lipid oxidation in 30% soybean oil-water emulsion system, increasing the concentrations of extracts from 200 to 500 µg/ml improved the potential of anti-rancidity. PV checking, *V. sprengelii, C. sphaerica, G. sphaerogynum, S. wallichii* and *C. formosum* extracts had effectiveness in inhibiting the primary oxidation products especially hydroperoxide better than TBHQ. For TBARS, only *G. sphaerogynum* extract was more effective in inhibiting aldehyde production than TBHQ while *C. formosum* extract had the potential as good as TBHQ. Anisidine value, 7 plants extracts including *V. sprengelii, C. sphaerica, G. sphaerogynum, C. formosum*, *C. sinensis*, *S. wallichii* and *F. auriculata* retarded secondary oxidation production better than TBHQ. Oil stability index by Rancimat, *C. formosum, C. sphaerica, V. sprengelii, S. wallichii* and *C. sinensis* extracts had higher induction time than those of 200 µg/ml of each extracts. In addition, they were as effective as TBHQ. However, there are two plant extracts, *E. trifoliatum* and *P. odorata* still showed accelerating lipid oxidation at both concentrations of extracts. The results concluded that increasing of plant extract concentrations increased the potential of inhibiting lipid oxidation reaction. *G. sphaerogynum* extract exhibited the greatest against lipid oxidation followed by *C. formosum* extract.

G. sphaerogynum and *C. formosum* extracts were chosen to study the effects of pHs and storage temperatures. pH of emulsion may be affected on charge of the extracts. It was demonstrated that at pH 3, the extract presented as prooxidant whereas pH 5 and 7 presented as antioxidative properties. Difference of storage temperatures affected the shelf life of emulsion. An increase in temperature caused to shorter shelf life due to instability of the extract. Emulsion added *C. formosum* extract exhibited lower antioxidive effect than *G. sphaerogynum* extract in all storage temperatures. Emulsion added *G. sphaerogynum* extract adjusted pH 5 and kept at 25 $^{\circ}$C was the best condition for longer shelf life.

For biological activities, different plant species demonstrated various types of bioactive compounds, which could be responsible for their antioxidant activities. *G. sphaerogynum* extract had the highest polyphenol content whereas *C. sphaerica*, *F. auriculata and P. odorata* extracts had the highest in tannin, saponin and flavonoid content

respectively. Antioxidant activities, the highest in ABTS assay was found in *G. sphaerogynum* extract, *C.inermis* extract had the most in DPPH and FRAP assay whereas the most potential in TBARS assay were found in *C. sinensis*, *C.inermis* and *F. auriculata* extracts. Cytotoxic activities by MTT assay, it was found that *P. odorata*, *S. wallichii*, *C. sinensis* and *V. sprengelii* extracts exhibited high antiproliferative activity in HT-29, HepG2 and KB cell lines. Moreover, cells treated with *S. wallichii*, *P. odorata, V. sprengelii* and *C. sinensis* extracts showed a smear pattern of DNA fragmentation when compared with the DNA ladder which indicated that four plant extracts can destroy cancer cell lines. Interestingly, *S. wallichii* and *P. odorata* extracts had the highest potential in DNA fragmentation for HT-29 and HepG2 respectively. However, four plant extracts also affected on normal cell line (TK6) by DNA fragmentation. Also apoptosis observation, *S. wallichii* and *P. odorata* extracts induced apoptotic cells in TK6 cell line at both concentrations (500 and 1000 μg/ml). It was also those observed in the control cells. Finally, anti-inflammatory activities of 10 plant extracts were tested. It was found that, *C. sphaerica* and *S. wallichii* extracts showed high activity in lipoxygenase inhibition while showed moderate activities in anti-hyaluronidase activities compared to the other extracts.

From the experiments, it could be concluded that plant extracts that were effective in inhibiting rancidity at a concentration of 200 μg/ml including *G. sphaerogynum*, *C. formosum*, *V. sprengelii,* and *C. sphaerica* extracts. While, 500 μg/ml of extracts were *G. sphaerogynum*, *C. formosum*, *V. sprengelii, C. sphaerica*, *S. wallichii*, and *C. sinensis* extracts. *G. sphaerogynum* extract had the greatest anti-rancidity. Therefore, it was appropriate for retarding lipid oxidation in o/w emulsion. *S. wallichii* and *P. odorata* extracts were suitable for colon, liver and oral cancer treatment. In addition, *C. sphaerica* and *S. wallichii* extracts can be use as natural anti-inflammatory agents to enhance defence systems. In the future, these plant extracts could be used as natural anti-rancidity for food industry, as nutraceutical supplement to increase antioxidant needed in the body also as natural anti-cancer agents.

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Appendix I

Statistical analysis of lipid oxidation

Table 4.10 Peroxide values of o/w emulsion added extracts at 200 µg/ml

Extracts	Peroxide Values (meq/kg)					
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
P. odorata	3.44±0.02^{f}	12.39±0.01^{i}	22.08±0.03^{h}	31.71±0.02^{h}	42.53±0.04^{h}	48.17±0.18^{h}
V. sprengelii	2.42±0.03^{e}	3.89±0.01^{c}	6.55±0.07^{f}	7.03±0.04^{e}	4.38±0.03^{a}	5.13±0.25^{a}
C. formosum	2.05±0.07^{d}	4.82±0.05^{d}	4.43±0.04^{d}	9.27±0.09	9.40±0.02^{d}	10.68±0.01^{d}
E. trifoliatum	3.56±0.01^{f}	13.38±0.03^{j}	22.05±0.07^{h}	34.84±0.23^{i}	38.92±0.12^{g}	48.15±0.49^{h}
C. sinensis	1.52±0.05^{c}	5.95±0.07^{f}	3.93±0.04^{c}	5.41±0.04^{c}	7.87±0.04^{c}	11.23±0.10^{d}
F. auriculata	3.55±0.07^{f}	7.05±0.07^{f}	6.55±0.07^{f}	8.23±0.04^{f}	15.50±0.71^{f}	17.90±0.14^{f}
C. sphaerica	1.46±0.01^{c}	3.95±0.02^{c}	2.05±0.07^{a}	4.43±0.04^{a}	5.05±0.07^{b}	5.99±0.16^{ab}
S. wallichii	2.43±0.04^{e}	12.15±0.21^{h}	24.88±0.17^{j}	35.40±0.15^{j}	42.79±0.30^{h}	48.80±0.42^{h}
G.sphaerogynum	1.05±0.07^{a}	3.32±0.16^{a}	2.53±0.11^{b}	4.85±0.07^{b}	5.13±0.18^{b}	6.39±0.02^{b}
C. inermis	1.10±0.14^{a}	5.48±0.04^{e}	6.88±0.03^{g}	12.44±0.09^{g}	15.10±0.14^{f}	20.82±1.15^{g}
TBHQ	0.97±0.02^{a}	3.14±0.01^{a}	4.84±0.05^{e}	6.68±0.04^{d}	8.05±0.07^{c}	8.86±0.08^{c}
Control	1.27±0.05^{b}	3.68±0.04^{b}	4.38±0.04^{d}	6.93±0.04^{e}	10.3±0.07^{e}	13.7±0.04^{e}

Table 4.11 TBARS values of o/w emulsion added extracts at 200 μg/ml

Extracts	TBARS (mg/kg)					
	Day 0[ns]	Day 7	Day 14	Day 21	Day 28	Day 35
P. odorata	1.08±0.15	8.26±1.17^{bc}	8.14±1.15^{d}	8.24±1.17^{d}	14.91±2.11^{d}	14.81±2.09^{d}
V. sprengelii	1.12±0.16	3.83±0.54^{a}	3.14±0.44^{ab}	4.20±0.59^{bc}	6.35±0.90^{c}	3.25±0.07^{ab}
C. formosum	1.02±0.14	2.48±0.35^{a}	2.25±0.32^{ab}	2.40±0.34^{ab}	2.63±0.37^{ab}	2.75±0.07^{ab}
E. trifoliatum	1.05±0.15	7.09±1.00^{b}	9.07±1.28^{d}	11.50±1.63^{e}	15.21±2.15^{d}	14.66±2.07^{d}
C. sinensis	0.89±0.13	3.17±0.45^{a}	2.28±0.32^{ab}	2.65±0.37^{ab}	3.11±0.44^{ab}	3.83±0.04^{abc}
F. auriculata	1.01±0.14	3.75±0.53^{a}	5.65±0.80^{c}	5.00±0.71^{c}	5.30±0.75^{bc}	5.53±0.78^{bc}
C. sphaerica	0.83±0.12	3.19±0.45^{a}	1.42±0.20^{a}	1.60±0.23^{a}	1.82±0.26^{a}	2.38±0.11^{a}
S. wallichii	1.04±0.15	8.75±1.24^{c}	11.54±1.63^{e}	11.28±1.60^{e}	18.46±2.61^{e}	18.57±2.63^{e}
G.sphaerogynum	0.80±0.11	2.89±0.41^{a}	1.55±0.22^{a}	1.58±0.22^{a}	1.55±0.22^{a}	1.60±0.28^{a}
C. inermis	0.90±0.13	3.48±0.49^{a}	2.87±0.41^{ab}	3.00±0.42^{ab}	3.56±0.50^{abc}	3.63±0.04^{abc}
TBHQ	1.00±0.14	2.31±0.33^{a}	2.44±0.35^{ab}	2.50±0.35^{ab}	2.50±0.35^{ab}	2.55±0.07^{a}
Control	1.10±0.16	3.51±0.50^{a}	4.03±0.57^{bc}	5.80±0.82^{c}	6.30±0.89^{c}	6.37±0.90^{c}

Table 4.12 Anisidine values of o/w emulsion added extracts at 200 μg/ml

Extracts	Anisidine values					
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**
P. odorata	0.91±0.13^{c}	1.15±0.16^{abc}	1.57±0.22^{cd}	1.87±0.27^{cd}	2.34±0.33^{d}	2.59±0.37^{c}
V. sprengelii	0.57±0.08^{b}	1.09±0.15^{abc}	1.18±0.17^{abc}	1.35±0.19^{abc}	1.98±0.28^{cd}	2.08±0.29^{abc}
C. formosum	0.77±0.11^{c}	1.05±0.15^{abc}	1.14±0.16^{ab}	1.14±0.16^{a}	1.35±0.19^{a}	1.72±0.24^{ab}
E. trifoliatum	0.57±0.08^{b}	1.35±0.19^{c}	1.88±0.27^{d}	2.09±0.30^{d}	2.17±0.31^{cd}	2.27±0.32^{bc}
C. sinensis	0.35±0.05^{a}	1.03±0.15^{abc}	0.90±0.13^{a}	1.31±0.18^{abc}	1.19±0.17^{a}	1.55±0.22^{a}
F. auriculata	0.44±0.06^{ab}	1.28±0.18^{c}	1.79±0.25^{d}	1.99±0.28^{d}	2.01±0.28^{cd}	1.89±0.27^{ab}
C. sphaerica	0.44±0.06^{ab}	0.78±0.11^{a}	1.28±0.18^{abc}	1.28±0.18^{ab}	1.36±0.19^{a}	1.60±0.23^{a}
S. wallichii	0.48±0.07^{ab}	1.20±0.17^{bc}	1.50±0.04^{bcd}	1.78±0.05^{bcd}	1.91±0.05^{bcd}	2.27±0.32^{bc}
G.sphaerogynum	0.58±0.08^{b}	0.90±0.13^{ab}	1.12±0.16^{ab}	1.17±0.17^{a}	1.20±0.17^{a}	1.52±0.21^{a}
C. inermis	0.54±0.08^{ab}	0.97±0.^{14abc}	1.14±0.16^{ab}	1.39±0.20^{abc}	1.69±0.24^{abc}	1.97±0.28^{abc}
TBHQ	0.52±0.07^{ab}	1.14±0.16^{abc}	1.07±0.15^{a}	1.37±0.19^{abc}	1.41±0.20^{ab}	1.93±0.27^{ab}
Control	0.58±0.08^{b}	1.11±0.16^{abc}	1.10±0.03^{ab}	1.60±0.45^{abcd}	1.67±0.05^{abc}	2.25±0.07^{bc}

Table 4.13 Peroxide values of o/w emulsion added extracts at 500 µg/ml

Extracts	Peroxide Values (meq/kg)					
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**
P. odorata	2.48±0.04^{g}	7.91±0.01^{k}	7.32±0.05^{h}	13.19±0.10^{k}	15.25±0.07^{k}	20.14±0.02^{l}
V. sprengelii	1.24±0.01^{c}	2.98±0.01^{d}	2.47±0.02^{a}	5.37±0.04^{e}	6.39±0.01e	8.52±0.02^{e}
C. formosum	1.46±0.01^{d}	2.03±0.04^{a}	2.95±0.01^{b}	4.74±0.04^{c}	6.02±0.03^{c}	6.04±0.01^{b}
E. trifoliatum	2.44±0.01^{g}	7.47±0.05^{j}	11.17±0.04^{i}	15.70±0.14^{l}	17.27±0.04^{l}	19.71±0.13^{k}
C. sinensis	2.19±0.02^{f}	2.77±0.05^{c}	4.08±0.04^{d}	6.3±0.00^{f}	14.36±0.01^{g}	10.20±0.01^{h}
F. auriculata	2.46±0.01^{g}	3.96±0.01^{i}	6.39±0.02^{g}	9.14±0.01^{j}	8.84±0.01^{j}	11.89±0.01^{i}
C. sphaerica	0.99±0.02^{b}	2.98±0.01^{d}	3.86±0.01^{c}	5.23±0.04^{e}	6.09±0.02^{d}	6.66±0.06^{c}
S. wallichii	0.77±0.02^{a}	2.97±0.04^{d}	2.44±0.05^{a}	4.39±0.02^{b}	5.01±0.01^{b}	6.03±0.04^{b}
G.sphaerogynum	0.74±0.01^{a}	2.26±0.01^{b}	2.51±0.23^{a}	2.87±0.01^{a}	3.38±0.01^{a}	3.4±0.07^{a}
C. inermis	1.74±0.02^{e}	3.48±0.01^{f}	4.18±0.02^{d}	7.36±0.01^{i}	9.78±0.04^{h}	9.34±0.02^{g}
TBHQ	0.99±0.01^{b}	3.16±0.01^{e}	4.88±0.01^{f}	6.66±0.01^{g}	8.02±0.03^{f}	8.92±0.01^{f}
Control	1.24±0.01^{c}	3.68±0.04^{h}	4.36±0.01^{e}	6.96±0.01^{h}	10.21±0.01^{i}	13.65±0.01^{j}

Table 4.14 TBARS values of o/w emulsion added extracts at 500 μg/ml

Extracts	TBARS (mg/kg)					
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**
P. odorata	2.52±0.02^{g}	4.34±0.04^{i}	5.84±0.06^{f}	6.84±0.03^{k}	7.23±0.04^{i}	7.45±0.05^{j}
V. sprengelii	1.75±0.06^{b}	2.1±0.02^{b}	2.11±0.03^{b}	2.79±0.04^{c}	2.99±0.01^{c}	3.53±0.04^{c}
C. formosum	2.42±0.03^{a}	2.22±0.02^{c}	2.41±0.03^{b}	2.64±0.03^{b}	2.84±0.01^{b}	3.15±0.02^{b}
E. trifoliatum	2.43±0.01^{f}	5.26±0.04^{j}	5.82±0.03^{f}	6.45±0.07^{j}	7.53±0.04^{j}	8.06±0.06^{k}
C. sinensis	1.84±0.02^{c}	2.72±0.03^{d}	3.39±0.04^{c}	3.8±0.04^{d}	4.58±0.04^{e}	4.94±0.07^{e}
F. auriculata	2.19±0.02^{e}	3.54±0.04^{h}	4.32±0.04^{e}	5.78±0.03^{i}	6.05±0.05^{g}	6.78±0.01^{h}
C. sphaerica	2.19±0.03^{e}	3.52±0.05^{h}	3.82±0.03^{d}	4.08±0.03^{e}	4.54±0.04^{e}	5.55±0.04^{f}
S. wallichii	2.42±0.01^{f}	2.94±0.03^{e}	3.27±0.03^{c}	4.19±0.04^{f}	4.23±0.04^{d}	4.31±0.04^{d}
G.sphaerogynum	1.67±0.01^{b}	1.75±0.03^{a}	1.59±0.01^{a}	1.75±0.03^{a}	1.83±0.03^{a}	1.86±0.03^{a}
C. inermis	2.42±0.04^{f}	3.27±0.03^{g}	3.16±0.04^{c}	4.11±0.03^{ef}	5.07±0.04^{f}	5.46±0.08^{f}
TBHQ	1.00±0.05^{a}	2.30±0.03^{c}	2.42±0.05^{b}	2.51±0.06^{c}	2.58±0.08^{b}	2.63±0.04^{b}
Control	1.20±0.01^{a}	3.50±0.02^{h}	4.07±0.02^{de}	5.80±0.09^{i}	6.32±0.04^{h}	6.44±0.08^{g}

Table 4.15 Anisidine values of o/w emulsion added extracts at 500 μg/ml

Extracts	Anisidine values					
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
P. odorata	0.53±0.02^{a}	1.32±0.01^{g}	1.53±0.04^{i}	1.89±0.02^{h}	2.23±0.03^{g}	2.51±0.04^{h}
V. sprengelii	0.69±0.01^{c}	0.89±0.01^{b}	1.18±0.02^{e}	1.49±0.01^{f}	1.67±0.02^{d}	1.57±0.02^{b}
C. formosum	0.71±0.02^{c}	1.05±0.02^{d}	0.95±0.04^{b}	1.41±0.04^{e}	1.59±0.01^{c}	1.66±0.02^{c}
E. trifoliatum	0.70±0.02^{c}	1.25±0.02^{f}	1.15±0.02^{de}	1.85±0.03^{h}	1.98±0.01^{e}	2.72±0.02^{i}
C. sinensis	0.57±0.02^{ab}	1.01±0.01^{c}	0.85±0.02^{a}	1.03±0.04^{a}	1.53±0.01^{b}	1.59±0.01^{bc}
F. auriculata	0.83±0.02^{e}	1.08±0.01^{d}	1.29±0.02^{f}	1.57±0.04^{g}	1.66±0.01^{d}	1.81±0.01^{d}
C. sphaerica	0.74±0.02^{cd}	1.13±0.01^{e}	1.24±0.02^{f}	1.29±0.02^{cd}	1.42±0.01^{a}	1.41±0.01^{a}
S. wallichii	0.72±0.02^{c}	0.74±0.01^{a}	1.13±0.04^{de}	1.21±0.02^{b}	1.66±0.02^{d}	1.63±0.01^{c}
G.sphaerogynum	0.77±0.02^{d}	0.72±0.01^{a}	1.46±0.02^{a}	1.22±0.02^{b}	1.55±0.04^{bc}	1.58±0.03^{bc}
C. inermis	0.71±0.01^{c}	1.05±0.01^{d}	1.35±0.01^{g}	1.23±0.03^{bc}	2.14±0.02^{f}	2.17±0.01^{f}
TBHQ	0.54±0.02^{a}	1.12±0.03^{e}	1.05±0.04^{c}	1.35±0.04^{de}	1.43±0.02^{a}	1.94±0.01^{e}
Control	0.59±0.01^{c}	1.13±0.03^{e}	1.09±0.01^{cd}	1.63±0.04^{g}	1.68±0.01^{d}	2.25±0.01^{g}

Appendix II

SPME-GC-MS

Table 4.16 Retention time and compounds in emulsion samples by GC-MS

RT	Compounds	Day 0	Day 35	Day 49	Day 0	Day 35	Day 49	Day 0	Day 35	Day 49
		Control	Control	Control	*G. phaerogynum*	*G. phaerogynum*	*G. phaerogynum*	*C. formosum*	*C. formosum*	*C. formosum*
1.64	Hexane	74379030	-	-	79472443	-	-	82640855	-	-
1.99	Acetone	-	-	23645482		-	37264849	-	-	27988723
2.76	Ethanol	225724604	261703418	175173236	259392219	328168874	196910709	289542787	267318291	208391119
3.09	3-Methylbutanal	-	-	-			9467626	-		7315457
3.93	Toluene	3958078	11375051	4853527	4615633	9652587	3678140	5415116	8625349	3781292
4.86	Hexanal	861025	9099108	89793037	1313108	9267400	14455791	2004889	18893106	2802032
5.83	unknow	-	17363851	8509379	-	15947321	9087621	-	6855144	12602662
7.25	1-penten-3-ol	-	936887	-	-		-	-	1386262	-
8	Limonene	-	1052677	674693	-	1579626	-	-	1520985	572717
9	2-Pentyfuran	-	616428	1431945	-		-	-	1411759	-
10.18	pentanol	-	1232644	2226254	-		-	-	1602864	-
13.36	1-Hexanol	-	-	4760160	-		-	-	-	-
16.3	Acetic acid	781539	-	-	-		-	-	-	-
16.48	Heptanal	-	-	-	-		-	-	-	-
21.01	Butyrolactone	-	-	-	-		-	-	-	-
28.6	Benzothiazole	-	-	440626	-	2042304	-	-	2075176	-
32.49	eugenol	-	-	-	-	-	-	-	6751456	-
37.5	Myristic acid	-	-	1115659	-	-	-	-	-	-

Appendix III

Cytotoxic Activity and Cytotoxic Concentration (CC_{50})

Table 4.17 Cytotoxic Activity and Cytotoxic Concentration (CC_{50}) of 4 plant extracts on HT-29, HepG2, KB and Vero cell lines

Cell lines	Concentration (µg/ml)	%Cytotoxicity			
		S. wallichii	*P. odorata*	*V. sprengelii*	*C. sinensis*
HT-29	4000	79.18±18.79	86.35±3.63	89.43±12.70	86.15±4.70
	2000	72.90±23.56	80.15±8.92	71.06±1.12	78.15±5.34
	1000	67.53±21.53	67.35±5.83	77.69±4.94	42.72±4.76
	500	67.21±19.27	29.16±8.73	53.00±9.65	21.01±5.94
	250	21.59±12.54	15.76±8.25	26.99±3.00	8.51±2.15
CC_{50}		**453.0±163.09**	**774.90±101.70**	**493.59±72.87**	**1,214.01±165.55**
HepG2	4000	90.81±7.10	84.31±7.91	82.6±6.55	89.77±1.47
	2000	83.2±3.93	67.67±11.08	62.07±14.48	82.31±2.46
	1000	70.97±11.55	19.09±2.30	29.2±4.82	66.40±4.44
	500	68.37±3.13	6.49±0.95	24.03±3.54	59.55±2.11
	250	35.02±9.72	2.88±0.65	16.54±2.98	37.96±2.20
CC_{50}		**346.6±77.9**	**1,666±117**	**1514±73.9**	**360.1±7.87**
KB	4000	80.75±10.40	86.38±8.44	77.44±8.53	96.62±0.10
	2000	76.42±9.67	79.15±12.3	76.81±10.6	75.62±2.69
	1000	72.09±1.72	51.04±13.3	63.85±15.2	54.78±1.82
	500	65.79±3.70	37.33±2.23	50.17±13.7	27.88±1.20
	250	40.76±10.0	26.84±9.53	10.24±3.76	10.93±0.35
CC_{50}		**286.8±62.6**	**495.6±22.8**	**530.0±117**	**889.3±83.0**
Vero	4000	87.57±3.29	83.04±1.32	92.91±0.34	96.94±1.73
	2000	82.39±5.99	66.08±2.42	87.57±0.50	71.78±1.49
	1000	61.60±1.77	46.86±3.23	85.42±1.70	63.59±1.65
	500	50.73±2.63	25.00±1.58	62.72±20.39	53.27±5.09
	250	29.53±3.29	3.950±1.58	54.42±2.03	40.34±5.99
CC_{50}		**487.8±22.01**	**1,140±22.83**	**217.9±25.32**	**388.0±33.01**

Appendix IV

Standard Curve

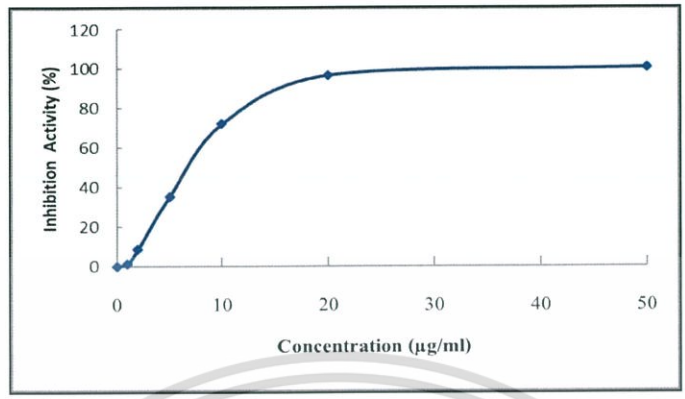

Figure 4.29 Standard curve of NDGA

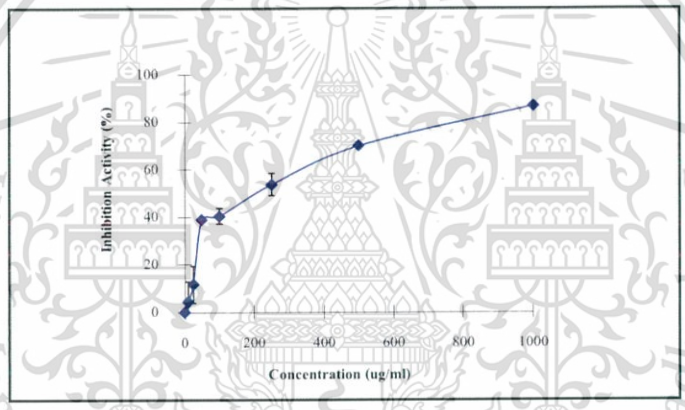

Figure 4.30 Standard curve of tannic acid

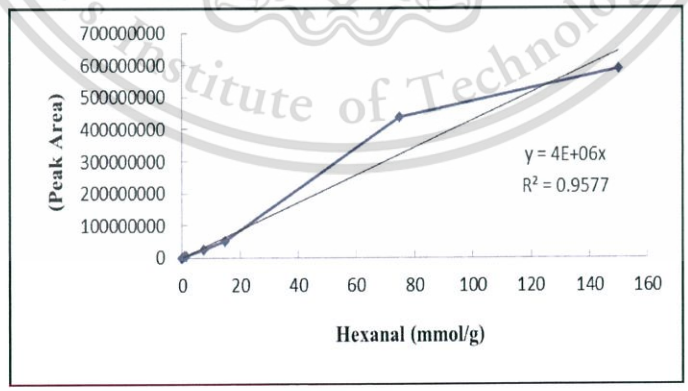

Figure 4.31 Standard curve of hexanal

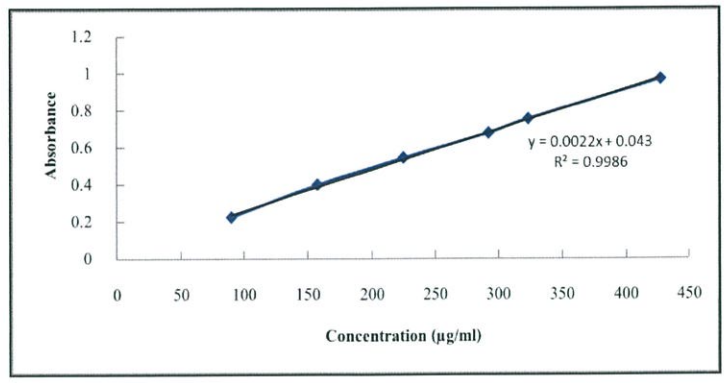

Figure 4.32 Standard curve of gallic acid

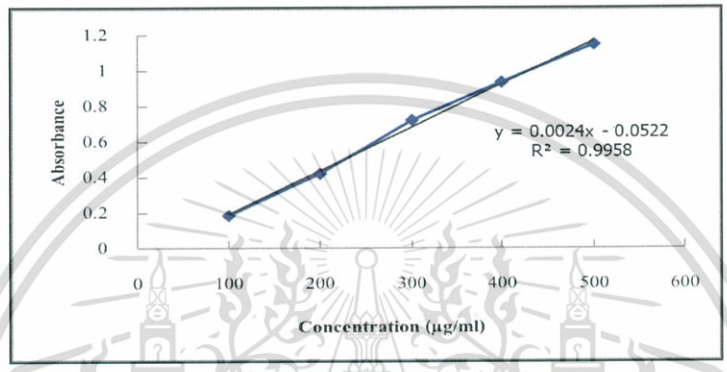

Figure 4.33 Standard curve of catechin

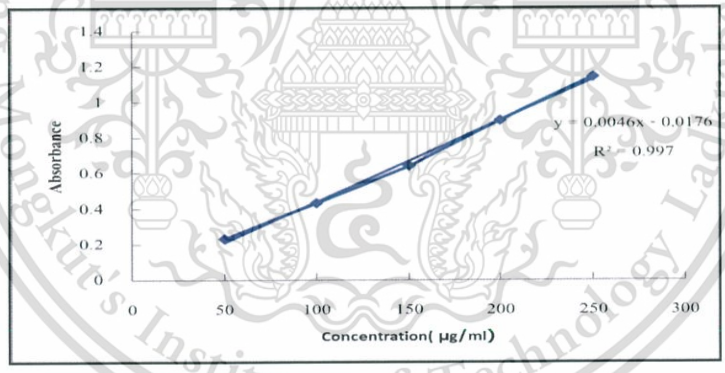

Figure 4.34 Standard curve of rutin

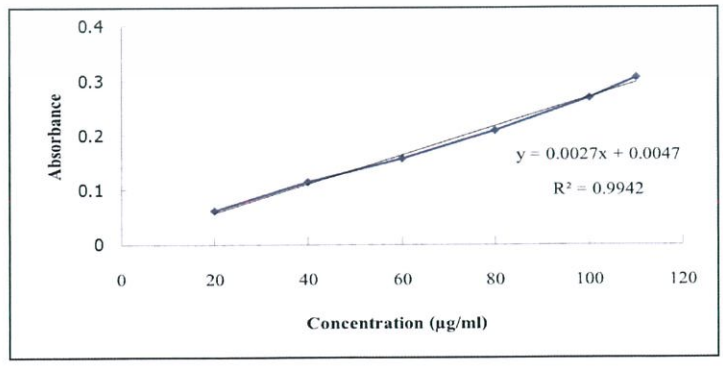

Figure 4.35 Standard curve of diosgenin

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7. Publications

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