PHYSIOCHEMICAL PROPERTIES AND BIOACTIVITIES OF TEA SEED (*Camellia oleifera*) OIL

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Master of Science
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ABSTRACT

Tea seed (*Camellia oleifera*) oil has been used for cooking in China and other Asian countries for more than a thousand years. This study determined the fatty acid composition and tocopherol content through chromatography-flame ionization (GC-FID) and high performance liquid chromatography-UV (HPLC-UV), respectively. The results showed that the tea seed oil (TSE) was mainly composed of 18.30% palmitic acid (C16:0), 54.95% oleic acid (C18:1), 22.41% linoleic acid (C18:2), and 0.17% linolenic acid (C18:3). Its α-tocopherol content was found the 21.13 mg/100 g oil. Moreover, the antioxidant capacities of methanolic extract of TSE and the remaining meal (Meal) were determined using the following four *in vitro* antioxidant methods, total phenolic content (TPC) assay, trolox equivalent antioxidant capacity (TEAC) assay, 2,2-di-phenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and metal chelating capacity. The TPC and the TEAC of the Meal were 23.3 mg gallic acid equivalent/g and 4.42 μM Trolox/g, respectively, 7 to 15 times higher than those of the TSE. In addition, the metal chelating capacity of Meal can reach 90% at 25 mg sample equivalent/mL. Moreover, the TSE and the Meal showed different levels of antiproliferative activities against the following three cancer cell lines: SiHa (human uterus cancer cell line), MCF-7 (human breast cancer cell line) and HT-29 (human colon cancer cell line). The IC_{50} values of TSE and Meal were 146.70 and 2.92 mg sample equivalent/mL for SiHa, 236.20 and 2.94 mg sample equivalent/mL for MCF-7, and 155.20 and 1.52 mg sample equivalent/mL for

HT-29. In addition, comparative analysis of the TSE and olive oil showed that both dietary edible oils had similar iodine values, though the former exhibited a lower PV-Fox value in the study. These results demonstrate TSE and Meal are rich in bioactive chemicals and provide health benefits.

DEDICATION

I dedicate this work to my parents, Ming-Fa Chen and Chin-Mei Fan, my lovely sisters, Ying-Li Chen and Ying-Chu Chen, my aunt, Karen Fan, my uncle, Paul Gao, and my dear friends, Anny and Nana, with love and pride.

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Tea seed (*Camellia oleifera*) oil, called Tea-oil in China, is well known as a sweet seasoning and cooking oil. It should not be confused with tea tree (*Melaleuca alternifolia*) oil, an essential oil extracted from the tea tree farmed in Australia (Gilman & Watson, 1993). Tea seed (*Camellia oleifera*) taxonomy as determined by Linnaeus is as follows (Integrated Taxonomic Information System, 2006):

Kingdom Plantae

Division Magnoliophyta

Class Magnoliopsida

Order Ericales

Family Theaceae

Genus *Camellia*

Species *C. oleifera*

In the genus *Camellia*, some species such as *Camellia chrysantha* (Golden Camellia), *Camellia japonica (*Japanese Camellia), *Camellia rusticana* (Snow Camellia) and *Camellia sasanqua* (Christmas Camellia) are usually used for decoration. In contrast, *Camellia sinensis* is well known and widely planted in order to make popular commercial tea drinks, while *Camellia oleifera* is the most important and widely distributed of the 18 species used for producing tea seed oil

in China (The American Camellia Society, 2006; The International Camellia Society, 2006; Weihrauch & Teter, 1994).

The *Camellia oleifera* plant is a large shrub approximately 15 to 20 feet tall with glossy, dark green leaves and fragrant, 2-inch-wide flowers as seen in **Figure 1.1**. *Camellias* are often grown in hilly areas from latitude 18° to 34° N, longitude 98° to 120° E, in an annual mean temperature of approximately 15 to 20℃ (Cilman & Watson, 1993), with an annual precipitation of 1,000 to 2,000 mm, and with acidic soils, such as red, yellow and grayish red-yellow (Shanan & Ying, 1982). Some species are also found present at high elevation, about 2000 m on the Yunnan-Guizhou Plateau (Shanan & Ying, 1982). However, the lower Piedmont and Coastal Plain regions of the southeastern United State are also within the suitable environments for *Camellia oleifera* as seen in **Figure 1.2** (Gilman & Watson, 1993).

Two primary methods for the commercial processing of the tea seed oil are mechanic expression and solvent extraction (**Figure 1.3**) (Zhou, Liu, Zhu & Zhou, 2006). The former method is free of solvents and inexpensive, while the latter is more efficient. Supercritical fluid extraction (SFE), a third method recently suggested for extracting tea seed oil, requires special expensive equipment to withstand the high pressure needed for the process (Rajaei, Barzegar & Yamin, 2005).

Camellia seeds have a long history, probably more than 1,000 years (Shanan & Ying, 1982). More than 50% of the population uses this oil as their primary cooking oil in Hunan Provinces, China (Zhou, Liu, Zhu & Zhou, 2006). It is also

considered an edible oil in such other countries as Taiwan, Japan, India, and Indonesia (Ravichandran & Dhandapani, 1992; Sahari, Atii & Hamedi, 2004; Tokue, Kataoka & Tanimura, 1989). In addition, tea seed oil has other potential usages in the food industry, for instance, as a coca butter equivalent (CBE) produced through insteresterification (Wang, Wu, Ho & Weng, 2006).

1.2 Relationship between Color and Stability of Tea Seed Oil

Shyu *et al*. found that tea seed oil exhibits a superior antioxidative stability, having an antioxidative index of 98 hours, when determined by the active oxygen method (AOM) (Shyu, Huang, Gyen & Chang, 1990). The longer the roasting time, the more stable and dark the oil becomes as seen in **Table 1.1**. This antioxidative quality and stability have been partially attributed to the presence of carotenoid pigments in the seed oils (Schmidt & Pokorny, 2005).

1.3 Health Benefits

1.3.1 Cardiovascular Disease Risk Reduction

The effect of sasanguasponins from tea seed on blood lipids was evaluated by using a hyperlipoidemia rat model, showing that the compounds played a significant role in the atherosclerosis prevention because it could markedly reduce serum total cholesterol (TCh), triglycerides (TG), low density lipoprotein cholesterol (LDL) and HDL3 (Chen, Qiu & Peng, 1998). In addition, it was found that the tea seed oil could inhibit aortic atherosclerosis (AS) as a consequence of lowering liver and blood lipids by decreasing Thromoboxane B2 (TXB2) and lipid peroxides products (LOP), while increasing antioxidative enzyme SOD and GSH-Px activities (Chen, Gu, Sun, Yang, ZHang & Liu, 1996). Other papers also

reported that the tea seed oil, which contained a high content of monounsaturated fatty acid (MUFA), exerted a beneficial effect on the prevention of cardiovascular diseases (Deng, Zhang, Huang, Huang, Zheng & Chui, 1993; Deng, Xie & Huan, 2002; Wang, Wang, Zhang & Fan, 1993).

Separated from the seed oil of *Camellia oleifera* Abel was a bioactive compound squalene (**Figure 1.4**) (Li, Wang, Bi & Zhao, 2006), which was also found in olive oils (Newmark, 1997). Squalene is a triterpenic hydrocarbon chemical with known naturally antioxidant activity. Its antitumor and anticarcinoma activities have also been reported (Newmark, 1997). Squalene from shark liver was suggested to be a medicine for chronic disease of the liver (Kelly, 1999).

1.3.2 Antioxidant Activities

Li *et al*. isolated seven compounds from tea seed, including dimethylterephthalate, p-hydroxybenzoic acid, kaemferol, kaemferol-3-O-α-L-rhamnopyranosyl(1→6)β-D-glucopyranoside, kaemferol-3-O-[2-O-β-D-glucopyranosyl-6-O-α-L-rhamnopyranosyl]-β-D-glucopyranoside, kaemferol-3-O-[2-O-β-D-xylopyranosyl-6-O-α-L-rhamnopyranosyl]-β-D-glucopyranoside, and kaemferol-3-O-[2-O-α-L-rhamnopyranosyl-6-O-β-D-xylopyranosyl]-β-D-glucopyranoside (**Figure 1.6**) (Li & Luo, 2003). Among these chemicals, kaemferol has been known as a strong antioxidant compound that may be comparable to Trolox, a standard antioxidant for the DPPH radical scavenging assay (Park, Rho, Kim & Chang, 2006). Recently, other two bioactive compounds were identified in tea seed oil, i.e., 2,5-bis-benzo[1,3]dioxol-5-yl-tetrahydro-furo[3,4-d][1,3]-dioxine and

sesamin (**Figure 1.5**) that also exhibited remarkable antioxidant activity (Lee & Yen, 2006). Ye *et al*. reported that the tea seed oil could effectively clean up free radicals in rat liver tissue and inhibit lipid peroxidation (Ye, Fang & Bao, 2001). Moreover, after a comparison of the tea seed oil and soybean oil on inhibiting oxygen free radicals and regulating the activity of antioxidative enzymes in rats, Zhang *et al*. found the former possessed a higher potential of antioxidation (Zhang & Zhou, 1995), indicating the tea seed oil could be considered a high-value edible oil.

1.3.3 Cancer Risk Reduction

Cancer may be defined as the development, growth and metastatic spread of a malignant neoplasm. Malignant tumors derived from epithelial cells are called carcinomas, and those derived from connective or mesenchymal cells are called sarcomas. Carcinogenesis is a prolonged multi-stage process and very complex, so many critical steps at which food-related substances or metabolic process may interact with the sequence of events can accelerate, delay or even reverse it. Diet-related anti-carcinogenesis can usefully be classified into "blocking mechanisms" that operate during the initiation phase of carcinogenesis, and suppressing mechanisms that delay or reverse tumor promotion at a later state (Johnson, Williamson & Musk, 1994). **Figure 1.7** shows a schematic illustration of these concepts and a summary of the mechanisms through which various compounds may act. Suppression of carcinogenesis may involve inhibition of mitosis and increase expression of the differentiated phenotype; this serves to reduce the clonal expansion of initiated cells, or an increases susceptibility to

programmed cell death or apoptosis, which can eliminate precancerous cells from the tissue (Chan, Morin, Vogelstein & Kinzler, 1998; Smith, Lund & Johnson, 1998). The classic epidemiological analysis of cancer indicated that diet was responsible for approximately 35% of cancers in the west (Doll & Peto, 1981).

The potential of using natural products as anticancer agents was recognized in the 1950s by the U.S. National Cancer Institute (NCI) under the leadership of the late Dr. Jonathan Hartwell. Since then, NCI has made major contributions to the discovery of new naturally occurring anticancer agents through its contract and grant support, including an important program of plant and marine collection (Gragg, Kingston & Newman, 2005) under the recognition of plants that can involve the production of a large number of diverse bioactive compounds (Johnson, Williamson & Musk, 1994).

Tea (*Camellia oleifera*) may be one of these valued plants. Several recent papers have provided evidence that tea seed and its oil have the antioxidant (Chen, Qiu & Peng, 1998), antimicrobial (Hou, Wan & Wu, 2006; Huang, Ao & Zhong, 2002; Ye, 2001), and other bioactivities (Chen, Qiu & Peng, 1998; Chen, Qiu & Peng, 1998; Deng, Xie & Huan, 2002; Xiong, Zhang & Yu, 2002; Zhang & Zhou, 1995).

1.3.4 Others

Some research groups reported that fatty acid compositions of edible oils were closely associated with changes of health status (Diniz, Cicogna, Padovani, Santana, Faine & Novelli, 2004; Kris-Etherton, Pearson, Wan, Hargrove, Moriarty, Fishell *et al.*, 1999). Feng *et al*. evaluated the influence of different composition of

unsaturated fatty acids of three oils, including tea seed oil, corn oil and fish oil, on the immune status in mice (Feng & Zhou, 1996). The result showed that tea seed oil had better benefits for the immune system due to high monounsaturated fatty acid composition. Bin *et al*. indicated that tea seed oil had a significantly protective effect on bile duct ligated rats by decreasing the cardiac damage in morphology and function (Hou, Peng & Mou, 2000). Besides, it was reported that the saponin extracted from tea seed had a remarkably inhibitive effect on *Escherichia coli*, *Penicillium citrinum*, *Candidauitilis*, and *Aspergillus niger* (Hou, Wan & Wu, 2006; Huang, Ao & Zhong, 2002).

1.4 Analytical Methods

1.4.1 Fat Characterization Tests

Many papers have reported the fatty acid composition of tea seed oil (Rajaei, Barzegar & Yamin, 2005; Ravichandran & Dhandapani, 1992; Sahari, Atii & Hamedi, 2004; Sengupta, Sengupta & Ghosh, 1976; Shyu, Huang, Gyen & Chang, 1990), of which quantitative analysis can be summarized into the following two steps: first, the oil is under saponification to free the bound fatty acyl side groups from their ester bond; second, each free fatty acid is methylated to a fatty acid methyl ester (FAME) (**Figure 1.8)** (Nielsen, 2003). FAMEs are easy to be volatilized at high temperature so that they may be quantified by GC coupled with different detectors, such as FID and MS. Some common methods and terms related with fat or oil are summarized in **Table 1.2** (Campbell, Baker, Bandurraga, Belcher, Heckel, Hodgson *et al.*, 1999).

1.4.2 Organoleptic Characterization Test

Organoleptic characterization tests (or sensory evaluation) generally include tests of color, flavor and aroma. The color of transparent food samples, such as vegetable oil and beverage, are measured by pouring the samples into a transmission cell and then placing them against the sphere port in the transmission compartment under daylight source. In food industries, three methods are often used, including the Commission International de'Eclariage (CIE) system, the Hunter *L*, *a*, *b* system, and the Munsell color solid system (Giese, 2003). Other methods measure the concentration of certain highly conjugate colored compounds such as carotenoids that are known to absorb light at known wavelengths in the visible spectrum.

The aroma of tea seed oil depends on its existing nonpolar compounds, which are low molecular weight chemicals, easily volatilized at room temperature, and can be determined by GC-FID and GC-MS (Morales, Luna & Aparicio, 2005). Ravichandran and Dhandani reported that tea seed oil was clear, free-flowing and had acceptable sensory properties (Ravichandran & Dhandapani, 1992).

1.4.3 Phytochemical Characterization Test

Edible plants contain a large variety of phytochemicals, such as phenolic derivatives, including simple phenols, phenylpropanoids, benzoic acid derivates, flavonoids, stilbenes, tannins, lignans and lignins. These chemicals are generally more hydrophilic or amphipathic in nature. Solvents frequently used for the extraction of polyphenolic compounds include methanol, ethanol, acetone, water,

ethyl acetate, propanol, dimethylformamide and their combinations (Antolovich, Prenzler, Robards & Ryan, 2000).

Spectrophotometric method is commonly used for determination of the total amount of phenolic contents (TPC) in samples, and performed exclusively by the Folin-Ciocalteu (FC) reagent that is subject to react with compounds containing phenolic structures (Huang, Ou & Prior, 2005; Rababah, Hettiarachchy & Horax, 2004; Yi, Fischer & Akoh, 2005; Yu, Haley, Perret, Harris, Wilson & Qian, 2002). The chemical reduction converts the yellow FC reagent to a blue species, of which absorbance can be measured at wavelength of 765 nm (Huang, Ou & Prior, 2005). The disadvantage of this test lies in its non-specificity as it that may be reduced by many nonphenolic compounds, such as vitamin C and Cu, etc. Besides, it detects all phenolic groups in the extracted compounds such as proteins. However, despite the undefined chemical nature of this test, the TPC assay is convenient, simple, and reproducible (Huang, Ou & Prior, 2005).

Chromatographic techniques have been employed for separation, preparative isolation, purification and identification of phytochemicals in tea seed (Lee & Yen, 2006). The polar compounds with high variation in their partition coefficients (K*p*) are easily separated by a reverse phase HPLC system. The small volatile compounds can be analyzed by the GC system (Wang, Yin & Liu, 1994). These compounds can also be separated by capillary zone gel electrophoresis. Identification of these compounds has been reported by several methods including: nuclear magnetic resonance (NMR), mass spectroscopy (MS), and ultraviolet spectroscopy (Shahidi & Naczk, 2003).

1.4.4 Extraction Methods of Antioxidants

There are two methods that are often used to extract antioxidants from food.

1.4.4.1 Organic solvents

Solvent extraction can give good recoveries of compounds with a wide range of polarities and volatilities (Zhou, Robards, Glennie-Holmes & Helliwell, 1999), more freedom of solvent selection (Zhou & Yu, 2004), and extraction methods (Ntonifor, Brown & Mueller-Harvey, 2002). In this research, methanol and the pear-shaped funnel were used to extract the antioxidants from tea seed oil and meal.

1.4.4.2 Supercritical fluid carbon dioxide extraction

The extraction of tea seed oil using carbon dioxide in the near or supercritical state (supercritical fluid extraction, SFE) has been reported (Rajaei, Barzegar & Yamin, 2005). This method was also used to remove phenolic substances from sunflower extracted meals (Andrich, Balzini, Zinnai, vitis, Silvestri, Venturi et al., 2001). SFE technology with carbon dioxide as the solvent allows an environmentally friendly process, but this technology is restrained by its request of high operation pressure, which requires expensive equipments regarding safety.

1.4.5 Antioxidant Assays

Methods used to determine antioxidant capacities can be classified into two types: assays based on hydrogen atom transfer (HAT) reaction and assays based on electron transfer (ET) reaction (Huang, Ou & Prior, 2005), which are listed in **Figure 1.9**. The HAT-based assays quantify the hydrogen atom donating capacity, while the ET-based assays determine an antioxidant's reducing capacity (Benzie & Strain, 1999). Dietary antioxidants often broadly include radical chain reaction

inhibitors, metal chelators, oxidative enzyme inhibitors, and antioxidant enzyme cofactors. In this research, the DPPH assay and Trolox equivalent antioxidant capacity assay were used to evaluate the antioxidant capacity of tea seed oil and its meal. In addition, their metal chelating ability was estimated by the ferrozine method.

1.4.5.1 Diphenylpicryl-hydrazyl (DPPH) free radical scavenging assay

The molecule of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also give rise to the deep violet color, characterized by an absorption band in ethanol solution centered at about 517 nm (Huang, Ou & Prior, 2005). When a DPPH stock solution is mixed with a compound that can donate an electron, the DPPH radical will be reduced and simultaneously with the loss of its violet color. However, it is expected there is a residual pale yellow color from the picryl group still present (Molyneux, 2004). The scheme for scavenging the DPPH radicals by an antioxidant RH is shown in **Figure 1.10** (Yamaguchi, Takamura, Matoba & Terao, 1998).

1.4.5.2 Trolox equivalent antioxidant capacity assay (TEAC)

$ABTS^{+}$ radicals can be generated by the oxidation of 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) for antioxidant determination, which was developed by Miller *et al.* (Miller, Rice-Evans, Davies, Gopinathan & Milner, 1993). This method was based on the activation of metmyoglobin with hydrogen peroxide in the presence or absence of antioxidant, and the method was improved

by Re *et al*. (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999) with the use of potassium persulphate to oxidize ABTS to a radical cation. The concentration of antioxidants that gives the same percentage change of absorbance of the $ABTS^{+}$ at 734 nm as that of Trolox is regarded as TEAC (Trolox equivalent antioxidant capacity) assay. Moreover, the ABTS radical cation is more reactive than the DPPH radical (Miller, Rice-Evans, Davies, Gopinathan & Milner, 1993). The scheme for scavenging the ABTS Radical is shown in **Figure 1.11**.

1.4.5.3 Metal chelating assay by using the ferrozine reagent

Proposed by Stookey (Stookey, 1970), the ferrozine (monosodium salt hydrate of 3-[2-pyridinyl]-5,6-diphenyl-1,2,4-iazine-p,p'-disulfonic acid) reagent (**Figure 1.12)** is used for measurement of iron-chelating capacity, because it can specifically react with ferrous ions to form a stable, magenta-colored solution. The complex (ferrozine-Fe^{2+} chromophore) has an absorptive peak at 562 nm so that it can be measured spectrophotometrically. (Viollier, Inglett, Hunter, Roychoudhury & Cappellen, 2000).

In this research, antioxidant extracts were pre-mixed with known amounts of ferrous ion solution and then added to the ferrozine solution. The metal chelating ability of antioxidants in samples was measured by inhibiting the ferrozine-Fe^{2+} form.

1.5 Significance of the Project

The camellia seeds used for making cooking oil and as medicines have a long history (Shanan & Ying, 1982). The objectives of this research were to find out other potential health beneficial characteristics of the tea seed (*Camellia oleifera*)

oil and its byproduct, meal, and consequently to promote its value-added utilization. Thus, the specific objectives of this research were:

1. To evaluate physical and chemical properties of the tea seed (*Camellia oleifera*) oil,
2. To compare the fatty acid composition and the tocopherol content of the tea seed (*Camellia oleifera*) oil with other commercial oils, and
3. To assess the antioxidant capacities and antiproliferative activities of the tea seed (*Camellia oleifera*) oil and its byproduct, meal.

1.6 Figures and Tables

A. Meddle-aged plant

B. Seed (seed / skin/ hull/ whole)

Figure 1.1 The Appearance of *Camellia oleifera*

Figure 1.1-A was authorized to be added in this thesis (from Gilman & Watson, 1993).

Figure 1.2 Shaded Area Represents Potential Planting Range of *Camellia oleifera*

This figure was authorized to be added in this thesis (from Gilman & Watson, 1993).

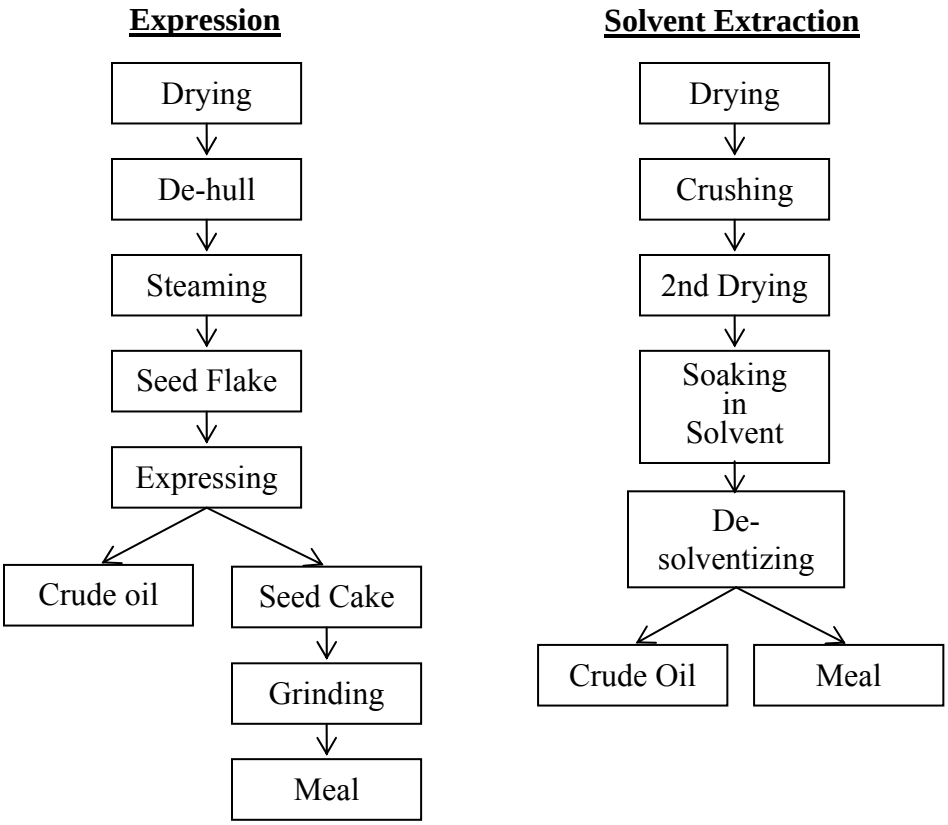


Figure 1.3 Flow Sheet for Crude Tea Seed Oil Extraction by Expression and Solvent

This figure is modified from report (Zhou, Liu, Zhu & Zhou, 2006)

Table 1.1 The Color and Active Oxygen Method (AOM) Result of Tea Seed Oil Prepared from Tea Seeds with Different Roasting Time

Tea seed oils	Lovibond color unit			AOM (hrs)
	R	Y	B	
Freeze dry	0.7	23.0	0	15.8
5 min	1.2	33.5	0	5.2
10 min	1.6	46.2	0	8.1
20 min	1.9	52.2	0.6	38.2
30 min	3.4	56.4	0.8	95.4
Commercial	1.7	39.5	0	9.2

Each sample was roasted at 200℃ for different time;
Commercial tea seed oil obtained from market in Taiwan.

AOM: An accelerated rancidity test measures the rate of oxidation of a fat or oil. The higher number of time, the more stable the product is to oxidation.

R: redness; Y: yellow; B: blue

This table is modified from the report (Shyu, Huang, Gyen & Chang, 1990)

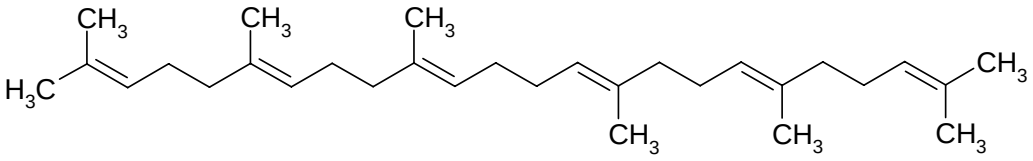
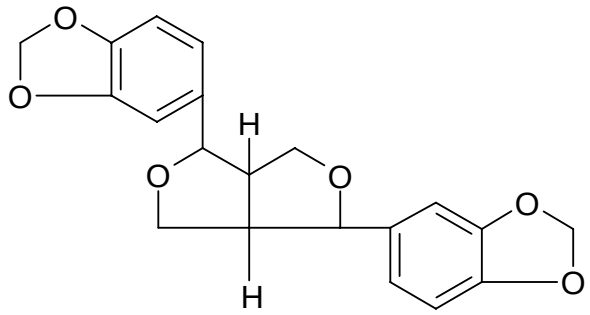
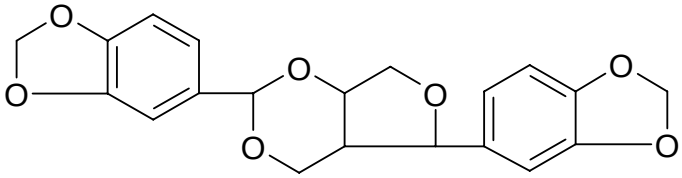


Figure 1.4 Chemical Structure of Squalene

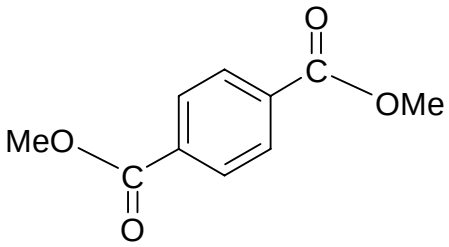


Sesamin

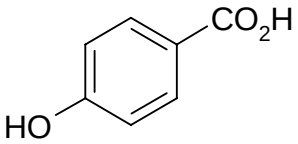


Compound B (2,5-bis-benzo[1,3]dioxol-5-yl-tetrahydro-furo [3,4-d][1,3]-dioxine)

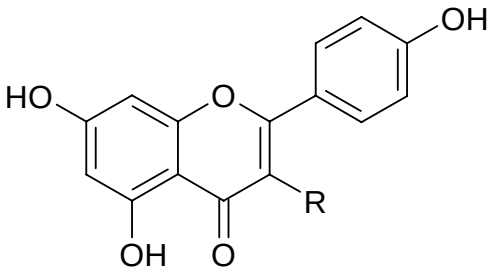
Figure 1.5 Structures of Sesamin and Compound B Isolated Form the Methanolic Extract of Tea Seed Oil.



Dimethylterephthalate



Hydroxybenzoic Acid



Kaempferol Glycosidic Flavonoids

Compound	R
Kaempferol	OH
1	Glu (1→2) Glu
2	Rha (1→6) Glu
3	Glu (1→2) [Rha(1→6)] Glu
4	Xyl (1→2) [Rha(1→6)] Glu
5	Rha (1→6) [Xyl (1→6)] Glu

Figure 1.6 Chemical Constituents of *Camellia oleifera*

Glu=Glucopyranoside; Rha=Rhamnopyranoside; Xyl=Xylopyranosid

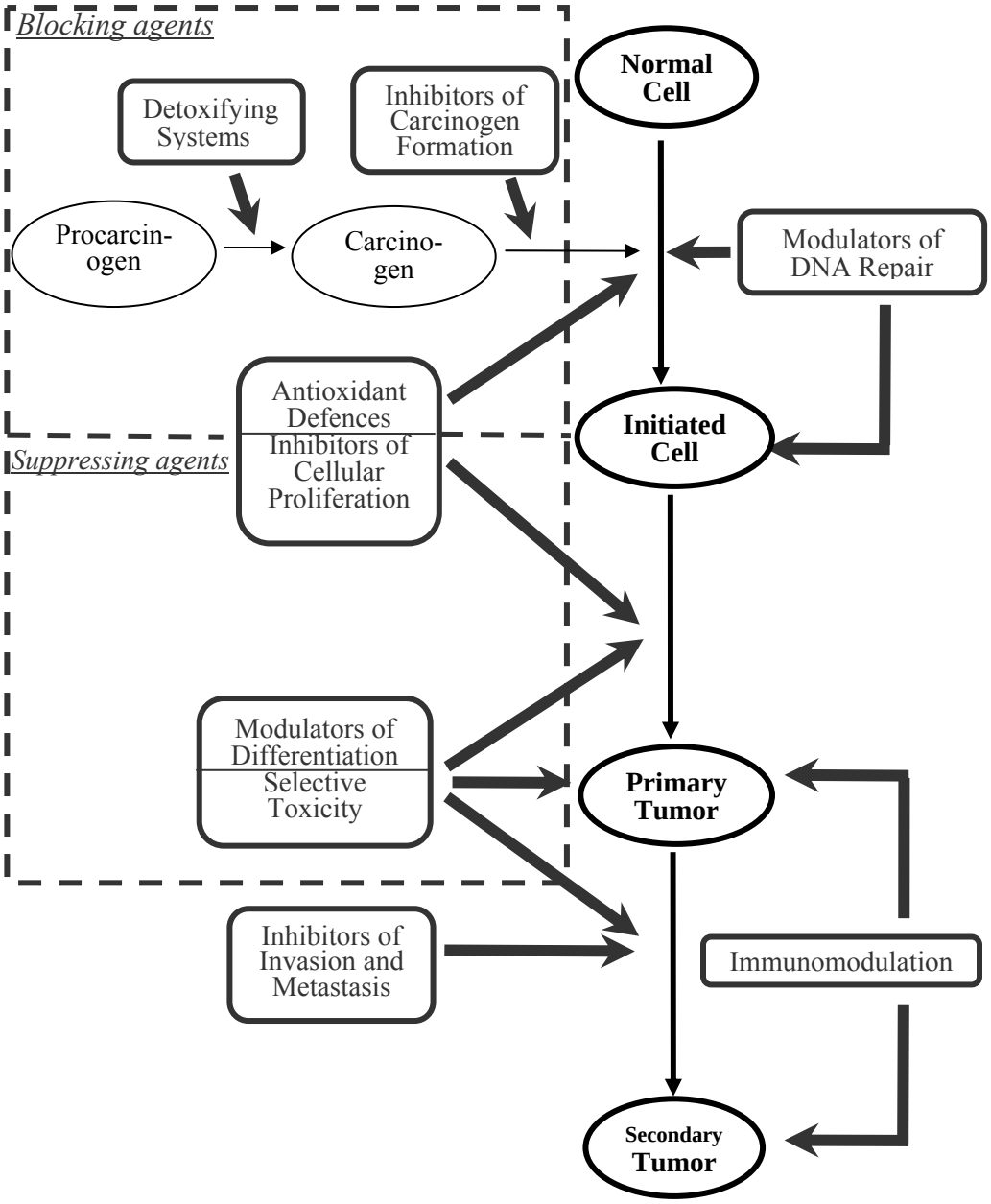


Figure 1.7 Mechanisms and Sites of Interaction Whereby Protective Factors May Inhibit the Carcinogenic Process

This figure is modified from the report (Johnson, Williamson & Musk, 1994)

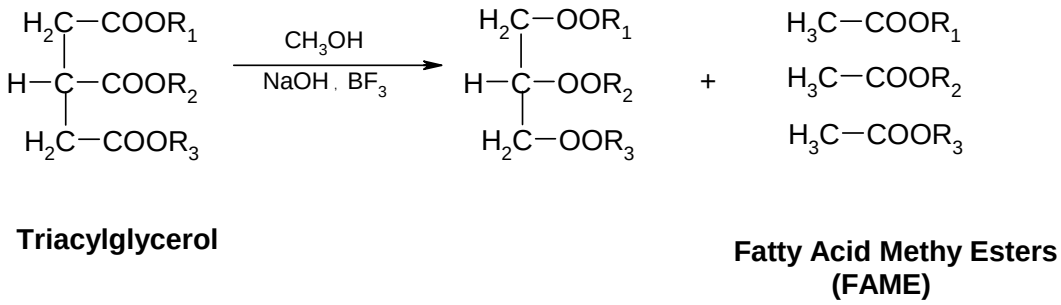


Figure 1.8 The Reaction of Fatty Acid Methylation

Table 1.2 Common Test Methods and Related Terms of Fat or Oil

Common test methods	Related terms
Cold Test	The evaluation in time that an oil remains free of visible solids when immersed in a 32 °F ice-water bath
Dropping Point	The temperature at which a solid fat softens to the point, where it will flow and drop out of a specially designed container. The dropping point is an indication of the chemical and crystalline nature of the solid fat
Free Fatty Acid (FFA)	The amount of free fatty acids present in an oil as determined by simple titration
Melting Point (MP)	The temperature at which a fat changes from solid to liquid
Oil Stability Index (OSI)	An accelerated rancidity test that measures the rate of oxidation of a fat or oil and it expressed as an index number. The higher the index number, at a given temperature, the more stable the product is to oxidation. The method replaces the Active Oxygen Method (AOM)
Phosphorus Content	A predictor of the total concentration of phospholipids in a sample
Solid Fat Index (SFI)	Provides and index or indication of the proportions of crystallized and molten fat at a given series of temperature checkpoints. Determined by measuring the change in volume that occurs when a solid fat partially melts to liquid at a temperature of interest
Solid Fat Content (SFC)	A measure of the crystallized fat content is determined by magnetic resonance (NMR) at a series of temperature check points

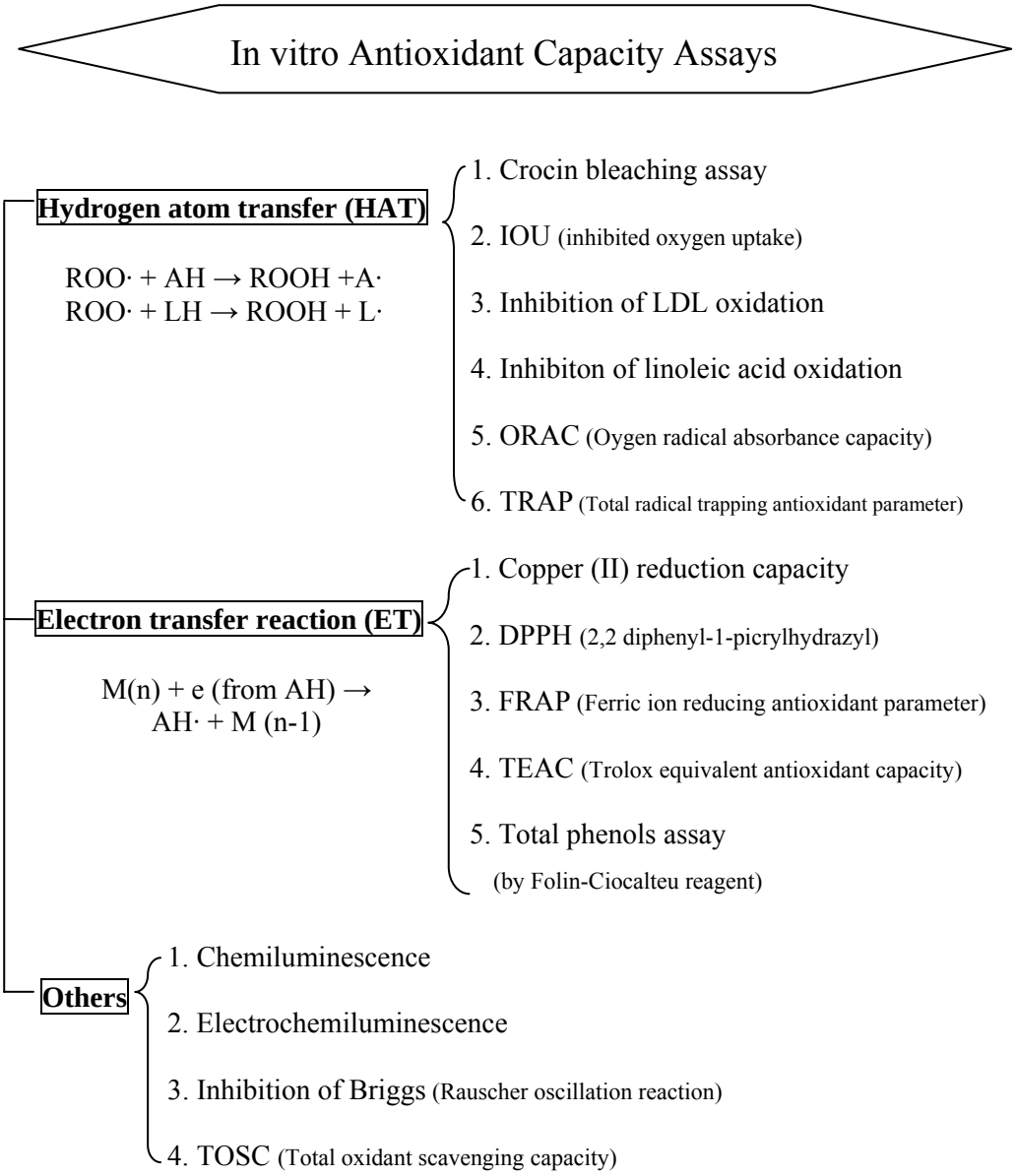
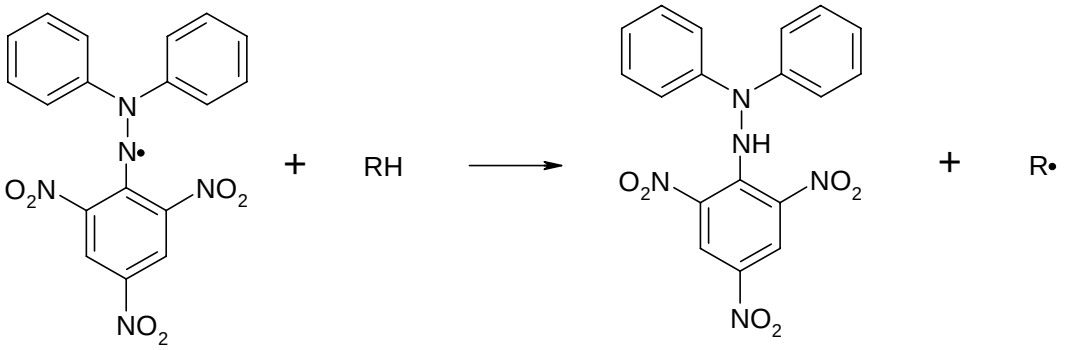


Figure 1.9 *In vitro* Antioxidant Capacity Assays

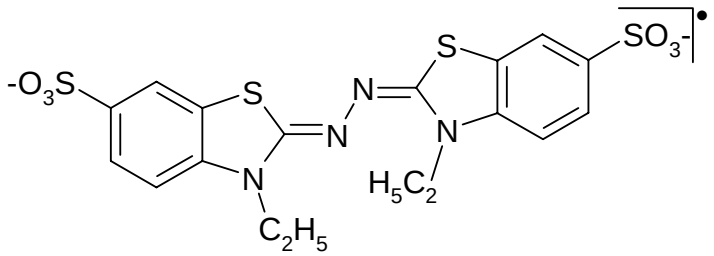
This figure is modified from the report (Huang & Prior, 2005)



DPPH -517 nm (Purple) (Colorless)

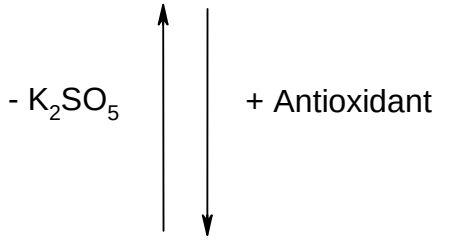
Figure 1.10 The Scheme for Scavenging the DPPH Radical by an Antioxidant.

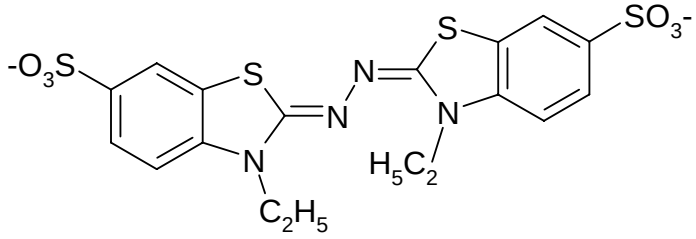
*RH. Antioxidant



$ABTS^{+}$-734nm

(Blue/Green)





$ABTS^{2-}$

(Colorless)

Figure 1.11 The Scheme for Scavenging the ABTS Radical by an Antioxidant.

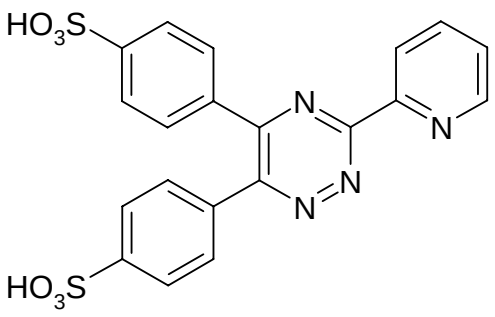


Figure 1.12 The Structure of Ferrozin

1.7 References

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CHAPTER 2

CHARACTERISTIC COMPARISON OF TEA SEED (*Camellia oleifera*) OIL WITH TEA SEED (*Camellia sinensis*), OLIVE AND CORN OILS

Abstract

Recent studies have found many unique nutritional values of the tea seed (*Camellia oleifera*) oil (TSE). In this study, it was further compared with three other commercially edible oils: including the tea seed (*Camellia sinensis*) oil (TSC), olive oil, and corn oil, in terms of their physical and chemical characteristics, fatty acid composition and tocopherol content. The tea seed (*Camellia oleifera*) sample could produce 27.21 % oil that had a relative density of 0.904 (25℃/water), with the lowest PV (peroxide value)-Fox value within the four tested oils, yet all four oils had similar iodine values. The tea seed (*Camellia oleifera*) oil (TSE) contained 18.3% palmitic acid (16:0), 54.95% oleic acid (C18:1), 22.41% linoleic acid (C18:2), and 0.17% linolenic acid (18:30). It has a higher content of oleic acid than the olive oil, but lower than the corn oil. The α-tocopherol content of the *C. oleifera* oil and the *C. sinensis oil* were 21.13 and 25.88 mg and / 100 g oil, respectively.

2.1 Introduction

China is the largest producer of tea seed oil (Tang, Bayer & Zhuang, 1993) that is principally produced from the plant *Camellia oleifera*, which is cultivated widely in 17 provinces of southern China with mild climates (Cilman & Watson, 1993). Some Chinese and Japanese farmers also plant *Camelia sinensis* for making the edible oil, though the plant is usually cultivated for making tea drinks (Tang, Bayer & Zhuang, 1993). In the late 1980s and early 1990s, the annual production of tea seed oil in China was estimated in 15,000 tons per year (Tang, Bayer & Zhuang, 1993). It is extensively used as a cooking oil, and also traditionally applied as a medicine for curing burn injuries (Chen, 2005).

Edible oils' physicochemical properties, such as color and oxidative stability, to a large extent determine their quality and potential application (Parker, Adams, Zhou, Harris & Yu, 2003; Parry & Yu, 2004). Particularly, the oxidative stability of oil samples is a very important index because lipid degradation results in unpalatable odors and toxic compounds such as malondialdehyde (Romero, Bosch-Morell, Romero, Jareno, Romero, Marin *et al.*, 1998). The oil stability can be expressed by several indices, such as iodine value, peroxide value (PV), and oxidative stability index (OSI).

Fatty acid composition may affect lipid oxidation and the final quality of oils and fats, as well as the human health (Yu, Scanlin, Wilson & Schmidt, 2002). For example, diets rich in high monounsaturated fatty acid may decrease the risk of cardiovascular disease (Kris-Etherton, Pearson, Wan, Hargrove, Moriarty, Fishell *et al*., 1999; Mangiapane, McAteer, Benson, White & Salter, 1999). Besides,

unsaturated fatty acid may provide other health benefits in preventing cancer, hypertension and autoimmune disorders (Iso, Sato, Umemura, Kudo, Koilke, Kitamura *et al.*, 2002; Tapiero, Ba, Gouvreur & Tew, 1992).

In addition to fatty acid composition, several other factors, such as presence of natural antioxidants and storage conditions, may affect lipid oxidation of oils and fats. Natural antioxidants, such as tocopherols, are potent lipid oxidation inhibitors for stabilizing seed oils (Schmidt & Pokorny, 2005). Moreover, tocopherols were found to possess antiproliferative (Azzi, Boscoboinik, Marilley, Ozer, Stauble & Tasinato, 1995) and anticlotting activities (Dowd & Zheng, 1995), and be able to reduce the LDL oxidation, platelet adhesives and thromobosis (Hodis, Mack, LaBree, Cashin-Hemphill, Sevanian, Johnson *et al.*, 1995; Jialal & Grundy, 1992; Princen, Poppel, Vogelezang, Buytenhek & Kok, 1992; Reaven, Khouw, Beltz, Parthasarathy & Witztum, 1993). Increasing the intake of tocopherols is inversely associated with lower risk of cardiovascular and coronary heart diseases (Losonczy, Harris & Havlik, 1996; Rimm, Stampfer, Ascherio, Giovannucci, Colditz & Willett, 1993; Stampfer, Hennekens, Manson, Colditz, Rosner & Willett, 1993).

Both olive oil and corn oil are considered health benefiting oils and used popularly in the United States. Thus, this study was conducted to (1) assess physical and chemical properties of the extracted tea seed (*Camellia oleifera*) oil (TSE); (2) compare the fatty acid compositions of TSE with the commercial tea seed (*Camellia sinensis*) oil (TSC), olive oil and corn oil; and (3) measure the tocopherol content in four oils aforementioned.

2.2 Materials and Methods

2.2.1 Materials and Chemicals

The tea seed (*Camellia Oleifera*) sample was obtained from Fujian province, China and tea seed (*Camellia sinensis*) commercial oil product was generously supplied by Lu Yu Tea Company (http://www.luyutea.com, Seattle, WA, USA). Extra virgin olive oil (World Finer Food, Inc., Bloomfield, NJ, USA) and corn oil (Wesson, ConAgra Food, NE, USA) were purchased at a local market in Clemson, SC, USA. Fatty acid methyl ester (FAME) and tocopherol standards were purchased from Supelco Co. (Bellefonte, PA, USA). Potassium iodide, *tert*-butyl peroxide, sodium methoxide, and acetyl chloride were purchased from Sigma-Aldrich chemical Co (St. Louis, MO, USA.) Sodium thiosulfate-anhydrous, sulfate acid, peroxide hydrogen and all other HPLC analytical grade solvents were purchased from Fisher Scientific (Suwanee, GA, USA). Hanus iodine solution was purchased from Ricca chemical company (Arlighton, TX, USA). Starch indicator was purchased from AQUA solution (Deer park, TX, USA). Ferrous ammonium sulfate was purchased from J. T. Baker chemicals, Inc (Phillipsburg, NJ, USA). Xylenol orange was purchased from MP biomedicals, Inc. (Fountain, OHIO, UAS).

2.2.2 Extraction and Determination of the Tea Seed (*Camellia Oleifera*) Oil

Lee and Yen' method was adopted with minor modifications (Lee & Yen, 2006) to extract the seed oil from the tea seed (*Camellia oleifera)*. The tea seed powder was roasted at 120 ℃ for 20 minutes. Then twenty grams of the roasted powder was extracted by 250 mL hexane for 12 hours using a Soxhlet extractor. The extract was filtered before hexane was evaporated to obtain the tea seed oil

using a rotary evaporator (Model R-200; Buchi, Switzeland). Tea seed (*Camellia oleifera)* oil content was determined according to the AOAC method Ba 3-38 (1973).

2.2.3 Relative Density

The relative density of each oil sample, which is the ratio of the density of the oil sample to the density of deionized water, was determined gravimetrically at the observed room temperature of 25 ℃ (Model PB 303; Mettler Toledo International Inc., Greifensee, Switzerland.)

2.2.4 Solubility in Different Solvents

Each oil sample was diluted by 100 times by methylene chloride, ethylacetate, acetone, reagent grade alcohol, and acetonitrile (Model K-500-G; Vortex-Genie Scientific Industries Inc., Springfield 3, MA, USA). After 5 minutes, the solubility of each oil sample in different solvents was evaluated visually and classified into miscible and immiscible categories.

2.2.5 Color Measurement

Color was measured by using a Minolta CR300 chroma meter (CR-300, Minolta Camera Co., Ltd., Japan) standardized with the white standard plate (CR-A43, Minolta Camera Co., Ltd., Japan). Twenty milliliters of each oils were transferred to a quartz cup (CR-A33a, Minolta Camera Co., Ltd., Japan). Their Hunter color values were obtained by using light source of D65 (daylight, 2° light angle) (Smith & Alvarez, 1988). The tests were conducted in triplicate for each oil sample.

2.2.6 Iodine Value

Iodine value was measured according to the AOAC Method 920.158 (Hanus method) (Cunniff, 1995) with minor modifications. This is one of two accepted official procedures for determination of the iodine value of oil or fat. Two hundred milligrams of each sample oils were placed into a 250 mL flask and dissolved in 10 mL chloroform. Ten mL of Hanus iodine solution was added to the flask and let it stand for 30 minutes in the darkness while shaking occasionally. Then 10 mL of 15% KI solution and 40 mL of freshly boiled and cooled H_2O were added sequentially. Iodine was titrated gradually with 0.1 N sodium thiosulfate solution with constant shaking until the yellow solution turned almost colorless. Then a few drops of starch indicator were added to turn the solution into a blue color. The titration continued until the blue color entirely disappeared. The determination was conducted in triplicate, and two blank determinations were also prepared and conducted. Number of mL of 0.1 N sodium thiosulfate solution required by blank minus mL used in the sample gives sodium thiosulfate equivalent of iodine absorbed by the oil. The iodine value was calculated by the following equation:

$$\text{The Iodine Value} = \frac{(B-S)*N*12.69}{\text{Weight of sample (g)}}$$

Where:

B = titration of blank

S = Titration of Sample

N =Normality of Sodium thiosulfate

2.2.7 Ferrous-Oxidation in Xylenol Orange (FOX) Peroxide Value (PV)

Amount of lipid peroxides present in the oil samples was determined according to the FOX version II assay described by Parry and Yu (Parry & Yu, 2004), of which the *tert*-Butyl peroxide was used as the standard. The oil samples were diluted in ratio of 1:100 with reagent grade alcohol. The final Fox reagent contained 90% methanol, 10% 250 mM sulfate acid, 4 mM butylated hydroxytoluene, 250 mM ferrous sulfate ammonium sulfate, and 100 mM xylenol orange. The Fox reagent was freshly prepared and used within 24 hours. The reaction mixture contained 950 μL of Fox reagent and 50 μL of either *tert*-Butyl peroxide solution or oil sample solution. Absorbance was measured at 560 nm by a UV spectrophotometer (Genesys 20 Model 400/4; ThermoSpectronic, Rochester, NY, USA) after a 10 min of reaction at ambient temperature. Measurements were conducted in duplicate for each *tert*-Butyl peroxide solution and in triplicate for each oil sample solution. The PV-FOX of each oil sample was calculated from the standard curve and expressed as concentration of *tert*-Butyl peroxide equivalent (mg TPOE/g oil).

2.2.8 Preparation of Fatty Acid Methyl Esters (FAMEs)

The preparation and analysis of fatty acid methyl esters (FAMEs) was modified from a procedure previously described by Kramern *et al*. (Kramer, G., Fellner, Vivek, Dugan, Micalael *et al.*, 1997). Fifty milligrams of oil samples were put into a screw capped (Teflon^{TM} lined caps) culture tube mixed with 1 mL internal standard of heptadecacanoic acid (2 mg/mL). Then 2 mL of sodium methoxide was added in the tube that was placed in a 50℃ water bath for 10

minutes, and then cooled down for another 5 minutes before adding 3 mL of 5% methanolic HCl. The mixture was again incubated in 80℃ water bath for 10 minutes and cooled down for 7 minutes. Finally, 1 mL of hexane and 7.5 mL of 6% K_2CO_3 were added to separate the polar phase into the 6% K_2CO_3. After centrifugation at 1200 rpm for 5 minutes, the organic layer was transferred into a vial by a capillary pipette and then sealed with a Teflon lined cap.

2.2.9 Gas Chromatographic (GC) Analysis of FAMEs

The fatty acid composition of sample oil was determined from the FAMEs by GC–FID (Model GC-17A, Shimadzu Scientific Instruments Inc., MD, USA). The results were expressed as % individual fatty acid of total fatty acids. Analysis was carried out by GC-FID with a fused silica capillary column DB-Wax (I.D. 0.25mm, Length 60m, Thickness 0.25μm, J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. Injection volume was 5 μL at a split ratio of 11:1. Initial oven temperature was set at 180℃ held for 2 minutes and then increased by 5℃/min to 250 ℃ and then held for another 10 minutes. Temperature of the injector and detector was set at 260℃. Fatty acids were identified by comparing the retention time of peaks in the sample chromatography with those of the standards. Determination of the retention time and area of peaks was performed by the software "Shimadzu ClassVP 7.0" (Shimadzu Scientific Instruments Inc., MD, USA).

2.2.10 Tocopherol Content

Oil samples were diluted with hexane to obtain a concentration of 0.1 g/mL before their tocopherol contents were determined by high performance liquid

chromatograph (HPLC) system (Model: LC-10 ATvp, Shimadzu Scientific Instruments Inc., MD, USA), that consists of a pump with a 5μ silica normal phase column (4.6mm x 250mm, Waters Spheriosorb 5μ silica, Waters cooperation, Milford, MA, USA) and a guard column (10 x 4.6, Waters cooperation, Milford, MA, USA). The method was modified from that described by Psomiadou and Tsimidou (Psomiadou & Tsimidou, 1998). The system was operated isocratically at a flow rate of 0.8 mL/min. Hexane / isopropanol (98:2) was used as the mobile phase. After membrane filtration (0.2μm, Gelman Science Inc, ANN Arbor, MI, USA), 20 μL of each sample was injected by an auto-injector (Model: SIL-10ADvp, Shimadzu Scientific Instruments Inc., MD, USA). Tocopherols were detected at 293 nm with a PDA detector (Model: SPD-M10Avp Photodiode Array Detector, Shimadzu Scientific Instruments In., MD, USA), while spectrum in the region 300-800 nm were also recorded. The tocopherol content was calculated from the standard curve (concentration versus peak area) which was calculated by linear regression analysis. The tocopherol content was expressed in mg/100 g oil.

2.2.11 Statistical Analyses

Data were reported as the mean ± standard deviation. All statistical analysis was conducted on the SAS V9.1 software for Windows (SAS Institute Inc., Cary, NC, USA). Differences among all sample means were determined by analysis of variance (one–way analysis of variance, AVONA) at $p<0.05$.

2.3 Results and Discussion

As shown in **Table 2.1**, oil content of the tea seed (*Camellia oleifera*) was 27.21%, and the relative densities of TSE, TSC, olive oil and corn oil at 25℃ were

0.904, 0.921, 0.916 and 0.916, respectively. The relative density of the TSE is significantly lower than that of the other three oils. Also, the solubility of TSE, TSC, olive oil and corn oil in different solvents is listed in **Table 2.1.** Though acetone is able to dissolve pure edible vegetable oils, it could not mix well with the TSE. This table can be used as a reference for selecting alternative solvents for oil extraction (Gandh, Joshi, Jha, Parihar, Srivastav, Raghunadh *et al.*, 2005), and these data lay down a foundation for further determination of different characteristics of the oil samples (Kahkonen, Hopia, Vuorela, Rauha, Pihlaja, Kujala *et al.*, 1999; Matthaus, 2002; Perez Jimenez & Saura Calixto, 2006).

Color is another important indicator of product composition, purity and degree of deterioration (Tan, Kuntom, Lee & Low, 2004). It may also be associated with antioxidant capacity due to different inherent pigments (Yu, Scanlin, Wilson & Schmidt, 2002). The HunterLab *L*, *a*, *b* is one of the most commonly used methods in the food industry. The system measures the degree of lightness(*L*), of redness or greenness (+/- *a*), and of yellowness or blueness (+/-, *b*) (Giese, 2003). **Table 2.2** lists the Hunter *L*, *a* and *b* values of the oil samples. The olive oil and the TSE had similar *a* values, but the TSE and TSC were significantly different in all of their *L*, *a* and *b* values. These data reflected the differences of different plant varieties, growing conditions, harvesting conditions and processing procedures.

The peroxide value (PV) reflects the content of primary oxidation products, i.e. peroxides, that are formed during both initiation and propagation phases of lipid oxidation. It is an important indicator because peroxides may act as inducers to

accelerate free radical-mediated lipid peroxidation. The PV was determined by the FOX method (Parry & Yu, 2004). As shown in **Table 2.3**, olive oil has the highest PV-Fox value (15.40 mg TPOE/g oil), followed by corn oil (7.58 mg TPOE/g oil), TSC (2.41 mg TPOE/g oil) and TSE (0.57 mg TPOE/g oil). Although PV-Fox values may differ from sample to sample, depending on extraction methods, storage conditions, and sample varieties, the PV of olive oils usually ranged from 5.89-9.49 mg TPOE/g oil with mean and median values of 7.18 and 7.06 mg TPOE/g oil (Ardo, 2005). In this study, the PV-Fox value of the olive oil was higher than that of olive oils in references. However, the PV-Fox values of the TSE and TSC were still significantly lower than that of the olive oil, which indicated the tested TSE and TSC might have a better oxidative stability than that of the olive oil. In contrast, iodine values of TSE, TSC, olive oil and corn oil were 83.45, 71.31, 75.18 and 126.25, respectively (Hanus method of Cunniff, 1995). Since these oils, except the corn oil, had iodine values below 100, they belonged to the non-drying oils for which monounsaturated fatty aids acting as the major components may be the critical reason. Sahari, Ataii, and Hamedi reported there were no significant differences in saponification values between tea seed (*Camellia sinenis*), sunflowerseed, and olive oils. Weihrauch and Teter reported that tea seed oil had comparable properties to olive oil (Weihrauch & Teter, 1997). The results from this paper confirmed both C*amellia sinenis* oil (TSC) and *Camellia oleifera* oil (TSE) were similar to olive oil.

Edible oils are often compared by their fatty acid profiles (Giacomelli, Mattea & Ceballos, 2006) because the fatty acid composition of oils may affect human

health (Lorgeril & Salen, 2004) and the quality of oil (Yu, Scanlin, Wilson & Schmidt, 2002). The fatty acid profiles of TSE, TSC, olive oil, and corn oil were determined by GC-FID, and the result is shown in **Table 2.4**. Edible vegetable oils seldom contain branched chains, odd-numbered fatty acids, or unsaturated fatty acids with carbon atoms less than sixteen or more than twenty.

As the result presented in **Figure 2.1**, the major fatty acid of the TSE, TSC and olive oil was the oleic acid (C18:1), a monounsaturated fatty acid (MUFA). In our samples, the mean values of the oleic acid (C18:1) in TSE, TSC, olive oil and corn oil were 54.95, 81.59, 80.82 and 25.01 (% FAME), respectively. It was reported that the oleic acid (C18:1) content of the olive oil ranged from 55 to 83% (Kiritsakis, 2002), and the content of corn oil ranged from 20.0 to 42.2 % (Moreau, 2002). In our samples, the TSC and olive oil had no significant difference in their oleic acid (C18:1) contents (**Table 2.5**). Our result confirmed the previous report that tea seed oil and olive oil could have similar fatty acid compositions (Weihrauch & Teter, 1997). However, Shari, Ataill, and Hamedi reported that the palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acid of the tea seed (*Camellia sinensis*) oil were 16.5, 3.34, 56.97, 22.17 and 0.3 (% FAME), respectively. These values were significantly different from those of the TSC, but close to those of the TSE (Sahari, Atii & Hamedi, 2004). These differences might be due to several factors such as different growing and harvesting conditions and extraction procedures.

A diet rich in oleic acid (C18:1) is recognized to be as effective in lowering serum cholesterol as a diet rich in linoleic acid (C18:2) (Kris-Etherton, Pearson,

Wan, Hargrove, Moriarty, Fishell *et al*., 1999; Mangiapane, McAteer, Benson, White & Salter, 1999). A comparative analysis to study the effects of olive oil (C18:1) (rich in oleic acid), safflower oil (rich in linoleic acid), and a high-oleic acid sunflower oil on immune cell function confirmed that the oleic acid rather than the non-lipid component of olive oil was the main contributor in adjustment of rat serum lipid levels and lymphocyte function (Jeffery, Yaqoob, Newsholme & Calder, 1996). In our samples, the mean values of the linoleic acid (C18:2), an essential fatty acid, of the TSE and TSC were 22.41 and 7.57 (% FAME), respectively, were comparable to that of the olive oil, ranging from 3.5 to 21.0 (% FAME) (Kiritsakis, 2002). Other desirable features of the tea seed oil rely on its relatively low levels (<15%) of saturated fatty acids and very low levels of linolenic acid (C18:3), which is especially more susceptible to oxidation, leading to rancidity.

Figure 2.2 shows the comparison of the polyunsaturated fatty acid (PUFA), monounsaturated fatty acid (MUFA) and total unsaturated fatty acid (TUFA) contained within TSE, TSC, olive oil and corn oil. These values are important because modern studies have shown that cell membranes require unsaturated fatty acid to maintain their structure, fluidity and function (Calder, 1997). Many studies have shown that the lipid profile of the diet affects membrane lipid content and phospholipids fatty acyl composition after the period of adaptation (Brasitus, Davidson & Schachter, 1985; Stubbs & Smith, 1984). Therefore, a diet rich in saturated fatty acids can decrease membrane fluidity, or, on the contrary, increase membrane fluidity if the diet is rich in polyunsaturated fatty acids (PUFA). The

mean values of PUFA of the TSE, TSC, olive oil and corn oil were 22.58, 7.82, 5.55, and 61.2 (% FAME), respectively. TSE and TSC have higher PUFA than olive oil, but lower than corn oil. Sahari, Ataii and Hamedi reported that the storage stability of the tea seed (*Camellia sinensis*) oil resembled that of olive oil (Sahari, Atii & Hamedi, 2004). They concluded that mixing the tea seed (*Camellia sinenis*) oil with other oils, such as sunflower seed oil, could increase other oils' stability. Both TSE and TSC had the MUFA over 55%, and there was no significant difference of MUFA between TSC and olive oil. The MUFA had a beneficial effect on endothelial function resulting in protective changes against thrombogenesis (Perez-Jimenez, Castro, Lopez-Miranda, Paz-Rojas, Blanco, Lopez-Segura *et al.*, 1999), providing an adequate fluidity to the biological membranes and diminishing the hazard of lipid peroxide of membrane (Galeotti, Borrello, Minotti & Masotti, 1986; Turini, Thomson & Clandinin, 1991). It may also help keeping oil stability because a lower number of double bonds in the fatty acid side chain will help lowering the probability of lipid oxidation.

Oxidative stability of oils also depends to a certain degree on the presence of tocopherols that consist of α, β, γ, and δ-tocopherols (**Figure 2.3**), which are naturally occurring minor compounds of oils. They function as the most effective lipid-soluble antioxidants protecting cell membranes from peroxyl radicals and mutagenic nitrogen oxide species (Christen, Woodall, Shigenaga, Southwell-Keely, Duncan & Ames, 1997; Van Acker, Koymans & Bast, 1993), yet the α-tocopherol was considered the most important and effective antioxidant in *in vivo* tests (Schmidt & Pokorny, 2005). The recommended daily requirements suggests 15 mg

tocopherols for men and women over 14 years old (Institute of Medicine, 2000). In TSE and TSC, only α- tocopherol was detected. As listed in **Figure 2.4**, the average content of the α-tocopherol of TSE, TSC, olive oil and corn oil are 21.13, 25.88, 4.31 and 31.28 mg/ 100g oil, respectively. The α-tocopherol content of TSE and TSC is higher than that of olive oil. However, the α-tocopherol in olive oil and corn oil are highly variable. Though normal values of tocopherols in good quality olive oils lie between 10 and 30 mg/100 g oil (Boskou, 2002), its concentration may change from 0.5 to 30 mg/100 g oil. For instance, tocopherol contents ranged from 0.98 to 37 mg/100 g in Greek olive oils (Psomiadou, Tsimidou & Boskou, 2000), and 3.6 to 31.4 mg/100 g in Italian olive oils (Boskou, 2002). The main component of the tocopherol mixture is α-tocopherol, which accounts for 95 % of the total (Boskou, 2002). Although corn oil had abundant γ- tocopherol, followed by α-tocopherol, the content of α-tocopherol of corn oil varied, and ranged from 0 to 57.3 mg/100 g oil (Firestone, 1996; Goffman & Bohme, 2001; Moreau, Hicks & Powell, 1996; Strecker, Bieber, Maza, Gorossberger & Doskoczynski, 1996; Wang, Ning, Krishnan & Matthees, 1998). Finally, the α-tocopherol content of TSE and TSE was comparable with that of olive oil, and similar to that of corn oil.

2.4 Conclusions

In summary, the TSE, TSC and olive oil had similar iodine values, and the TSE had the lowest PV (peroxide value)-Fox value within the four tested oils. The experiments confirmed the TSE contained 18.3 % palmitic acid (16:0), 54.95% oleic acid (C18:1), 22.41% linoleic acid (C18:2), and 0.17% linolenic acid (18:3), or 22.58% PUFA and 56.46% MUFA. In contrast, the TSC contained 7.95 %

palmitic acid (16:0), 81.59% oleic acid (C18:1), 7.57% linoleic (C18:2), 0.25% linolenic acid (18:3), and 7.82% PUFA. The MUFA of TSC was comparable with that of olive oil. The content of α-tocopherol of TSE and TEC were 21.31 and 25.88 mg / 100 oil g, respectively. From these results, tea seed oils, TSE and TSC, were confirmed to have the best stability against oxidation than the olive and corn oils, and have suitable nutritional properties (higher oleic, medium linoleic, low linolenic acid contents). They should be considered health-benefiting, edible oils.

2.5 Figures and Tables

Table 2.1 Relative Density and Solubility of Extracted Tea Seed, Commercial Tea Seed, Olive and Corn Oil.

	Oil Content (%)	Relative Density (25℃/water)	Solubility				
			Methylene chloride (3.1)	Reagent alcohols (4.3)	Ethyl acetate (4.4)	Acetone (5.1)	Acetone-nitrile (5.8)
TSE	27.21±0.82	0.904[a]±0.008	miscible	miscible	miscible	immiscible	immiscible
TSC	–	0.921[b]±0.006	miscible	miscible	miscible	miscible	immiscible
Olive	–	0.916[b]±0.006	miscible	miscible	miscible	miscible	immiscible
Corn	–	0.916[b]±0.008	miscible	miscible	miscible	miscible	immiscible

Data expressed as means ± standard deviations

TSE = Extracted tea seed oil; TSC = Commercial tea seed oil

The relative density of each oil sample, which is the ration of the density of the oil sample to the density of deionized water at 25 ℃

Each oil sample was diluted 1:100 with different solvents

(number): polarity parameter of solvent; e.g.: hexane(0.1), and water (10.2)

Table 2.2 Hunter Color Measurements for Extracted Tea Seed, Commercial Tea Seed, Olive and Corn Oil

	L	*a*	*b*
TSE	51.99 ^{c}±0.02	(-)2.14 ^{c}±0.01	15.35^{b}±0.01
TSC	52.82 ^{b}±0.04	1.89 ^{a}±0.02	1.23^{d}±0.01
Olive	47.05^{d}±0.03	(-)2.57^{d}±0.01	23.05^{a}±0.02
Corn	55.32^{a}±0.08	1.16^{b}±0.02	3.35^{c}±0.03

Data expressed as means ± standard deviations

TSE = Extracted tea seed oil; TSC = Commercial tea seed oil

L value = measure of lightness and varies form 100 for perfect white to zero for black;

a value = measure of redness when positive, gray when zero, and greenness when negative;

b value = measure of yellowness when positive, gray when zero, and blueness when negative.

Table 2.3 Iodine Value and Peroxide Value- FOX of Extracted Tea Seed, Commercial Tea Seed, Olive and Corn Oil

	Iodine value	PV-Fox (mg TPOE/ g oil)
TSE	83.45^{b}±1.64	0.57^{d}±0.02
TSC	71.31^{d}±1.10	2.41^{c}±0.78
Olive	75.18^{c}±1.22	15.40^{a}±0.82
Corn	126.25^{a}±1.66	7.58^{b} ±0.50

Data expressed as means ± standard deviations

TSE = Extracted tea seed oil; TSC = Commercial tea seed oil

PV-Fox is a measurement of peroxide concentration in an oil sample and the PV-Fox value was expressed as milligrams of t-butyl peroxide equivalent (TPOE) per g oil.

Iodine value is a measurement of unsaturation level of oil and iodine value is defined as the grams of iodine absorbed per 100 g oil

Table 2.4 Fatty Acid Composition (% of methyl esters) of Extracted Tea Seed, Commercial Tea Seed, Olive and Corn Oil

	Fatty acid composition (% of methyl esters)			
Fatty Acid	TSE	TSC	OLIVE	CORN
C14:0	0.07±0.01	0.03±0.01	nd	0.03±0.00
C16:0	18.30±0.21	7.95±0.10	10.31±0.44	10.68±0.45
C16:1	0.01±0.00	nd	0.67±0.06	0.10±0.01
C18:0	2.25±0.04	1.74±0.26	2.09±0.99	1.08±0.10
C18:1	54.95±0.09	81.59±0.23	80.82±1.43	25.01±0.58
C18:2	22.41±0.55	7.57±0.05	5.08±0.17	61.20±1.54
C18:3	0.17±0.01	0.25±0.01	0.47±0.08	0.40±0.01
C20:0	0.09±0.01	0.05±0.01	0.34±0.04	nd
C20:1 n9	1.05±0.02	0.63±0.06	0.10±0.02	0.21±0.01
C22:0	0.20±0.02	nd	0.07±0.03	0.14±0.01
C22:1 n9	0.30±0.03	0.04±0.02	nd	nd
C24:0	0.15±0.01	0.03±0.00	0.03±0.01	0.15±0.01
C24:1	0.15±0.04	0.06±0.01	nd	nd

Data reported as percentage of total fatty acid methyl esters and expressed as means ±standard deviations

TSE = Extracted tea seed oil; TSC = Commercial product of tea seed oil

nd = not detected

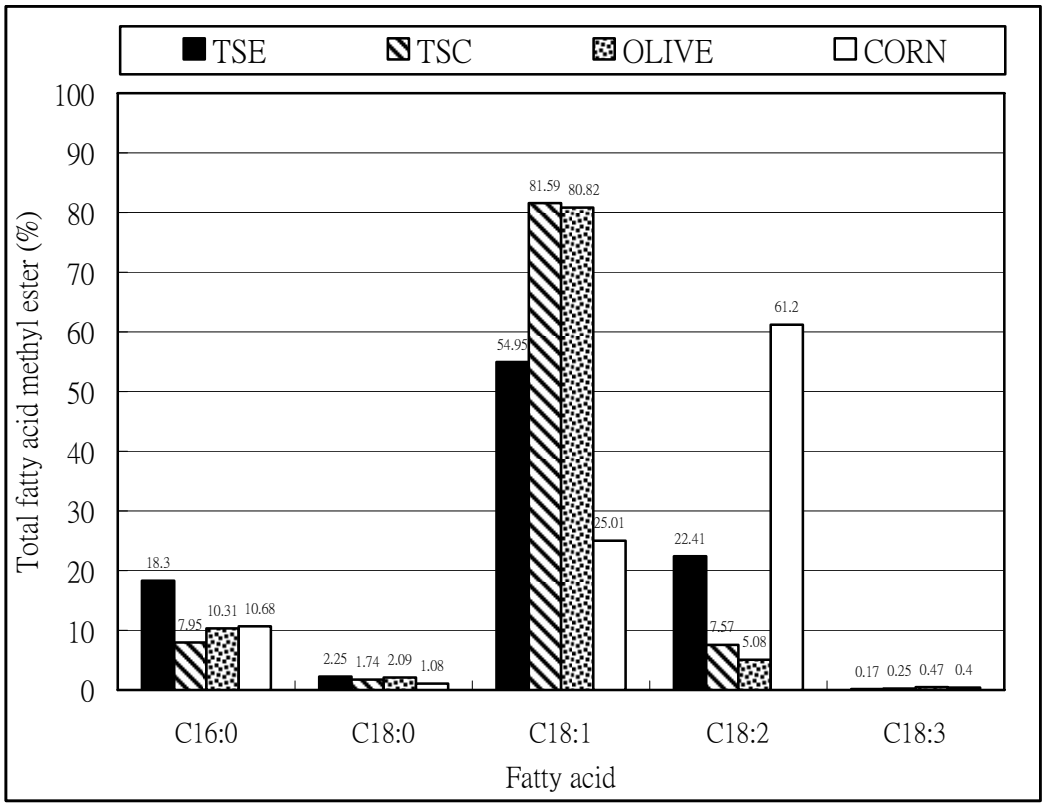


Figure 2.1 Comparison of Fatty Acid Profile within Extracted Tea Seed, Commercial Tea Seed, Olive and Corn Oils

TSE = Extracted tea seed oil; TSC = Commercial tea seed oil

Table 2.5 ANOVA for Oleic acid (C18:1), Linoleic acid (C18:2), and Linolenic acid (C18:3) in Extracted Tea Seed, Commercial Tea Seed, Olive and Corn Oil

Source of variation	DF	Sum of squares (SS)			Mean square (MS)			Observed F		
		C18:1	C18:2	C18:3	C18:1	C18:2	C18:3	C18:1	C18:2	C18:3
Treatment	3	19327.15	18143.42	0.50	6442.38	6047.81	0.17	10613.90	8818.86	100.72
Error	32	19.42	21.94	0.05	0.61	0.69	0.00	(P<.0001)	(P<.0001)	(P<.0001)
Total value	35	19346.57	18165.37	0.55				BAAC[a]	BCDA[b]	DCAB[c]

*There is no significant difference of C18:1 between TSC and Olive oil.
BAAC[a]: The content of oleic acid - Corn < TSE < TSC ≒ Olive
B=TSE; A=TSC; A=Olive; C=Corn

*For the four kinds of oils, differences were at $p<0.05$ in C18:2 and C18:3
BCDA[b]: The content of linoleic acid - Olive < TSC < TSE < Corn
B=TSE; C=TSC; D=Olive; A=Corn
DCAB[c]: The content of linoleic acid – TSE < TSC < Corn < Olive
D=TSE; C=TSC; A=Olive; B=Corn

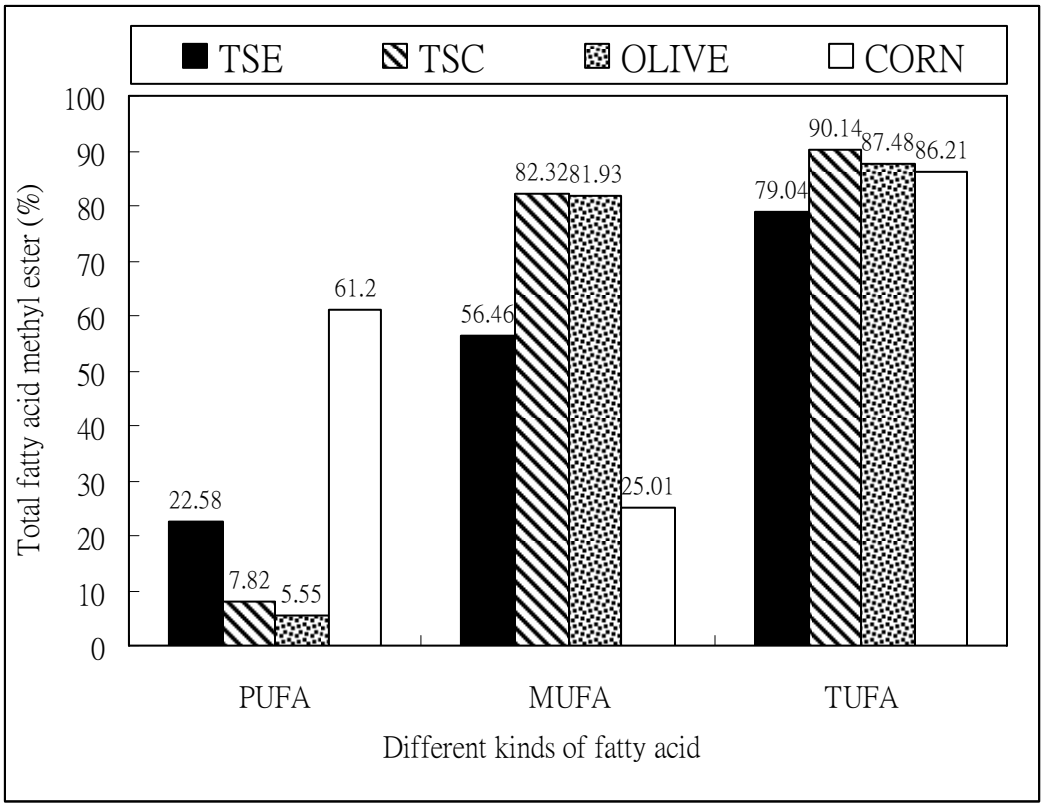
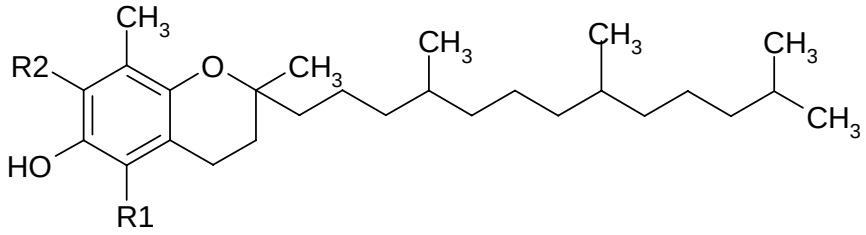


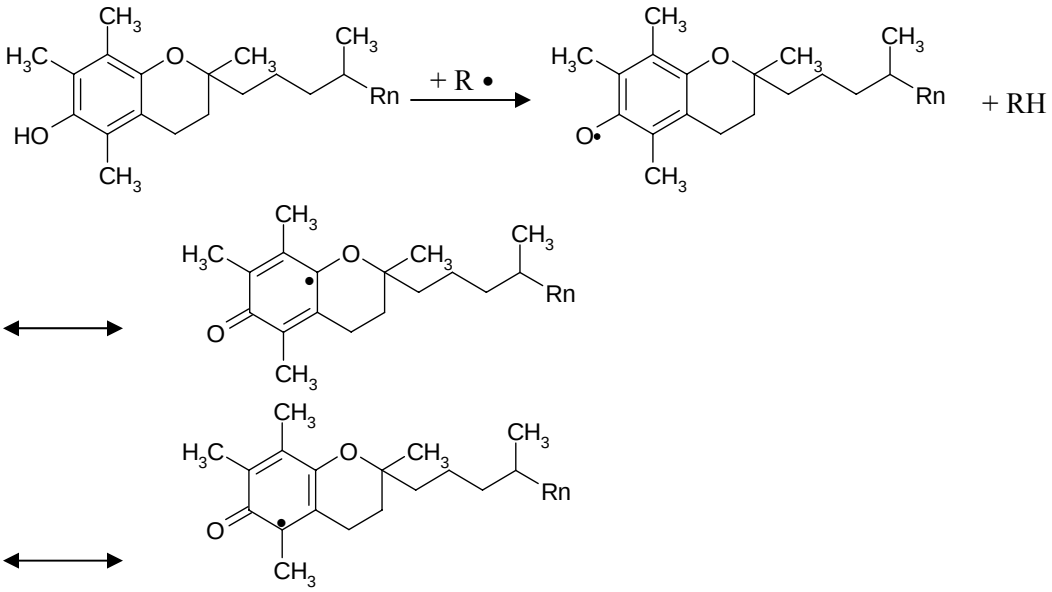
Figure 2.2 Comparison of Polyunsaturated Fatty Acid (PUFA), Monounsaturated Fatty Acid (MUFA) and Total Unsaturated Fatty Acid (TUFA) within Extracted Tea Seed, Commercial Tea Seed, Olive and Corn Oil

TSE = Extracted tea seed oil; TSC = Commercial tea seed oil



		R1	R2
α (alpha)	- tocopherol	$-CH_3$	$-CH_3$
β (beta)	- tocopherol	$-CH_3$	-H
γ (gamma)	- tocopherol	-H	$-CH_3$
δ (delta)	- tocopherol	-H	-H

A. Structures of Tocopherols



B. The Scheme for Scavenging the Radical by α – Tocopherol

Figure 2.3 Structures of Tocopherols and the Scheme for Scavenging the Radical by α -Tocopherol

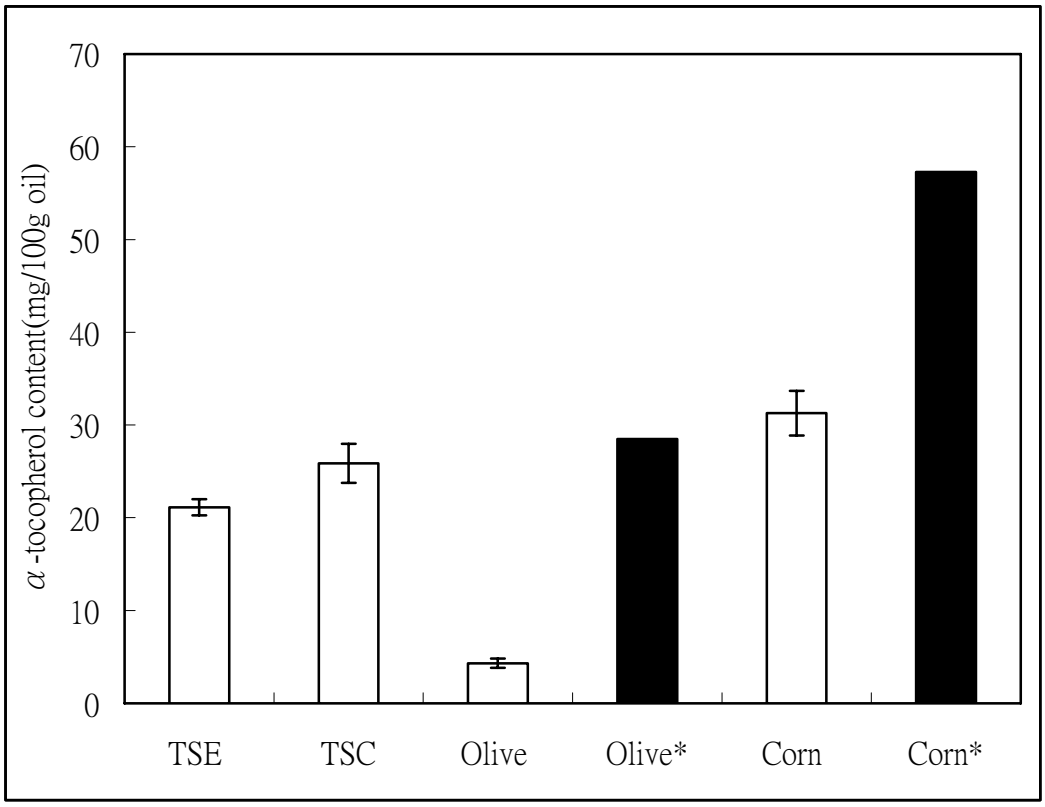


Figure 2.4 Comparison of α-Tocopherol Content within Extracted Tea Seed, Commercial Tea Seed, Olive and Corn Oil

TSE = Extracted tea seed oil; TSC = Commercial tea seed oil

Olive*: reference (Boskou, 2002)

Corn*: reference (Moreau, 2002)

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CHAPTER 3

ANTIOXIDANT CAPACITIES AND ANTIPROLIFERATIVE ACTIVITIES OF TEA SEED (*Camellia oleifera*) OIL AND MEAL

Abstract

Tea seed (*Camellia oleifera*) oil (TSE) and its meal (Meal) and commercial tea seed (*Camellia sinensis*) oil (TSC) were compared by the following antioxidant and anticancer activities in terms of their total phenolic content (TPC), trolox equivalent antioxidant capacity (TEAC), DPPH free radical scavenging capacity, and Fe^{2+} metal chelating capacity, as well as antiproliferative activities using three cancer cell lines: SiHa (human uterus cancer cell line), MCF-7 (human breast cancer cell line) and HT-29 (human colon cancer cell line). The TPC and TEAC of the Meal were 23.3 mg gallic acid equivalents/g and 4.42 μM Trolox /g, respectively, which were 7 to 15 times higher than that of the TSE. The Meal also exhibited a stronger DPPH free radical scavenging activity than TSE and TSC. By using the same DPPH assay, TSE at concentrations from 75 to 100 mg oil equivalent/mL showed stronger radical scavenging activity than 250 μM BHT (55.68 μg BHT/mL), but there was no significant difference between the Meal at concentrations of 50 to 100 mg Meal equivalent/mL and BHT at a concentration of 1 mM BHT (0.22 mg BHT/mL). The Fe^{2+} metal chelating capacity of the Meal at the level of 25 mg Meal equivalent/mL can reach 90%. In addition, both the TSE and Meal had antiproliferative activities against three cancer cell lines, SiHa,

MCF-7 and HT-29. The IC_{50} values of TSE and Meal were 146.70 and 2.92 mg sample equivalent/mL for SiHa, 236.20 and 2.94 mg sample equivalent/mL for MCF-7, and 155.20 and 1.52 mg sample equivalent/mL for HT-29, respectively. The data from this study suggests the potential of developing the TSE and Meal into value-added products rich in natural antioxidants and antiproliferative agents for benefiting human health.

3.1 Introduction

Tea seed (*Camellia oleifera)* oil has been used as one of the major edible oils in China for more than 1000 years (Shanan & Ying, 1982; Liao, Ji & Tong, 2005). It is also a good industrial raw material for producing manufactured soap, margarine, and hair oil. Residues of tea seed oil have also been used to feed livestocks (Ruter, 2002).

Some investigations were conducted to study the health beneficial characteristics of tea seed and its products. The tea seed oil was found to possess antioxidant activities capable to reduce liver reactive oxygen species (ROS) in rats (Zhang & Zhou, 1995). Some saponins in tea seed could decrease the cholesterols, triglycerides, and low density-lipoproteins (LDL) in the blood of rats (Chen, Qiu & Peng, 1998), and had gastroprotective effects (Morikawa, Li, Nagatomo, Matsuda, Li & Yoshikawa, 2006). Besides, tea seed contained some five-ring triterpenes that could suppress the microbial growth (Huang, Ao & Zhong, 2002).

Since oxygen-derived free radicals were found to be closely related to the pathogenesis of various diseases such as cancer, inflammation, atherosclerosis, ischemia-reperfusion injuries, shock, aging, Alzheimer's disease, cardiovascular diseases, diabetes, hypertension, cataracts, infertility and exercise-associated muscle damage (Halliwell, 1996). Antioxidants, particularly the natural antioxidants, have attracted much attention because they possess many bioactivities, such as antioxidant, antimutagenic, anti-inflammatory and anticarcinogenic activities (Yeh & Yen, 2003), that can help preventing the development of some chronic diseases as mentioned above, (Neff, 1997; Yu, Perret, Harris, J. & Haley,

2003) and thus provide health benefits for consumers (Andreasen, Landbo, Christensen, Hansen & Meyer, 2001; Halliwell, 1996; Wang & Lin, 2000; Yu, Perret, Harris, J. & Haley, 2003). Many research studies have focused on anticancer properties as protective adjuncts against a hot of diseases (Yang, Chen, Chang, Lu, Lai, Chen *et al.*, 2006; Yi, Fischer & Akoh, 2005). Although various synthetic agents also exhibit anticancer and antioxidative activities, they are not preferred by many consumers because they are not only expensive, but there is also concern about their possible long-term, hazardous effects. Therefore, natural antioxidants, such as natural phenolics, instead of the synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are intentionally kept in edible oils to prevent the lipid peroxidation, although natural phenolics are not considered the nutritive compounds (Branen, 1975; Tappel, 1995).

Cultured human cancer cell lines are employed extensively in screening putative anticancer agents. For example, the use of *in vitro* cellular models is crucial with regard to the acquisition of stable and reproducible results when screening anticancer agents. Also, the *in vitro* approach constitutes a crucial prerequisite to *in vivo* trials. Meanwhile, in the search for effective anticancer treatments for humans, edible plants and/or their oils may be a good source of new antitumor agents with reduced side effects (Chen, Montanari & Widmer, 1997; Miller, Porter, Binnie, Guo & Hasegawa, 2004). Lee *et al.* found that tea seed oil contained two bioactive compounds that may act as a prophylactic agent to prevent free radical related diseases (Lee & Yen, 2006).

My research work was conducted to evaluate the antioxidant capacity and antiproliferative ability of the extracted tea seed (*Camellia oleifera*) oil (TSE), commercial tea seed (*Camellia sinensis*) oil (TSC), and the tea seed meal (Meal). Its aims were to find out more potential health benefiting characteristics of the tea seed oil and its byproduct meal, and to help increase the profitability through value-added utilization.

3.2 Materials and Methods

3.2.1 Materials and Chemicals

The sample of tea seed (*Camellia oleifera*) was obtained from Fujian province, China. Commercial tea seed (*Camellia sinensis*) oil (TSC) was supplied by Lu Yu Tea Company (Seattle, WA, USA). The tea seed meal was obtained after oil extraction from the tea seed (*Camellia oleifera*). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), gallic acid, Folin-Ciocalteu reagent, butylated hydroxytoluene (BHT), sodium pyruvate, sterile cell culture penicillin, streptomycin, Rosewell Park Memorial Institute (RPMI) 1640, sodium bicarbonate, and trypsin EDTA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Trolox was purchased from ACROS Organics Co. (Morris, NJ, USA). 2,2'-azobis(2-aminopropane)dihydrochloride was purchased from Roche siagnostics crop. (Indianapolis, IN, USA). Ferrous chloride and 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine) were purchased from Fluka Chemical Co. (Milwakee, WI, USA). Ethylenedinitrile tetraacetic acid (EDAT), peroxide hydrogen, peroxidase, and all HPLC analytical grade solvents were purchased from Fisher Scientific (Suwanee, GA, USA). Tissue culture plates were from Costar

(Cambridge, MA). Comic calf serum and fetal bovine serum were purchased from Hyclone (Logan,Utah). MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazo, was provided by Promega (Madison, WI).

3.2.2 Oil Extraction of Tea Seed (*Camellia Oleifera*)

The oil extraction method is modified from Lee and Yen (Lee & Yen, 2006). Tea seed (*Camellia oleifera*) powder was roasted at 120 ℃ for 20 minute. Then twenty grams of the tea seed powder was extracted by 250 mL hexane for 12 hours using a Soxhlet extractor. After filtration, hexane was evaporated to obtain the tea seed oil by using a rotary evaporator (Model R-200; Buchi, Switzeland).

3.2.3 Preparation of Antioxidant Extracts

Preparation of antioxidant extracts was modified from the method described by Parry and Yu (Parry & Yu, 2004). One gram of TSE and TSC were dissolved in 10 mL hexane and extracted respectively with 5 mL methanol by using a pear-shape funnel at room temperature for 30 min. Five grams of the tea seed meal were extracted with 50 mL methanol. The mixtures were shaken at room temperature in the dark room for 24 hours and then filtered. The final concentration was expressed as mg of sample equivalent per mL of methanol. All antioxidant extracts were flushed with nitrogen and kept in the refrigerator for further analysis.

3.2.4 Total Phenolic Contents (TPC)

Determining TPC of the tea seed oil and meal used the Folin-Ciocalteu method (Yu, Haley, Perret, Harris, Wilson & Qian, 2002). Gallic acid was used as the standard and prepared in a serial of concentrations at 1, 10, 25, 50, and 100 mg/mL in methanol. The reaction mixture contained 50 μL of antioxidant extract or

gallic acid solution, 250 μL Folin-Ciocalteu reagent, 0.75 mL of 20% sodium carbonate, and 2 mL of distilled water. After 2 hours of the reaction in the darkness at room temperature, the mixture was measured at 765 nm by a UV spectrophotometer (Genesys 20 Model 400/4; ThermoSpectronic, Rochester, NY, USA). The TPC was calculated and expressed in gallic acid equivalent.

3.2.5 Trolox Equivalent Antioxidant Capacity Assay (TEAC)

The method is based on the ability of antioxidant molecules to quench the long-lived $ABTS^+$, a blue-green chromophore with characteristic absorption at 734 nm, and compared with that of Trolox, a water-soluble vitamin E analog. A stable stock solution of $ABTS^+$ was produced by reacting a 7 mM aqueous solution of $ABTS^+$ with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the darkness at room temperature for 12-16 hours before use (Pellegrini, Serafini, Colombi, Rio, Salvatore, Bianchi *et al.*, 2003). At the beginning of the analysis, the $ABTS^+$ working solution was diluted by methanol from the stock solution. The final reaction mixture contained 1.0 mL $ABTS^+$ solution and 100 μL of sample (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999). Its absorbance was measured at 734 nm by a spectrophotometer to an absorbance of 0.70 ± 0.02 AU after 5 min reaction. The radical cation $ABTS^+$ scavenging capacity of each antioxidant extract was calculated and expressed as concentration of Trolox equivalents (TEAC) (μM/g oil or g meal).

3.2.6 Determining Antioxidant Capacity by the DPPH Assay

3.2.6.1 The kinetics of DPPH-antioxidant reaction

The DPPH free radical scavenging activity of antioxidant extracts were measured by the Yamaguchi method with minor modification (Yamaguchi, Takamura, Matoba & Terao, 1998). DPPH radical is a stable free radical in purple color with an absorbance at 517 nm. When the odd electron of the radical pairs off in the presence of a hydrogen donor, its absorption strength decreases and is stoichiometric with respect to the number of electrons captured. The kinetics of the DPPH-antioxidant reaction was examined for each antioxidant samples at 1, 2, 5, 10, 20, 30, 40, 60 min and used to estimate the remaining radical levels. In this assay, the beginning concentration was 125 μM for DPPH radicals in all reaction mixtures. The concentration of meal extracts was 25 mg sample equivalent/mL of reaction mixture, and the concentration of oil extracts was 50 mg sample equivalent/mL reaction mixture.

3.2.6.2 DPPH radical scavenging activity

DPPH free radical scavenging assay was adopted from the method described by Yamaguchi *et al* (Yamaguchi, Takamura, Matoba & Terao, 1998). An aliquot of 0.5 mL of antioxidant extract solution was mixed with 0.5 mL of 250 μM DDPH solution dissolved in methanol. After incubation for 30 min in the darkness at room temperature, the absorbance of the reaction mixture was spectrophotometrically measured at 517 nm and compared with standard antioxidant BHT. Lower absorbance of reaction mixture indicated higher free radical scavenging activity. Scavenging activity of free radical was determined by:

$$\text{Scavenging Effect (\%)} = (1 - \frac{\text{absorbance of sample at 517 nm}}{\text{absorbance of control at 517 nm}}) \times 100$$

3.2.7 Metal Chelating Capacity of Meal

The metal chelating capacity of the methanolic extract of the tea seed meal was determined by the ferrous ions chelating assay described by Kim *et al*. (Kim, Chen, Wang, Chung & Jin, 2005). Reaction solution composed of 800 μL of sample and 10 μL of 2 mM Ferrous chloride was initially activated by addition of 20 μL of 5 mM Ferrozine. The reaction mixture was incubated at room temperature for 10 min, and its chelating activity was spectrophotometrically measured at 562 nm. The metal chelating effect was calculated by using the following equation:

$$\text{Metal Chelating Effect}(\%) = (1 - \frac{\text{absorbance of sample at 562 nm}}{\text{absorbance of control at 562 nm}}) \times 100$$

The EC_{50} value used to evaluate antioxidant capacities of the sample antioxidant extracts and standards were the effective concentration at which DPPH radicals were scavenged and metal ions were chelated by 50% respectively.

3.3.8 Antiproliferative Activities

Three cancer cell lines, SiHa (human uterus cancer cell line), MCF-7 (human breast cancer cell line) and HT-29 (human colon cancer cell line) were purchased from American Type Culture collection (ATCC) (Rockville, MD). All cell lines were cultured in the RPMI-1640 medium with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 unit/mL), streptomycin (0.1 mg/mL), 0.1 mM non-essential amino acids, 1.5 g/L sodium bicarbonate and 10% cosmic calf serum, and incubated at 37^{o}C with 5% CO_2 and 90-100% relative humidity. After 48h, cancer cells were fed with fresh medium, and cells were subcultured when they were about 80-90% confluence. The cells were determined with tyrpan blue.

Prior to chemical treatment, 10^4 cells/well (100 μL) were seed into a 96-well tissue culture plate, and allowed to attach for 24 hours, then treated with defined concentrations of the tested chemicals in methanolic extracts of samples. The sample concentration was kept at 2% per well, and control wells were treated with methanol only. After 24 hour incubation, cell proliferation was determined using the MTS bioassay according to the manufacture's recommendations (Promega, Madison, WI) and recorded at 490 nm. The MTS assay was based on the reduction of a soluble tetrazolium salt, by mitochondrial dehydrogenase of viable tumor cells, into an insoluble colored formazan product, which can be measured spectrophotometrically. The enzymatic activity and the number of formed formazan were proportional to the number of living cells. This can generally be explained by cell inhibition or cell viability (Endrini, Rahamt, Ismail & Hin, 2002). Cell inhibition was calculated by the following formula;

$$\text{Cell inhibition (\%)}=(1-\frac{\text{absorbace of sample at 490 nm}}{\text{absorbance of control at 490 nm}})*100$$

The IC_{50} was defined as the chemical concentration causing 50% inhibition of cell growth and calculated from a four-parameter curve. Four-parameter curves were expressed as:

$$Y = \frac{(A - D)}{[1 + (\frac{X}{C})^B]} + D$$

where X is the concentration, Y is the corresponding absorbance, A is the response at zero concentration of sample, B is the curvature parameter, C is the concentration giving 50% reduction (IC_{50}) and D is the response at infinite concentration.

Triplicates were performed for each concentration of the tested sample. All the experiments were done at least three times on different days.

3.2.9 Statistical Analyses

Data were reported as the mean ± standard deviation. All statistical analysis was conducted on the SAS V9.1 software for Windows (SAS Institute Inc., Cary, NC, USA). Differences among all sample means were determined by analysis of variance (one–way analysis of variance, AVONA), $\rho > 0.05$.

3.3 Results and Discussion

Though the contents of bioactive compounds in samples change with different extraction methods, the Soxhlet extraction method is often recommended and used. Selected extraction solvents also play critical roles. For example, Li *et al.* found that tea seed oil contained more bioactive compound squalene when extracted by hexane than by other solvents such as petroleum ether, acetone and chloroform, or the expression method (Li, Wang, Bi & Zhao, 2006). However, methanol was more commonly used to extract bioactive compounds (Owen, Giacosa, Hull, Haubner, Spiegelhalder & Bartsch, 2000). Lee *et al.* indicated that the methanolic extract of tea seed exhibited the highest yield of antioxidant content and the strongest antioxidant activity compared with other three extracts extracted by acetone, ethyl acetic and acetonitrile (Lee & Yen, 2006).

Plant phenolics constitute one of the major classes of natural antioxidants. These phenolics occur in all plant parts, including seeds, leaves, flower, roots, and barks (Dimitrios, 2006). Phenolic compounds can react with the molybdenum-containing Folin-Ciocalteu reagent and be reduced by an electron

transfer. The electron transfer, in turn, converts the deep yellow color to blue: a color which can be measured spectroscopically (Singleton & Rossi, 1965). As shown in **Table 3.1**, total phenolic contents of the samples were expressed as gallic acid equivalents per gram of sample of the extract. The TPC of the TSE and Meal were 1.63 and 23.30 milligrams of gallic acid equivalents per gram of sample (mg GAE/g sample). However, the phenolic compound could not be detected in TSC. The TSE was comparable to the cold-press berry seed oil (0.09-2.00 mg GAE/ g oil) (Parker, Adams, Zhou, Harris & Yu, 2003; Parry & Yu, 2004) and olive oil (0.55-0.81 mg GAE/ g oil) (Ardo, 2005) regarding their TPC values. The result suggests that TSE and Meal can potentially serve as natural sources of dietary phenolic compounds.

Antioxidant activities of the TSE, TSC and Meal were measured and compared on their free radical scavenging activities against radical cation $ABTS^{+}$ (**Table 3.1**). Meal had the greatest ability to quench the radical $ABTS^{+}$, followed by TSE and TSC. The mean values of the TSE, TSC and Meal were 0.70, 0.01 and 4.42 μM Trolox equivalent/g sample, respectively. The latter was comparable to the values of some plant foods and beverages (Pellegrini, Serafini, Colombi, Del Rio, Salvatore, Bianchi *et al*., 2003). Moreover, the Trolox equivalent of TSE was higher than that of the olive oil (0.63 μM Trolox/g) and peanut oil (0.61 μM Trolox/g). The DPPH scavenging activities of the TSE, TSC and Meal were measured to help gathering a full picture of their free radical scavenging capabilities. In addition, 50 milligrams Meal equivalent/mL reaction mixture and 100 mg oil equivalent/mL reaction mixture were measured by the DPPH assay in

terms of its remaining value (%). There was a significant difference within those three samples. After 5 minutes of the antioxidant reaction, the DPPH remaining % of TSE, TSC and Meal were 45.15%, 90.15% and 15.05%, respectively (**Figure 3.1**). After 10 minutes, their DPPH remaining % approached stable. In all tested periods, the TSE showed a higher level of DPPH scavenging ability than the TSC. Such different scavenging rates and antioxidant capabilities of the TSE, TSC and Meal were attributed to their different contests of phytochemicals. In comparison with the standard antioxidant BHT, free radical scavenging activity of TSE at concentration of 75 to 100 mg oil equivalents/mL was stronger than that of 250 μM of BHT (55.68 μg BHT/mL) (**Figure 3.2**). In addition, TSE had an $EC_{50\text{-DPPH}}$ value of 57.38 mg sample equivalent/mL (**Table 3.2**). As mentioned in **Table 2.2** of **Chapter 2**, the TSE and TSC significantly differed in their *L*, *a* and *b* values. Particularly, the TSE showed more yellowish color than the TSC. The color was attributed to the higher content of pigments or colorful phytochemicals in the TSE. These phytochemicals may be the major contributor for the oil stability against oxidation. In addition, the antioxidant capacity of TSE may be ascribed to other antioxidants such as sesamin, 2,5-bis-benzol[1,3]dioxol-5-yl-tertrahydro-furo [3,4-d][1,3]-dioxine (Lee & Yen, 2006) and squalene (Li, Wang, Bi & Zhao, 2006; Newmark, 1997). Due to the existence of these antioxidants in the TSE, the TSE had a higher antioxidant capacity than the TSC, and was comparable with the standard antioxidant BHT at specific concentration.

Oil seeds have been exploited as potential sources of natural antioxidants (Parry & Yu, 2004). As a byproduct of the tea seed oil, the tea seed Meal also

contained a high level of DPPH scavenging agents and had an $EC_{50\text{-}DPPH}$ value of 22.34 mg sample equivalent/ mL, approximately 2.5 times lower than that of the TSE (**Table 3.2**). In the DPPH free radical scavenging assay, there was no significant difference between the Meal at concentration of 50 to 100 mg sample equivalent/mL and BHT at concentration of 1 mM (0.22 mg BHT/mL), though the Meal at 25 mg sample equivalent/mL had higher DPPH antioxidant activity (61.05%) than at 250 μM BHT (55.68 μg BHT/mL) (**Figure 3.3a**). **Figure 3.3b** shows the Meal also possesses a strong Fe^{+2} metal chelating capacity at the level of 0.614 mg EDTA equivalents/g Meal; however, TSE and TSC did not show metal chelating ability. These results indicated that TSE was a moderately competitive free radical scavenging agent compared to the well known synthetic antioxidants BHT, but it may not be a good material to prevent free radical reactions initiated by metal ions. As compared to EDTA, the well known metal chelating agent, the Meal at a level of 10 mg sample equivalent/mL reached 78.15 % metal chelating activity, which was greater than 10 μM EDTA (2.9 μg EDAT/mL). When the concentrations of 25 to 100 mg sample equivalent/mL of Meal were tested, their metal chelating activity could approach 95 %, which was comparable to 50 μM EDTA (14.5 μg EDTA/mL). Moreover, the Meal had an $EC_{50\text{-}DPPH}$ value of 5.98 mg sample equivalent/mL (**Table 3.2**). The metal chelating capability of the Meal may provide complimentary protection against oxidations induced by metal ion.

The TSE and Meal methanolic extracts with a stronger DPPH radical scavenging capacity also exhibited a higher TEAC value; however, no significant

correlations could be found between the TPC and the DPPH scavenging activities (ρ<0.05) of the antioxidant extracts. It is known that different phenolic compounds have different responses in the Folin-Ciocalteu method because they do not react with tyrosine side chains even though these groups have free radical scavenging capacities (Huang, Ou & Prior, 2005). Thus, the result confirmed some conclusions in previous studies that the antioxidant activity of an extract, e.g., wheat extract (Yu, Haley, Perret, Harris, Wilson & Qian, 2002) and plant extracts (Kahkonen, Hopia, Vuorela, Rauha, Pihlaja, Kujala *et al*., 1999), could not be predicted on the basis of its total phenolic content.

Many studies have focused on antiproliferative and antioxidative properties as protective adjuncts against a host of diseases (Institute of Medicine, 2000; Yang, Landau, Huang & Newmark, 2001). In my investigation, three different human cancer cell lines, i.e., MCF-7 (human breast cancer cell line), SiHa (uterus cancer cell line) and HT-29 (human colon cancer cell line), were selected to evaluate the inhibitive activities of TSE and TSC at concentrations ranging from 150 to 500 mg sample equivalent/mL, and of Meal ranging from 1 to 10 mg sample equivalent/mL. The TSC didn't show any antiproliferative activities even at a high concentration, 500 mg sample equivalent/mL, and there was no antiproliferative ability of the TSE at the concentration of lower than 150 mg sample equivalent/mL. Nevertheless, the Meal showed a greater potential for inhibiting cancer cells at concentrations higher than 1 mg sample equivalent/mL.

As shown in **Figure 3.4**, there is no significant difference of the antiproliferative activity of TSE at concentration of 250, 300 and 500 mg oil

equivalent/mL within those three cancer cell lines. However, at concentrations of 150 and 200 mg oil equivalent/mL, the percent of inhibition of HT-29 and SiHa were higher than that of MCF-7. The result indicated that SiHa and HT-29 might be more sensitive to TSE at lower concentrations. **Figure 3.5** shows that, at concentrations from 3.25 to 10 mg Meal equivalent, no significant difference was found in the percent of inhibition with those three cancer cells, and the percentage of inhibition reached around 80-90%. The results point out that the Meal at concentration of 3.25 mg sample equivalent/mL may be the lowest effective concentration for the antiproliferative ability. Besides, HT-29 showed more sensitivity to the Meal at a concentration of 2 mg sample equivalent/mL. The antiproliferative and antioxidative bioactivities of the Meal may be attributed to kaemperol, kaemperol glycosideic flavonoids (Li & Luo, 2003; Park, Rho, Kim & Chang, 2006), saponins (Chen, Qiu & Peng, 1998), and five-ring triterpenes (Huang, Ao & Zhong, 2002). Also noted was that the order of the antiproliferative ability on these three cancer cell lines was the same as that of TPC, suggesting that total phenolic contents may be an important indicator for their antiproliferative activity. In **Table 3.3**, the IC_{50} values of the TSE for SiHa, MCF-7 and HT-29 were 146.70, 236.20 and 155.20 mg sample equivalent/mL, and the IC_{50} values of the Meal for SiHa, MCF-7 and HT-29 were 2.92, 2.94 and 1.52 mg sample equivalent/mL. Although the experiments demonstrated that the TSE could inhibit the cancer cells' growth, its inhibitive ability was much weaker than that of the Meal. The results indicated that TSE and Meal contained different

levels of antiproliferative compounds. Moreover, HT-29 showed more sensitivity to both TSE and Meal.

To explore their potential utilization in cancer prevention, further investigation is required to identify the bioactive components in the TSE and Meal, evaluate their antiproliferative activities, and investigate the underlying mechanisms.

3.4 Conclusions

The TPC and TEAC of Meal were 23.3 mg gallic acid equivalents/g and 4.42 μM Trolox/g, respectively. These were 7 to 15 times higher than those of the TSE. The Meal also exhibited a stronger DPPH free radical scavenging activity than TSE and TSC. In the same DPPH assay, TSE at concentrations from 75 to 100 mg oil equivalent/mL was stronger than 250 μM BHT (55.68 μg BHT/mL). Besides, there was no significant difference between Meal at concentrations of 50 to 100 mg Meal equivalent/mL and BHT at concentration of 1 mM BHT (0.22 mg BHT/mL). The Fe^{2+} metal chelating capacity of Meal can reach 90% at the level of 25 mg Meal equivalent/mL. In addition, both the TSE and Meal had antiproliferative activities against three cancer cell lines, SiHa, MCF-7 and HT-29. Those results from this paper demonstrate that both the TSE and the Meal had potent antioxidant activities and antiproliferative capabilities, which were attributed to their inherent contents of natural bioactive components. However, further investigation is required to separate the antioxidants and antiproliferative compounds in order to promote their utilization in food and dietary supplemental products for health promotion and disease prevention.

3.5 Figures and Tables

Table 3.1 Total Phenolic Content (TPC) and Trolox Equivalent Antioxidant Capacity (TEAC) of Tea Seed Oil and Meal

Sample	TPC (mg GAE/g)	TEAC (μM Trolox /g)
TSE	1.63^{b}±0.25	0.70^{b}±0.030
TSC	nd	0.01^{c}±0.005
Meal	23.30^{a}±2.37	4.42^{a}±0.345

TSE = Extracted tea seed oil

TSC = Commercial tea seed oil

Meal= Tea seed meal

TPC= total phenolic content and expressed as gallic acid equivalent (GAE)

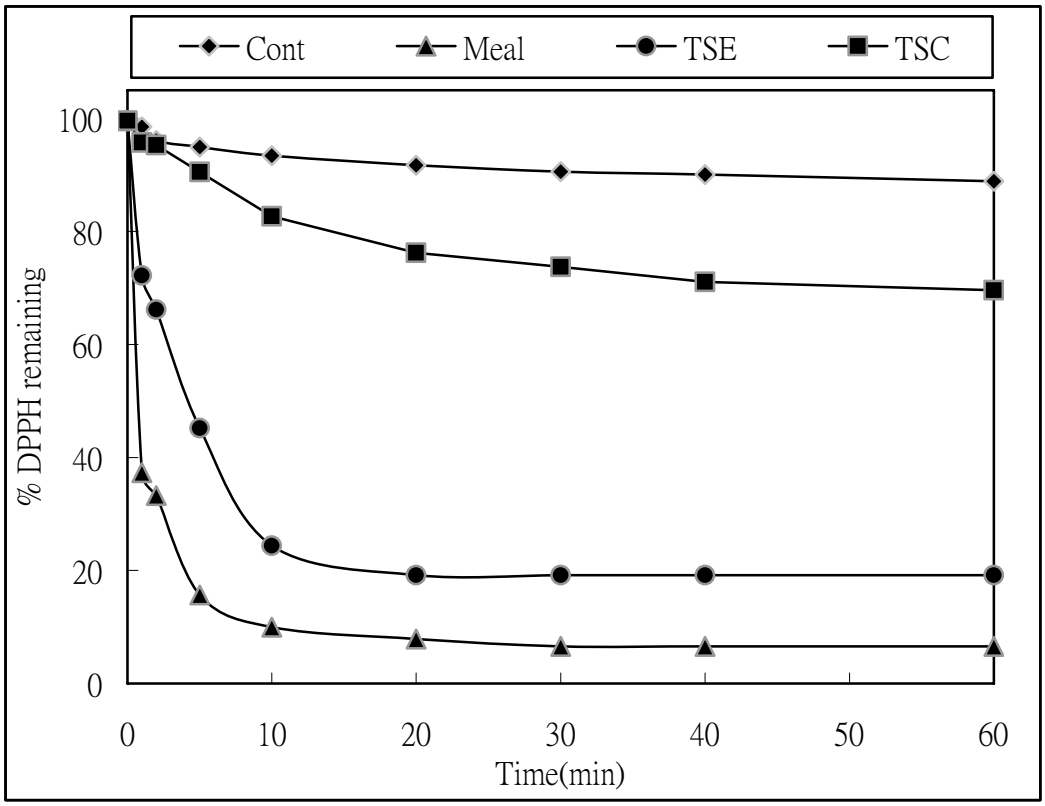


Figure 3.1 Reaction Kinetics of Tea Seed Oil and Meal - DPPH Assay

Cont = Control

Meal= Tea seed meal

TSE = Extracted tea seed oil

TSC = Commercial tea seed oil

The beginning concentration of DPPH was 125 μM for DPPH in all reaction mixture.

The concentration of meal extracts was 25 mg sample equivalent/mL of reaction mixture.

The concentration of oil extracts was 50 mg sample equivalent/ mL reaction mixture.

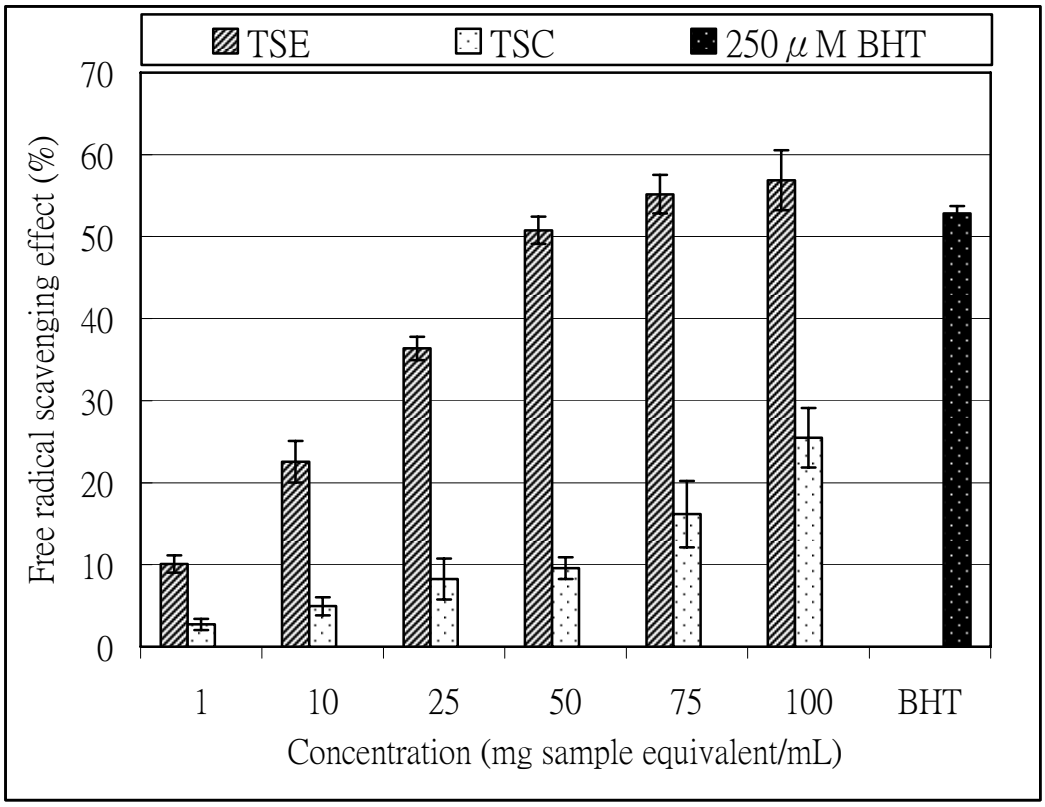
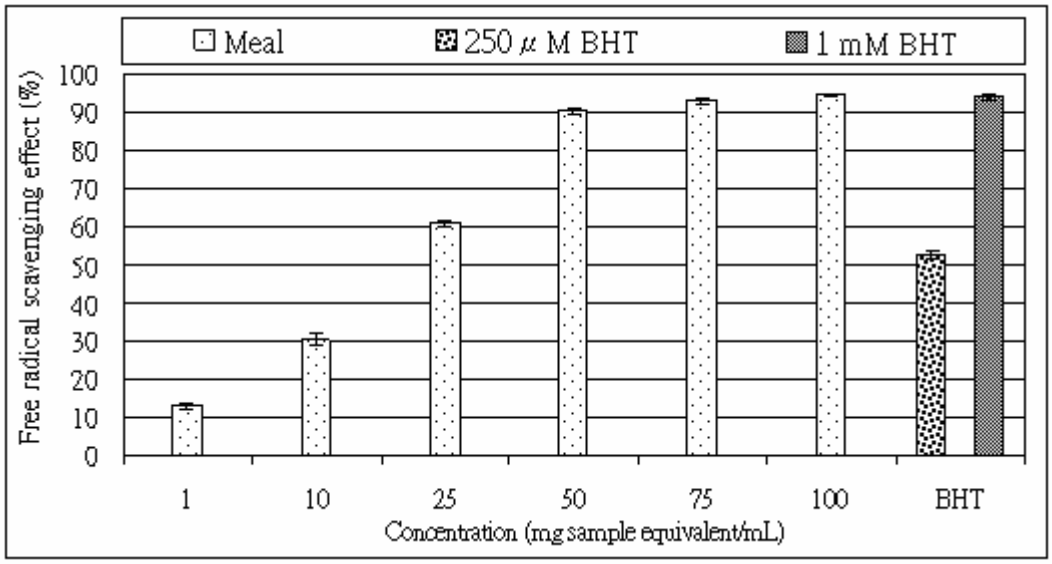


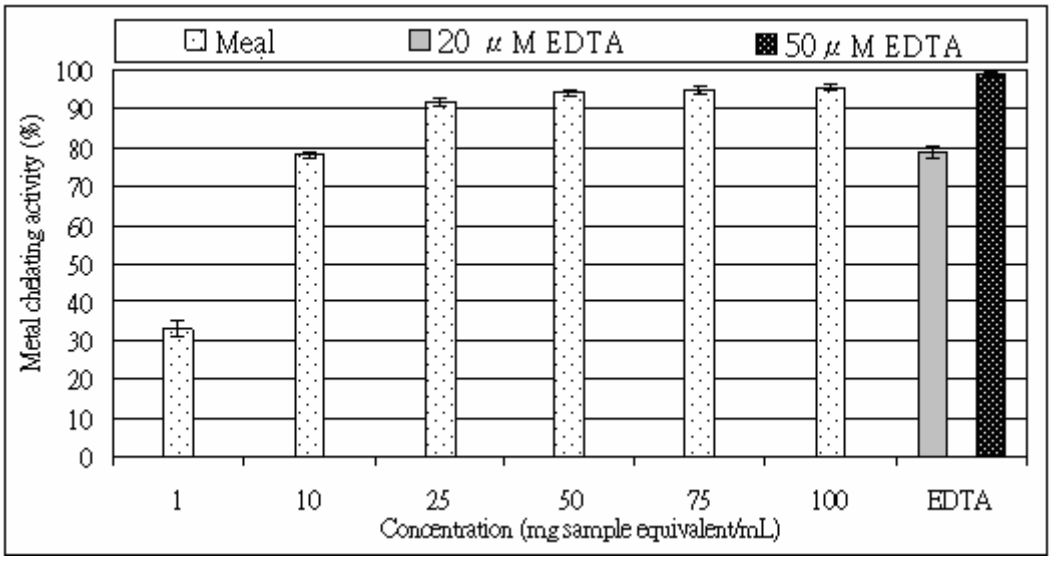
Figure 3.2 Comparison of Free Radical Scavenging Activity of Tea Seed Oil and Butylated Hydroxytoluene (BHT) - DPPH assay

TSE = Extracted tea seed oil

TSC = Commercial tea seed oil



a. Free radical scavenging capacity - DPPH assay



b. Metal chelating activity (%)

Figure 3.3 Antioxidant Capacity of Tea Seed Meal (Meal)

a. Free radical scavenging effect (%) - DPPH assay

b. Metal chelating activity (%)

Table 3.2 The EC_{50} Value of Tea Seed Oil and Meal against DPPH Radicals and Chelating Metal Ions

Sample	Antioxidant Capacity EC_{50} (mg sample equivalent /mL)	
	DPPH	Metal Chelating
TSE	57.38	-
TSC	-	-
Meal	21.37	5.98

The EC_{50} value used to evaluate antioxidant capacities of antioxidant extracts were the effective concentration at which DPPH radicals were scavenged and metal ions were chelated by 50% respectively.

TSE = Extracted tea seed oil

TSC = Commercial tea seed oil

Meal= Tea seed meal

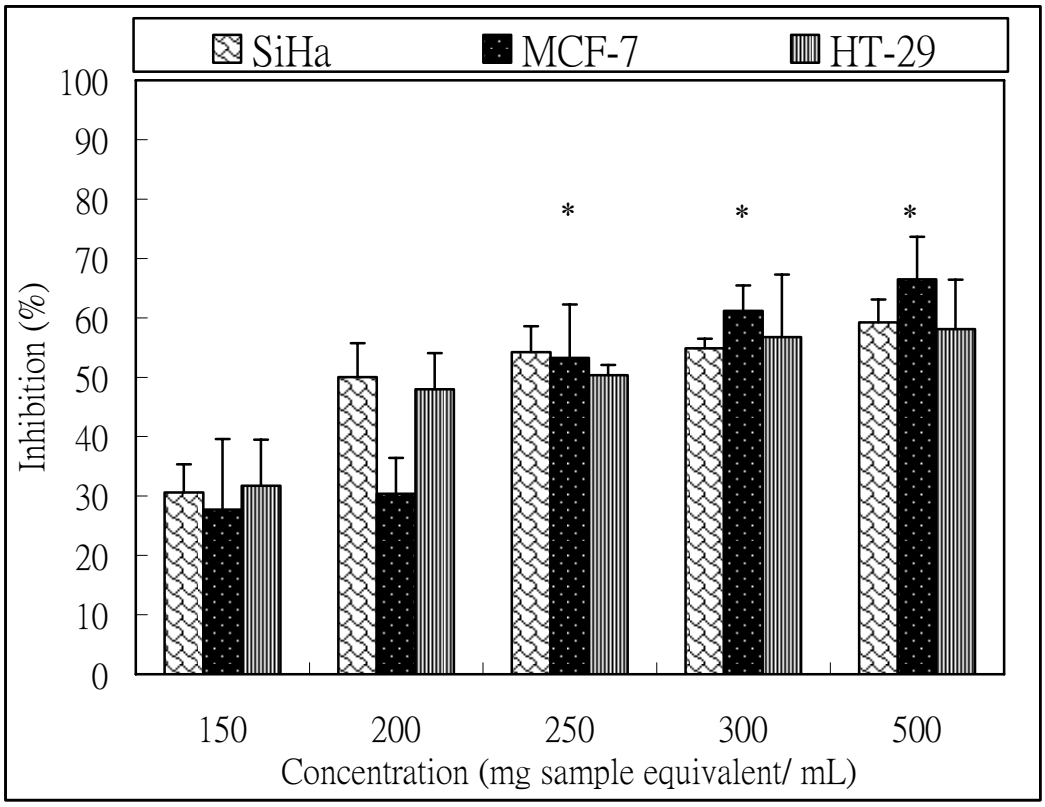


Figure 3.4 Antiproliferation Activity of Extracted Tea Seed Oil (TSE)

Antiproliferative effects of the tea seed oil antioxidant extracts were expressed as percent inhibition of cancer cells after exposure to treatment for 24 hours.

SiHa : Human uterus cancer cell line

MCF-7 : Human breast cancer cell line

HT-29 : Human colon cancer cell line

*: Asterisk indicates no significant difference at different concentrations within these three different cancer cell lines

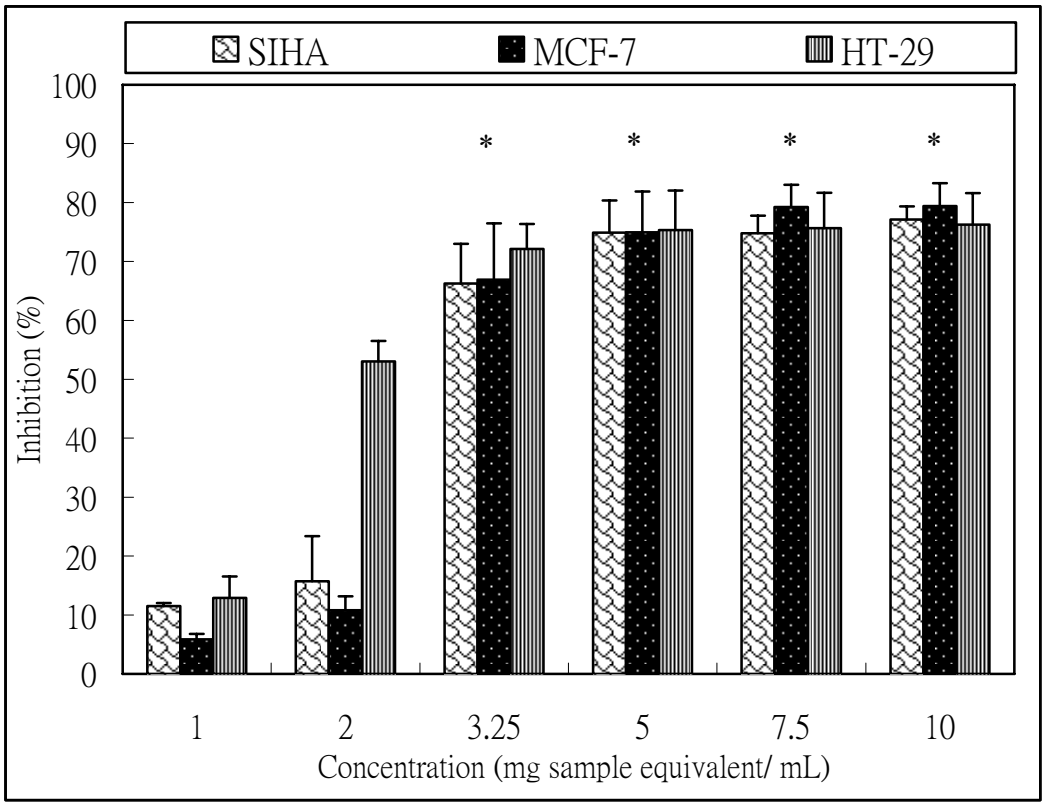


Figure 3.5 Antiproliferation Activity of Tea Seed Meal (Meal)

Antiproliferative effects of the tea seed meal antioxidant extracts were expressed as percent inhibition of cancer cells after exposure to treatment for 24 hours.

SiHa : Human uterus cancer cell line

MCF-7 : Human breast cancer cell line

HT-29 : Human colon cancer cell line

*: Asterisks indicate no significant difference at different concentrations within these three different cancer cell lines

Table 3.3 The IC_{50} Values of Extracted Tea Seed Oil (TSE) and Tea Seed Meal (Meal) to Inhibit Human Cancer Cell Growth

Human cancer cells	Antiproliferative Activities IC_{50} (mg sample equivalent /mL)	
	TSE	Meal
SiHa	146.70	2.92
MCF-7	236.20	2.94
HT-29	155.20	1.52

IC_{50} was expressed as the concentration resulting in a 50% inhibition of cell growth and calculated from four parameter curve

SiHa : Human uterus cancer cell line

MCF-7 : Human breast cancer cell line

HT-29 : Human colon cancer cell line

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APPENDICES

Appendix A

Ferrous-Oxidation in Xylenol Orange (FOX) Peroxide Value (PV) Assay

The final Fox reagent contained 90% methanol, 10% 250 mM sulfate acid , 4 mM butylated hydroxytoluene, 250 mM ferrous sulfate ammonium sulfate, and 100 mM xylenol orange.

Fox reagent A: dissolve 38 mg xylenol and 440 mg BHT in 450 ml methanol
Fox reagent B: dissolve 49 mg ferrous ammonium sulfate in 50 ml 250 mM sulfate acid

Appendix B

Color Measurement-Hunter Lab

The Hunter Lab Color scale evolved during the 1950s and 1960s. In a uniform color scale, the differences between points plotted in the color space correspond to visual differences between the colors plotted (Hunter Associates Laboratory, 1996). The Hunter Lab color space is organized in a cube form. The *L* axis runs from top to bottom. The maximum for *L* is 100, which would be a perfect reflecting diffuser. The minimum for *L* would be zero, which would be black. The *a* and *b* axes have no specific numerical limits. Positive *a* is red, and negative *a* is green. Positive *b* is yellow, and negative *b* is blue. **Figure 4.1** is a diagram of the Hunter Lab color space.

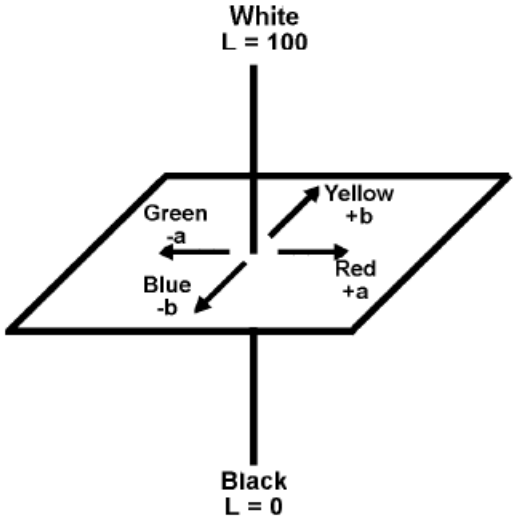

Figure 4.1 A Diagram of the Hunter Lab Color Space

Appendix C

MTS Assay

MTS assay is a colorimetric method for determining the number of viable cells in proliferation or chemosensitivity assays (Cory, Owen, Barltrop, & Cory, 1991). This assay works on the principle that the mitochondrial dehydrogenase of viable cancer cells reduces MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, to a colored formazan product which can be measured spectrophotometrically at 490 nm **(Figure 4.2)** (O'Toole, Sheppard, McGuinness, Gleeson, Yoneda & Bonnar, 2003). The enzymatic activity and the number of formed formazan were proportional to the number of living cells.

*The CellTiter 96 AQ_{ueous} Assay is composed of solutions: (Promega Technical Bulletin, 2005)

MTS- tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4- sulfophenyl)-2H-tetraolium, inner salt

PMS- phenazine methosulfate, an electron coupling reagent

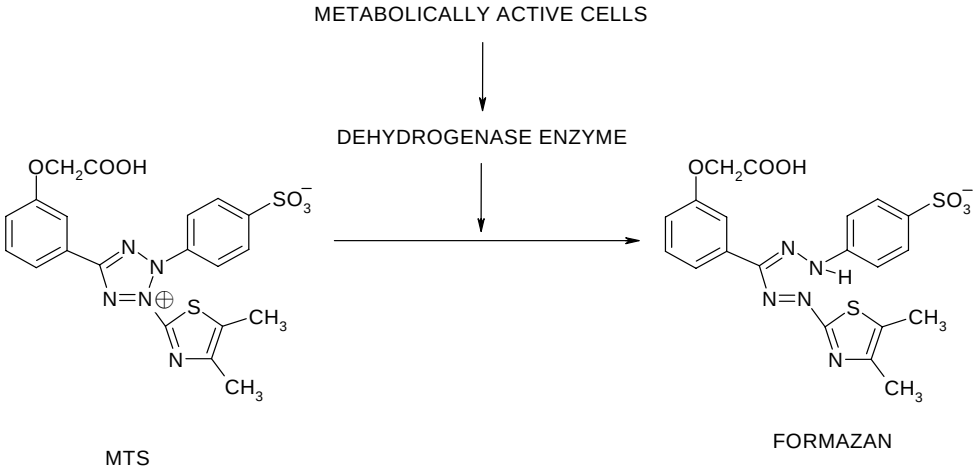

Figure 4.2 Structures of MTS Tetrazolium Salt and Its Formazan Product

Appendix D

A Letter of Authority

From : Dr. Edward F. Gilman, Professor of Environmental Horticulture, University of Florida, USA. (egilman@ufl.edu)

To : Yen-Hui Chen, Master Student, Graduate Research Assistant, Food Science and Human Nutrition, Clemson University, USA. (ychen@clemson.edu)

Original e-mail:

From: Gilman, Edward F [mailto:egilman@ufl.edu]
Sent: Mon 1/29/2007 6:48 AM
To: ychen@CLEMSON.EDU
Subject: RE: Request permission to use some figures; Form: Yen-Hui Chen (Clemson University, SC)

YEs sure.
Ed Gilman

From: ychen@CLEMSON.EDU [mailto:ychen@CLEMSON.EDU]
Sent: Sun 1/28/2007 10:07 PM
To: Gilman,Edward F
Subject: Request permission to use some figures; Form: Yen-Hui Chen (Clemson University, SC)

Dr. Gilman,

My name is Yen-Hui Chen, and I am a master student in Food Science (Clemson university, SC), and I come from Taiwan.

Can I obtain permission from you to add these figures to my thesis? One is from Camellia oleifera Tea-Oil Camellia-Figure 2 Shaded area represents potential planting range (Fact Sheet ST-116, Nov 1993, University of Florida), and the other is the pic of tea tree from www.horticopia.com/hortpix/html/pc1192.

I really appreciate your time and help.

Have a wonderful day!

Sincerely yours,
Yen-Hui Chen

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