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COMPARISON OF BIOACTIVITIES AND COMPOSITION OF CURCUMIN-FREE TURMERIC (*CURCUMA LONGA* L.) OILS FROM DIFFERENT SOURCES

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Food, Nutrition and Culinary Science

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by
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December 2006

Accepted by:
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Dr. Jeff Adelberg

ABSTRACT

Composition, antioxidant capacities and cell inhibition properties of curcumin-free turmeric (*Curcuma longa* L.) oils from different sources were evaluated by chromatographic method, two different *in vitro* antioxidative activity assays (DPPH* free radical scavenging assay and reducing power assay) and two different cancer cell lines (Caco-2 and MCF-7). Turmeric oil A (TOA) contains zingiberene, turmerone, and ar-turmerone, while turmeric oil B (TOB) contains 1-phellandrene and α-terpinolene as the major compounds. The antioxidant tests showed that both turmeric oils possessed strong free radical scavenging activities in the DPPH* free radical scavenging assay and high reducing powers in the reducing power assay compared with standard antioxidants such as BHT and commercial rosemary oil (RO). The free radical scavenging effect of 20 μL/mL TOA is comparable to that of 70 μL/mL TOB, comparable to that of 10 mM BHT and better than that of 100 μL/mL RO ($p<0.0001$). The order of reducing powers is: 100 μL/mL TOA > 100 μL/mL TOB > 10 mM BHT > 100 μL/mL RO ($p<0.0001$). Among the complex constituents in the crude TOA, ar-turmerone, turmerone, curlone and α-terpineol were isolated and found with strong antioxidant activities. The anticancer activity results showed that both turmeric oils possessed high inhibitive capacity against cancer cell lines (ie. Caco-2 and MCF-7) at 20 μL/mL.

DEDICATION

I would like to dedicate this work to my husband, Shenghua Fan and my lovely daughter, Jiejie, with great thanks, love, and pride.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Turmeric (*Curcuma longa* L.) is a tropical herb indigenous to Southern Asia, and probably originated on the slopes of the tropical forests of the west coast of South India. Turmeric is a sterile triploid and has been propagated vegetatively for thousands of years. Turmeric powder is used in food because of its spicy flavor and appealing bright yellow color. It is used to brine pickles and to some extent in mayonnaise, relish, mustard, and curry formulations; in non-alcoholic beverages such as orangeades and lemonades; in breading of frozen fish sticks, etc. In all these cases, it functions predominantly as an alternative of synthetic colors to decorate the products, as well as a flavoring ingredient to enhance the food taste (Govindarajan, 1980).

Turmeric is an erect perennial herb and grown as an annual crop. Its above-ground morphology is mainly represented by an erect pseudo stem bearing leaves and inflorescence. There may be 2-3 pseudostems (tillers) per plant. The height of the pseudostem varies from 90-100 cm depending on varieties. Leaf number ranges from 7-13 (Sasikumar, 2001). The underground rhizome that is commercially processed into the spicy powder, consists of two distinct parts. The egg-shaped primary or mother rhizome is an extension of the stem. Several long cylindrical multi-branched secondary rhizomes grow downward from the primary rhizome (Govindarajan, 1980). **Figure 1.1** shows the leaves and rhizomes of turmeric.

Turmeric, like ginger, belongs to the family *Zingiberaceae* that contains 49 genera and 1400 species. The taxonomic position of turmeric (*Curcuma longa* L.) is as follows:

Kingdom Plantae

Subkingdom Tracheobionta---Vascular plants

Superdivision Spermatophyta---Seed plants

Division Magnoliophyta---Flowering plants

Class Liliopsida--Monocotyledons

Subclass Zingiberidae

Order Zingiberales

Family *Zingiberaceae*---Ginger family

Genus *Curcuma*

Species *Curcuma longa* L.

A wild ancestor of turmeric is called *C. aromatica*, while the domestic species is called *C. longa* L. (Chattopadhyay, 2004). In addition, *C. zedoaria Rosc* and *C. xanthorrhiza Roxb* are also minor crops grown for curcumin color (Sasikumar, 2001).

Four products of turmeric

Ground turmeric

Ground turmeric is made by milling the clean, dry fingers followed by disc-type attrition mills to obtain 60-80 mesh powder. There is not much loss of quality from oxidation of grinding turmeric (Sasikumar, 2001; Govindarajan, 1980).

Curry powder

Curry powder is a blend of a number of spices and herbs, in which turmeric powder is the major component (about 40-50%) (Sasikumar, 2001) that provides desirable color and background aroma (Govindarajan, 1980). In Asia, curry powder is a spicy food ingredient used for seasoning dishes such as vegetables, meat, fish or eggs.

Turmeric oleoresin

Turmeric oleoresin is a mixture of curcumin, volatile oil, non-volatile fatty and resinous material, and other active ingredients, which are extracted from ground turmeric by solvents, used singly, in sequence or in combination (Govindarajan, 1980). For example, acetone is a good solvent for oleoresin extraction (Sasikumar, 2001). Turmeric oleoresin is in orange-red color and consists of an upper oily layer and a lower crystalline layer. The content of curcumin determines the quality of turmeric oleoresin. Turmeric oeloresin is the industrial starting material to produce pigment curcumin (Jayaprakasha, 2006).

Turmeric oil

Turmeric rhizome contains 3-5% volatile oil, which is obtained by steam distillation of turmeric powder for about 8-10h (Sasikumar, 2001). Turmeric oil is in pale yellow color with peppery and aromatic odor. Various sources of turmeric oils have been reported with different chemical composition and content ascribed to the different cultivars, different soil and climate, and age of plants that influenced the composition (Lawrence, 2003; Cooray, 1998; Chatterjee, 2000; Hu, 1998). Because it is a byproduct

of curcumin industry (Saju, 1998) and has less commercial importance, the chemistry of turmeric oil has not received much attention (Jayaprakasha, 2005).

Composition of turmeric oil

Nearly 100 chemicals have been reported in turmeric essential oils (**Table 1.1**) (Raina, 2002; Jayaprakasha, 2001; Garg, 2002; Braga, 2003). Among these are terpenes and oxygen derivative terpenoids that are believed to contribute "the character-impact" turmeric flavor. Other minor aromas include short chain alcohols, ketones, and fatty acids, which are degraded products of fatty acids.

Main compounds in turmeric

Curcumin and curcuminoids

Curcumin (1, 7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the most important compound in turmeric. It was first isolated in 1815 and its chemical structure was determined in 1973 (Roughley, 1973). It is a yellowish crystalline, odorless powder (mp 184-186˚C), poorly soluble in water, petroleum ether, and benzene; soluble in ethyl alcohols, glacial acetic acid, and in propylene glycol; very soluble in acetone and ethyl ether. Absorptive spectra of curcumin and curcuninoids are very similar, with their maximum values at 429 and 424 nm, respectively (Govindarajan, 1980; Sharma, 2005). In addition, curcumin is considered a non-nutritive and non-toxic chemical to mammals even at very high doses (5-10%) by weight of diet (Weber, 2005; Samaha, 1997).

Extraction and separation of curcumin and curcuminoids

Extraction

Many methods have been reported for extraction of curcumin and curcuminoids (Chatterjee, 1999; Chowdhury, 2000; Surh, 2002). The most common method to extract these compounds from turmeric powder involves sequential solvent extraction by, firstly, using hexane to remove the non-polar volatiles and fatty compounds, and then followed by alcohol or benzene extraction. After comparing the extraction efficiency of several solvents, Gupta *et al*. confirmed that acetone was suitable for curcumin extraction since this solvent can yield the highest recoveries of curcuminoids (Gupta, 1999). At the same time, Chassagnez-Méndez *et al.* studied the feasibility of using supercritical fluid extraction (SFE) method and confirmed that ethanol could increase the recovery of curcumionoids in this method (Chassagnez-Méndez, 2000). Schieffer reported that curcuminoids could be completely extracted by pressurized liquid extraction, which was better than multiple ultrasonically-assisted extraction, although the latter was simpler (Schieffer, 2002). Braga *et al.* also compared various techniques, including hydro-distillation, low-pressure solvent extraction, Soxhlet extraction, and supercritical fluid extraction using carbon dioxide and co-solvents (e.g., ethanol, isopropyl alcohol, and their mixture in equal proportion) on the extraction of curcumin. It was found that the largest yield (27%) was obtained by the Soxhlet extraction using ethanol, while the lowest yield resulted from the hydrodistillation process (2.1%) (Braga, 2003).

Quantification by spectrophotometer

Spectrophotometric methods are most often reported for the quantification of the curcuminoids. Usually, the detective wavelength was set at 420-430 nm, at which

curcuminoids have their maximal spectrophotometric absorption (Ruby, 1995; Deshpande, 1997; Braga, 2003; Leal, 2003; Manzan, 2003). A linear relationship between the absorbance and curcumin concentration was obtained in the range of 0-15 μg/mL with a detective limit as low as 0.076 μg/mL (Tang, 2002). Although the spectrophotometric method can quantify curcumin precisely within range mentioned above, it is not able to quantify each curcuminoid individually.

Separation of curcuminoids by TLC

Regarding the quantitative limitation of spectrophotometric method, high-performance thin-layer chromatography was suggested as an alternative method for the determination of individual curcuminoid in turmeric (Gupta, 1999). This method used chloroform-methanol (95:5) as the developing solvent to separate curcuminoids that were visualized at 430 nm. Quantitative linearity was found in the concentration range between 1 and 20 μg. At nearly the same time, Rasmussen *et al.* reported another simple but efficient method using the dihydrogen phosphate impregnated silica gel TLC plate to separate curcuminoids (Rasmussen, 2000). However, both TLC methods were restricted by their lower resolution than HPLC.

Separation and quantification by HPLC

Compared with the spectrophotometric and TLC methods, high performance liquid chromatography (HPLC) coupled with mass spectrometer can provide more powerful analytical capabilities in terms of quantitation and qualification. For example, curcuminoids can be easily separated by reverse phase HPLC column under the following condition: using the reverse phase Supelcosil LC-18 column, and mobile phase A: 1%

citric acid (pH adjusted to 3.0 with dilute NaOH) and B: acetonitrile (Hiserodt, 1996). A gradient mobile phase was controlled at 1 mL/min from 50% acetonitrile with an initial holding time of 10 min to 80% acetonitrile in 30 min. Although this method yielded good resolution and desirable peak shape, some components in the mobile phase (citric acid, NaOH) might clog the mass spectrometer interface leading to high back pressures, and thus contaminate the MS ion source (Hiserodt, 1996; Schieffer, 2002). HPLC-PDA (photo-diode array) was also used to determine curcuminoids and co-existing sesquiterpenes (He, 1998) by mobile phase A: water (0.25% HOAc) and B: acetonitrile at a flow rate of 0.2 mL/min. Jayaprakasha *et al*. used methanol as an additional mobile phase, which included solvents A: methanol; B: 2% acetic acid; and C: acetonitrile. Linearity was found in the concentration range between 0.0625 and 2.0 μg, with high reproducibility and accuracy (Jayaprakasha, 2002). In these methods, curcuminoids and sesquiterpenoids were detected at wavelengths at 426 nm and 240 nm, respectively (Nishiyama, 2005). In addition to the gradient method, acetonitrile at isocratic flow rate of 0.75 mL/min on LiChrosorb RP-8 column (Chowdhury, 2000) and ethanol/water (96:4) on Spheri-5 amino column were tested (Manzan, 2003). Both have demonstrated desirable resolution. Recently, Pak *et al*. reported the HPLC method could provide a highly sensitive separation that could reliably determine the curcumin in plasma at a concentration as low as 2.5 ng/mL (Pak, 2003).

Other methods

Besides the methods mentioned above, capillary electrophoresis with amperometric detection (CE-AD) pretreated by solid-phase extraction (SPE) was also reported to quantify curcumin. CE-AD with SPE exhibited a low detection limit at 3×10^{-8}

mol within a linear range of $7x10^{-4}$ to $3x10^{-6}$ mol/L for curcumin extracted in light petroleum (Sun, 2002). Flow-injection analysis (FIA) with on-line UV and fluorescent detector can provide detective limits at 30.0 ng/mL and 2.0 ng/mL, respectively (Inoue, 2001). The same research group also reported that LC/electrospray-MS could successfully determine the trace amounts of curcuminoids in food samples with a detective limit at 1.0 ng/mL (Inoue, 2003). Although these methods are much more sensitive compared with the traditional TLC method, they are not efficient in separating quantities of curcumin or curcuminoids larger than 10-20 mg. Therefore, Patel suggested using pH-zone-refining high-speed countercurrent chromatography to separate curcumin in large quantities (2g curcumin or 20g of turmeric powder) (Patel, 2000).

Antioxidant activity of curcumin

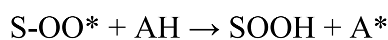
The antioxidant activity of curcumin was found with equivalent activity to butylated hydroxylanisole (BHA) and butylated hydroxyltoluene (BHT) (Sharma, 1976). For example, 40 ppm curcumin could completely prevent aldehyde formation in fermented cucumber tissue that was exposed to oxygen (Zhou, 2000). Like curcumin, demethoxycurcumin and bisdemethoxycurcumin also exhibited antioxidant activity (Chatterjee, 1999). In *in vitro* model systems, such as the phosphomolybdenum and linoleic acid peroxidation, antioxidant capacities were in the order of curcumin > demethoxycurcumin > bisdemethoxycurcumin (Jayaprakasha, 2006). Curcumin can act as a scavenger of oxygen free radicals (Ruby, 1995; Rukkumani, 2004; Das, 2002). It can protect hemoglobin from oxidation (Unnikrishnan, 1995). In an *in vitro* test, curcumin could significantly inhibit the generation of reactive oxygen species (ROS) such as superoxide anions and H_2O_2, and reactive nitric species (RNS), which play an important

role in inflammation (Joe, 1994). Also, curcumin exerted powerful inhibitory effect against H_2O_2-induced damage in human keratinocytes and fibroblasts (Phan, 2001). Oral administration of hydroalcoholic extract of *C. longa* decreased the susceptibility of LDL to lipid peroxidation in a dose-dependent manner (Ramírez-Tortosa, 1999). Curcumin can reduce the inflammatory response of ethanol by decreasing prostaglandin synthesis (Rajakrishnan, 2001). Thus, curcumin helps maintain the membrane structure integrity and function. It also protects against lead- and cadmium-induced lipid peroxidation in rat brain homogenates and against lead-induced tissue damage in rat brain through metal binding mechanism (Daniel, 2004). Administration of turmeric or curcumin to diabetic rats reduced the blood sugar, hemoglobin (Hb) and glycosylated hemoglobin levels significantly. Curcumin supplementation also reduced the oxidative stress encountered by the diabetic rats (Arun, 2002). Dietary supplementation of curcumin (2%, w/v) to male ddY mice for 30 days significantly increased the activities of glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and catalase as compared with the same type mice fed normal diet. This may be one of the possible mechanisms of cancer chemopreventive effects associated with curcumin in several animal tumor bioassay systems (Iqbal, 2003). Since ROS have been implicated in the development of various pathological conditions (Lee, 2004), curcumin has the potential to control these diseases through potent antioxidant activity.

The antioxidant capacity of curcumin is attributed to its unique conjugated structure, which exists in an equilibrium between the diketo and keto-enol forms that are strongly favored by intramolecular H-bonding (**Figure 1.2**) (Weber, 2005). Since demethoxycurcumin and bisdemethoxycurcumin have similar structures like curcumin

(**Figure 2.1**), they have similar bioactivities. Their respective amounts needed for 50% inhibition of lipid peroxidation were 20, 14, and 11 μg/mL, for 50% inhibition of superoxides were 6.25, 4.25, and 1.9 μg/mL, and those for hydroxyl radical were 2.3, 1.8 and 1.8 μg/mL (Ruby, 1995).

Curcumin shows typical radical-trapping ability as a chain-breaking antioxidant. Generally, the nonenzymatic antioxidant process of the phenolic material is thought to be mediated through the following two stages:



S-OO* + AH → SOOH + A*

A* + X* → Nonradical materials

Where S is the substance oxidized, AH is the phenolic antioxidant, A*is the antioxidant radical and X* is another radical species or the same species as A*. A* and X* dimerize to form the non-radical product (Chattopadhyay, 2004). Masuda *et al*. further studied the antioxidant mechanism of curcumin using linoleate as an oxidizable polyunsaturated lipid, and proposed that the mechanism involved oxidative coupling reaction at the 3' position of the curcumin with the lipid and a subsequent intramolecular Diels-Alder reaction (Masuda, 2001). Curcumin was also confirmed to have metal binding ability. FT-IR spectrometric analysis showed that both the hydroxyl groups and the β-diketone moiety of curcumin were involved in a metal-ligand complexation, either directly bonding to the metal, or in intermolecular hydrogen bonding (Daniel, 2004).

Chemopreventive and anticancer activity of curcumin

Recent studies on several animal tumor bioassays have shown that curcumin has a dose-dependent chemopreventive effect against colon, duodenal, stomach, esophageal and oral carcinogenesis (Narayan, 2004; Ruby, 1995; Hastak, 1997). It has been shown

that administration of turmeric powder in the diet reduced tumors induced by carcinogenic chemicals such as benzo[α]pyrene (BP) and 7, 12-dimethyl benz[α]anthracene (DMBA) (Li, 2002). Curcumin can inhibit the growth of estrogen positive human breast MCF-7 cells induced individually or by mixture of estrogenic pesticides, such as endosulfane, DDT and chlordane or 17-beta estradiol (Verma, 1997). Alcoholic extracts of turmeric (TE) and turmeric oleoresin (TOR) decreased the number of micronucleated cells both in oral mucosal cells and in circulation lymphocytes (Hastak, 1997).

Curcumin acts as a potent anticarcinogenic compound. Among various mechanisms, induction of apoptosis plays an important role in its anticarcinogenic effect. Apoptosis is an orchestrated series of events through which the cell precipitates its own death. The stages of apoptosis include cell shrinkage, chromatin condensation, nuclear segmentation and internucleosomal fragmentation of DNA, resulting in the generation of apoptotic bodies (Aratanechemuge, 2002). The antiproliferative effect of curcumin is mediated partly through inhibition of protein tyrosine kinase, *c-myc* mRNA expression and *bcl-2* mRNA expression (Chen, 1998). Nuclear factor (NF) - κB is known to control cellular proliferation and apoptosis. Curcumin can also inhibit the cell proliferation and induce apoptosis in human malignant astrocytoma cell lines and head and neck squamous cell carcinoma (HNSCC) by inhibition of NF-κB activity (Nagai, 2005; LoTempio, 2005). For HNSCC, curcumin can induce cell apoptosis both *in vitro* and *in vivo*. Curcumin caused lung cancer cell death by induction of apoptosis, which was independent of p53 status of the cell lines (Pillai, 2004). Other research showed that curcumin induced apoptosis in melanoma cell lines in a manner that was also

independent of p53 and the *bcl-2* family (Bush, 2001). Moreover, recent research found that curcumin had potent antiproliferative and proapoptotic effects in melanoma cells by suppression of NF-κB and IKK activities but were independent of the B-Raf/MEK (mitogen-activated)/ERK (extracellular signal-regulated protein kinase) and Akt pathway (Siwak, 2005).

Other bioactivities of curcumin

Curcumin has anti-inflammatory effects (Prasad, 2004). It can prevent rheumatoid arthritis in animal model (Funk, 2006). Oral administration of 5 and 10 mg/kg curcumin significantly reduced the duration of immobility in depressive-like behaviors (tail suspension and forced swimming) in mice (Xu, 2005). Pretreatment with curcumin significantly enhanced the rate of wound contraction, decreased mean wound healing time, increased synthesis of collagen, hexosamine, DNA and nitric oxide, and improved fibroblast and vascular densities (Jagetia, 2004).

Ar-turmerone and turmerone

Turmeric oil contains nearly 100 compounds (**Table 1.1**). Most of them are sesquiterpenes. Among them, ar-turmerone and turmerone account for nearly 50% of the oil (Govindarajan, 1980; Negi, 1999). Ar-turmerone [(*S*)-2-methyl-6-(4-methyl-phenyl)-2-hepten-4-one] is a colorless oily chemical with specific UV absorption at 221, 238, and 273 nm. In contrast, turmerone [2-methyl-6-(4-methyl-1,4-cyclohexadien-1-yl)-2-hepten-4-one] is a pale yellow oily chemical with a maximal UV absorption at 234-235 nm. Turmerone was thermally unstable and at ambient temperature in the presence of air,

yielding its dimmer, the more stable ar-turmerone (Su, 1982). Their structures and physical characters are shown in **Figure 1.3**.

Extraction and separation

Before 2000, little research was done on ar-turmerone and turmerone. The former compound was identified by NMR after its extraction from turmeric powder by petroleum ether and elution by chloroform in silica gel column (Su, 1982). However, Manzan *et al*. found that petroleum ether extraction process produced less ar-turmerone and turmerone than the steam distillation process (Manzan, 2003). Therefore, most of the turmeric oil is now prepared by steam distillation. Recently, SFE with carbon dioxide as solvent at 320K and 26MPa was also found to give a desirable production of turmeric oil (Chang, 2006). The same author found the reverse-phase column Purospher RP-18 (5 μm, 125 mm x 4 mm) could successfully separate the ar-turmerone and α + β-turmerone with following mobile phase with solvent A: 0.0025% TFA solution and solvent B: acetonitrile at a flow rate of 1 mL/min in the following gradient program: the initial solution of 80:20 (A:B) was held for 5 min, increased to 48% B in 5 min, to 60% in 10 min, held for 10 min, and to 100% in 10 min. The column temperature was maintained at 40˚C and UV detection was set at 254 nm.

Bioactivities of ar-turmerone and turmerone

Early in 1992, Ferreira reported that ar-turmerone had antivenom effect (Ferreira, 1992). Later, ar-turmerone was reported with other biological activities such as anti-mosquito effect (Roth, 1998), antibacterial activity (Negi, 1999) and antifungal activity (Jayaprakasha, 2001). Turmeric oil rich in ar-turmerone, turmerone, and some other

oxygenated compounds showed antioxidant and antimutagenicity (Jayaprakasha, 2002). Further research has focused on turmeric oil, ar-turmerone and turmerone. It was reported that hexane extract from turmeric powder had antiproliferative activity, for which ar-turmerone was a contributor (Aratanechemuge, 2002). Recent research also revealed that ar-turmerone could induce the apoptotic activity in the K562, L1210, U937 and RBL-2H3 cancer cell lines (Ji, 2004). In 2006, a new function was reported that ar-turmerone had antiplatelet property. Its 50% inhibitory concentration (IC_{50}) values for effectively inhibiting platelet aggregation induced by collagen and arachidonic acid were 14.4 μM and 43.6 μM, respectively (Lee, 2006).

Other bioactive compounds

Another important compound isolated from the aqueous extract of turmeric is a protein, called turmeric anti-oxidant protein (TAP). It is a heat stable protein and has antioxidant activity. Its maximal absorbance is 280 nm. The antioxidant activity may be mediated through the protection of the –SH group of the enzyme (Selvam, 1995).

Importance of the project

According to the Food and Agriculture Organization of the United Nation, over 2400 tons of turmeric is imported annually in the USA for consumer use in recent years. Since turmeric oil is the major by-product of curcumin production, it is important to identify more bioactive chemicals in the curcumin-free turmeric oil and explore their bioactivities. The current usage of turmeric oil as fuel (Saju, 1998) and for aromatheraphy (Sasikumar, 2005) may not fully utilize this undervalued resource. Thus, the specific objectives of this study were:

(1) To profile the composition of turmeric oil;

(2) To assay the bioactivities, such as antioxidant, anti-cancer activities of turmeric oil;

(3) To separate and identify the individual bioactive compounds.

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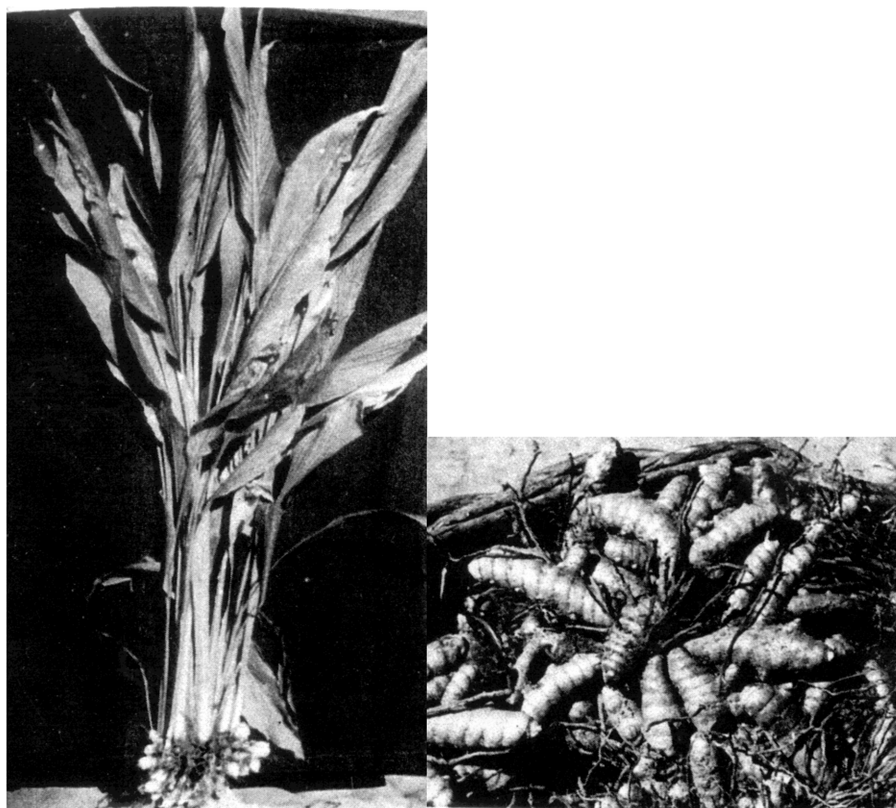


Figure 1.1 The leaves and rhizomes of turmeric.

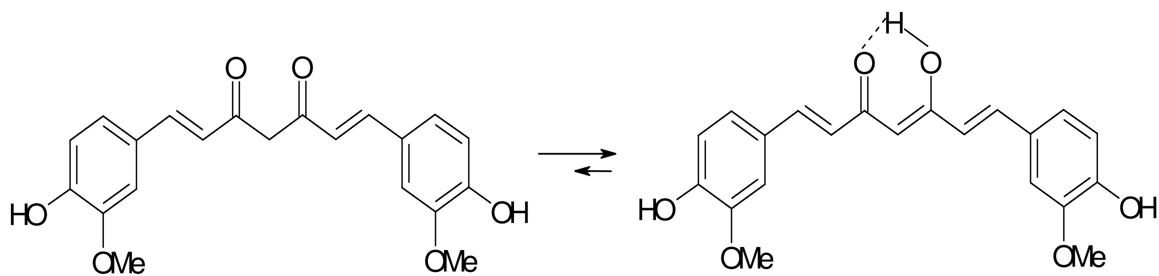
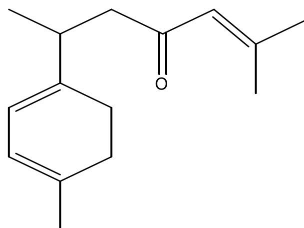
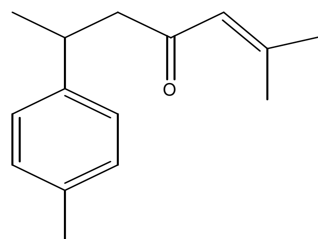


Figure 1.2 Curcumin in solution.

Curcumin exists in solution as an equilibrium mixture of the symmetrical diketo and the keto-enol tautomers stabilized by intramolecular H-bonding.





	Turmerone	ar-Turmerone
Molecular Formula:	$C_{15}H_{22}O$	$C_{15}H_{20}O$
Molecular Weight:	218	216
Boiling Point:	125-126˚C / 10 mm	159-160˚C / 10 mm
Absorption:	λ_{max}, 234-235 nm	λ_{max}EtOH, 239 nm
Melting Point:	Semicarbazone, 110-120˚C	Semicarbazone, 109˚C

Figure 1.3 Structures and physical characters of turmerone and ar-turmerone.

Table 1.1 Turmeric (*Curcuma Longa* L.) oil components.

Chemicals	KI[a]	Sources[b]
(E)-*α*-atlantone	1743	1
(E)-Nerolidol	1550	1
(E)-β-Farnesene	1448	1
(E)-β-Ocimene	1042	1
(E)-γ-Atlantone		4
(Z)-α-Atlantone		4
(Z)-β-Ocimene	1030	1
(Z)-γ-Atlantone		4
1,8-Cineole	1023	1, 3, 4
10-*epi*-γ-Eudesmol	1617	1
1-Bisabolone	1714	1
1-*epi*-Cubenol		4
1-Hexen-3-ol	772	1
2-Decanol	1190	1
2-Nonanol	1092	1
3-Buten-2-ol	810	1
6S, 7R-Bisabolone		4
α-Bisabolol	1673	1
α-Cadinene	1436	1
α-*cis*-Bergamotene		2
α-Fenchol	1099	1
α-Guaiene	1451	1
α-Humelene	1445	1, 3
α-Longefoline	1399	1
α-Muurolene	1492	1
α-Patchouline	1456	1
α-Phellandrene	999	1
α-Pinene	935	1, 4
ar-Curcumene	1471	1, 3,4
ar-Turmerol	1559	1, 3, 4
ar-Turmerone	1640	1, 3, 4
α-Selinene	1496	1
α-Terpinene	1010	1, 4
α-Terpineol	1176	1
α-Thujene	925	1, 4
α-Turmerone	1650	1, 4
Borneol	1157	1, 3, 4
Camphene	946	1
Camphene hydrate	1142	1
Camphor	1130	1
Capric acid	1355	1
Carvacrol	1288	1

Table 1.1 Turmeric (*Curcuma Longa* L.) oil components (continued).

Chemicals	KI[a]	Sources[b]
Carvone	1217	1
Caryophyllene oxide	1566	1
Cinnamyl cinnamate	2053	1
cis-3-Hexenol	849	1
cis-Carveol	1206	1
cis-Carvotanacetol	1199	1
cis-Carvyl acetate	1321	1
cis-Linalool oxide (furanoid)	1062	1
cis-Sabinol	1178	1, 3
cis-Sesquisabinene hydrate	1539	1
cis-β-Elemenone	1579	1
Curdione	1689	1
Curlone		2
Curzerene	1480	1
Elimicin	1524	1
epi-Curzerenone	1584	1
Furanodienone	1752	1
Geramacrene-B	1543	1
Geranial	1252	1
Geraniol	1242	1
Geranyl acetate	1359	1
Geranyl butyrate	1531	1
Geranyl formate	1278	1
Geranyl hexanoate	1722	1
Germacrene-D	1461	1
Germacrone	1669	1
Heptadeconic acid		2
Heptan-2-ol	887	1
Heptan-2-one	875	1
Hexadecanoic acid		2
Humilene epoxide II	1612	1
iso-Borneol	1148	1
iso-Bornyl acetate	1268	1
Linalool	1082	1, 3
Linalyl acetate	1247	1
Linalyl propionate	1314	1
Methyl eugenol	1366	1
Myrcene	984	1
Myrtenal	1173	1, 3
Myrtenol	1184	1
n-Heptyl salicylate	1789	1
n-Hexan-2-ol	787	1

Table 1.1 Turmeric (*Curcuma Longa* L.) oil components (continued).

Chemicals	KI[a]	Sources[b]
p-Cymene	1014	1, 2, 3
p-Cymene-8-ol		3
Perillaketone	1230	1
p-Methyl acetophenone	1124	1
Sabinene	966	1
Sabinyl acetate	1289	1
Safrole	1262	1
T-cadinol	1624	1
Terpinen-4-ol	1166	1, 3
Terpinolene	1084	1
Tetradecane	1393	1
Thymol	1281	1
Thymol acetate	1348	1
trans-Linalool oxide (furanoid)	1077	1
trans-*p*-Menth-2-en-1-ol	1109	1
trans-Sesquisabinene hydrate	1602	1
Undecanol	1299	1
Undecanone	1273	1
Virdifloral	1591	1
γ-Elemene	1423	1
γ-Terpinene	1053	1
Zingiberene	1487	1, 3, 4
β-(Z)-Farnesene		2
β-Bisabolene	1501	1, 3, 4
β-Bisabolol	1659	1
β-Caryophyllene	1418	1, 3, 4
β-Curcumene	1510	1
β-Elemene	1385	1
β-Eudesmol	1629	1
β-Patchouline	1378	1
β-Pinene	974	1, 3
β-Sesquiphellandrene	1516	1, 3, 4
β-Turmerone	1681	1, 3
δ-3-Carene	1005	1
δ-Elemene	1331	1

[a] from source 1;

[b] 1 (Raina, 2002), 2 (Jayaprakasha, 2001), 3 (Garg, 2002), 4 (Braga, 2003).

CHAPTER 2

COMPOSITION AND BIOACTIVITIES OF CURCUMIN-FREE TURMERIC (*Curcuma longa* L.) OILS FROM DIFFERENT SOURCES

Abstract

Composition, antioxidant capacities and inhibitive anticancer activities of curcumin-free turmeric (*Curcuma longa* L.) oils from different sources were evaluated by GC-MS, two different *in vitro* antioxidative assays (i.e., DPPH* free radical scavenging assay and reducing power assay), and MTS bioassays against two cancer cell lines (i.e., Caco-2 and MCF-7), respectively. The commercial turmeric oil A (TOA) was mainly composed of zingiberene, ar-curcumene, turmerone, ar-turmerone and curlone; while turmeric oil B (TOB) contained 1-phellandrene, cymene, 1,8-cineole, and α-terpinolene as the major components. The antioxidative tests showed that both the two curcumin-free turmeric oils possessed strong free radical scavenging activities and reducing powers compared with the standard antioxidant products such as BHT and rosemary oil (RO). The free radical scavenging effect increased with the increasing concentrations of TOA or TOB and then leveled off as the concentration further increased. The free radical scavenging effect can be saturated by 20 μL/mL TOA or 70 μL/mL TOB (90%) when scavenging 0.25 mM DPPH* solution, which was comparable to 10 mM BHT (86%), and was better than 100 μL/mL rosemary oil (68%) at the same reaction condition. The reducing power is also dose-dependent for both TOA and TOB. There is a significant difference between TOA, TOB, BHT and RO in reducing power ($p<0.0001$) in the order of 100 μL/mL TOA > 100 μL/mL TOB > 10 mM BHT > 100 μL/mL RO. The anticancer

experiments showed that both TOA and TOB had antiproliferative activities against human colon cancer cell (Caco-2) and human breast cancer cell (MCF-7). The IC_{50} values of TOA and TOB were 1.66 μL/mL and 21.5 μL/mL for Caco-2 cell line, 0.122 μL/mL and 1.219 μL/mL for MCF-7 cell line, respectively.

Introduction

Research on bioactive principles of essential oils extracted from various herbs and spices has become increasingly popular. Essential oils have been discovered to have many functional properties such as antimicrobial and anti-inflammatory activities (Vardar-Ünlü, 2003; Hammer, 1999; Griffin, 1999; Güllüce, 2003). Besides these activities, many essential oils have been qualified as natural antioxidants (Kim, 2004; Ruberto, 2000; Lin, 2003; Domínguez, 2005; Zhang, 2006) and proposed as potential substitutes of synthetic antioxidants in food preservation. Currently, many research groups are focusing their investigation on the pharmacological actions of essential oils from aromatic and medicinal plants. Many studies have shown that natural antioxidants in various aromatic and medicinal plants are closely related to the reduction of chronic diseases such as cancer, cardiovascular disease, diabetes, arthritis (Fridovich, 1999; Zhu, 2002; Covacci, 2001). Among them, turmeric is one of the most important studied subjects because of its demonstrated broad spectrum of activities.

The main compounds in turmeric include curcumin (1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadien-3, 7-dione) and two curcuminoids, demethoxycurcumin and bisdemethoxycurcumin (**Figure 2.1**). They contribute the yellow color to turmeric and have received increasing attention because of their many bioactivities. Current research shows that curcumin and curcuminoids have antioxidant, antivirus,

antimutagenicity, anti-arthritic and anti-thrombotic activities (Braga, 2003; Apisariyakul, 1995; Taher, 2003; Funk, 2006). Turmeric oil is usually obtained from turmeric powder by steam distillation or from curcumin removed turmeric oleoresin (CRTO) that was extracted with hexane and then concentrated. Turmeric oil accounts for 3-5% of the total weight of raw turmeric rhizome. Turmeric oil is pale yellow with peppery and aromatic odor. The reported major compounds in turmeric oil include some terpenic aromas such as α-phellandrene, 1,8-cineol, zingiberene, ar-turmerone, turmerone, curlone, β-sesqui-phellandrene, dehydro-zingerone, etc (Sasikumar, 2001). Because turmeric oil is the by-product of curcumin industry and has long been considered with very little commercial value (Saju, 1998), it was neglected in an extent of degree in its potent biological activities, including its antioxidant activity. So it is our current interest to explore the antioxidant activity and other bioactivities of curcumin-free turmeric oil.

Turmeric and turmeric oil from different sources may have different chemical profiles with different bioactivities. However, the production of these bioactive compounds is controlled by plant genotypes, postharvest processing (e.g., drying, extraction, etc) and environmental conditions, such as temperature, humidity, light, soil, etc. In this study, we compared two turmeric oils from two commercial sources for their chemical profiles and antioxidant activities, as well as anti-cancer activities.

Materials and methods

Materials and chemicals

Two commercially available turmeric oils were designated as TOA (Aromaland Co. USA) and TOB (Bianca Rosa Co. Canada). 2, 2-diphenyl-1-picrylhydrazyl ($DPPH^*$), butylated hydroxytoluene (BHT), β-caryophyllene, Minimum Essential Medium (Eagle),

MEM non-essential amino acids, Rosewell Park Memorial Institute 1640 (RPMI 1640) medium, curcumin standard, rosemary oil, sodium bicarbonate, trypsin EDTA solution, sodium pyruvate, α-terpinolene and sterile cell culture penicillin were purchased from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid and all solvents were obtained from Fisher Scientific (Suwanee, GA). Potassium ferricyanide and ferric chloride were obtained from J. T. Baker Chemical Co. (Phillipsburg. NJ). New born calf serum and fetal bovine serum were purchased from Hyclone (Loga, Utah). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was provided by Promega (Madison, WI). Human breast carcinoma cell lines (MCF-7) and human colon carcinoma (Caco-2) were obtained from the American Type Culture Collection (Rockville, MD).

HPLC analysis

A Waters C_{18} reverse phase column (Xterra^{TM}, 4.6 x 150 mm, 5 μm) and a photodiode-array (PDA) detector were equipped in a Shimadzu LC-10AT HPLC system (Kyoto, Japan). UV spectra were recorded in the region of 200-800 nm. The mobile phase consisted of solvents (A) water (0.25% acetic acid) and (B) acetonitrile was programmed in the following gradient condition: 0-17 min, 40-60% B; 17-32 min, 60-100% B; 32-38 min, 100% B; 38-40 min, 100-40% B; 41 min, stop. Total flow rate of the mobile phase was controlled at 0.6 mL/min. Injection volume was 20 μL. Curcumin standard (dissolved in ethanol, 0.15 mg/mL), TOA [dissolved in DCM/methanol (v/v 1:2), 100 μL/mL], TOB (dissolved in ethanol, 100 μL/mL) were analyzed by the same method described above.

GC-MS identification

A Shimadzu GC-MS system consisting of a GC-17A with a QP5050 Mass Spectrometer (Kyoto, Japan) was equipped with a DB-5 capillary column (60 × 0.25 mm, thickness 0.25 µm; J&W Scientific, Folsom, CA, USA) for all chemical quantitative and qualitative analyses in this research. The oven temperature was programmed from 60 to 280˚C at a ramp rate of 8˚C/min and held at 280˚C for 30 min. The injector and ion source temperatures were set at 180 and 290˚C, respectively. The detector voltage was 70eV, and the scanning mass range was *m/z* 43-350. Helium was used as the carrier gas at a column flow rate of 1.2 mL/min. Samples in volume of 2 µL was injected with a split ratio of 1:1. Identification of compounds was based on comparison of their mass spectra and retention indices (RIs) with those of the authentic standards. RIs were calculated using series of *n*-alkanes (C_8-C_{26}). If standard compounds were not available, each unknown compound was tentatively identified by comparing the mass spectrum with that of the Wiley and NIST mass spectral databases and the previously published RIs obtained under the same conditions (Negi, 1999; Chatterjee, 2000; Adams, 2001).

Antioxidative capacity

The antioxidant capacities of turmeric oils were determined by two methods: the DPPH* free radical scavenging assay and the reducing power assay. BHT and rosemary oil (RO) were used as standards for above *in vitro* bioassays in antioxidant comparison.

DPPH* free radical scavenging assay

DPPH* free radical scavenging assay followed the procedure described by Yamaguchi *et al.* (1998) with minor modification. In this antioxidant method, DPPH* is a

stable free radical and shows a characteristic absorption at 517 nm due to its odd electron. As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, or a free radical scavenging antioxidant, the absorption strength decreased resulting in decolorization that is stoichiometric with the number of electrons captured. In this test, 0.4 mL of different concentrations of samples was mixed with 0.4 mL of 0.25 mM DPPH* solution. The mixtures were shaken vigorously and left in the dark at room temperature for 30 min. A control consisted of 0.4 mL solvents instead of sample. Because both TOA and TOB have colors, so we also add 0.4 mL turmeric oil (at each concentration) to 0.4 mL solvent as blank, respectively. The absorbance of the mixtures was measured spectrophotometrically at 517 nm. The scavenging effect of DPPH* free radical was calculated by using the following formula:

$$\text{Scavenging effect (\%)} = \left(1 - \frac{\text{absorbance of sample at 517 nm} - \text{blank}}{\text{absorbance of control at 517 nm}}\right) \times 100 \qquad (1)$$

Reducing power assay

The reducing power of two turmeric oils was determined by the method described by Yen *et al*. (1995) and Chung *et al*. (2002) with minor modification. TOA, TOB and RO were dissolved in acetone to prepare solutions in concentrations of 1, 5, 10, 20, 40, 70, 100 μL/mL. BHT was prepared in concentrations of 0.1, 0.5, 1, 2, 4, 7, 10 mM. Solutions of 0.5 mL samples were mixed with 1 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50˚C for 20 min. Then 1 mL of trichloroaceteic acid (10%) was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (1 mL) was mixed with distilled water (1 mL) and

$FeCL_3$ (0.2 mL, 0.1%) to read the absorbance at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power.

Triplicates were performed for each concentration of the tested samples and standards in these two methods. The experiments were repeated three times on different days.

MTS assay

Both MCF-7 and Caco-2 cells were plated at 10,000 cells/cm^2 in 75 cm^2 cell culture flasks. The medium for MCF-7 was Minimum Essential Medium with 10% newborn calf serum, 1% sodium pyruvate, 1% non-essential amino acid and 1% penicillin. The medium for Caco-2 cells was formulated by RPMI with 10% fetal bovine serum and 1% sodium pyruvate, and 1% penicillin. After 48h, cancer cells were fed with fresh medium. The cells were determined with trypan blue. Exponentially growing cells were harvested, counted, diluted and seeded into the 96-well tissue culture plate at 10^4 cells/well (100 μL). Then the plate was incubated in a 5% CO_2 incubator at 37˚C for 24h. The diluted TOA and TOB were added to obtain the final concentrations 0.02, 0.2, 2, 20 μL/mL, respectively. The solvent ethyl acetate was added as the control. The plates were incubated in 5% CO_2 incubator at 37˚C for 48h. Then, 20 μL of MTS reagent was added into each well. These plates were incubated again in the CO_2 incubator at 37˚C for 2h. 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 after dissolution. 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, 2002).

Cell inhibition can be reflected by the spectrophotometrical absorbance recorded at 490 nm and calculated by the following formula:

$$\text{Cell inhibition (\%)} = (1 - \frac{\text{absorbance of sample at 490 nm}}{\text{absorbance of control at 490 nm}}) \times 100\% \qquad (2)$$

The 50% inhibition concentration (IC_{50}) was used to compare the inhibitive activity of TOA and TOB. It was defined as the chemical concentration causing 50% inhibition of cell growth. Triplicates were performed for each concentration of the tested samples and the experiments were repeated three times on different days.

Statistical analysis

The data of the antioxidant activities and cell inhibitions of TOA, TOB and standards were subjected to the analysis of variance (ANOVA). Treatment means were separated by the least significant difference (LSD at $p<0.05$). Analyses were performed using the statistical software SAS 9.1 operated on the Windows system (SAS Institute Inc., Cary, NC).

Results

Confirming absence of curcumin or curcuminoids in TOA and TOB

Curcumin standard separated by the HPLC showed three separate peaks (**Figure 2.2**), i.e., (1): bisdemethoxycurcumin; (2): demethoxycurcumin; and (3): curcumin. Their maximum absorbent wavelength was around 420 nm (Sasikumar, 2001). Since TOA and TOB did not show absorbance after 300 nm, and there were no matching peaks with the curcumin standards, it was concluded that there were no curcumin or curcuminoids in the TOA or TOB, which excluded the possible interference from those chemicals on the antioxidant and anticancer tests.

Identification of volatile compounds in TOA and TOB

GC-MS analyses of the chemical profiles of the turmeric oils indicated that the two oils had quite different chemical compositions (**Figure 2.3 A** and **B**). There were 9 important volatile compounds with larger RIs (1400~1800) identified in TOA which contained trans-caryophyllene, farnesene, ar-curcumene, zingiberene, β-bisabolene, β-sesquiphellandrene, ar-turmerone, turmerone, and curlone (**Figure 2.3 A, Table 2.1**). In contrast, there were only 4 major components with small RIs (less than 1100) found in TOB which include 1-phellandrene, cymene, 1,8-cineole, and α-terpinolene (**Figure 2.3 B**, **Table 2.1**). 1-phellandrene was found to be the major compound (58%) in TOB (**Table 2.1**).

Comparing antioxidant activity of TOA and TOB

Since the major compounds in two turmeric oils were remarkably different, it was of our interest to investigate their antioxidant activities regarding their free radical scavenging activity and the reducing power. The DPPH* free radical scavenging activity of both TOA and TOB increased with the increase of concentrations and then leveled off after 20 µL/mL TOA or 70 µL/mL TOB. 20 µL/mL TOA or 70 µL/mL TOB could scavenge the free radicals at 90%, which was comparable to the scavenging effect of 10 mM BHT (86%) and higher than that of 100 µL/mL rosemary oil (68%) at the same reaction condition ($p<0.0001$) (**Figure 2.4 A**).

In the reducing power assay, the presence of reductants (antioxidants) would result in reducing Fe^{3+}/ferricyanide complex to the ferrous form. The Fe^{2+} could be monitored at 700 nm by measuring the formation of Perl's Prussian blue. **Figure 2.4 B** shows the reducing power of the turmeric oils, BHT and rosemary oil. The reducing

powers of TOA and TOB are in a dose-dependant manner that is similar to their scavenging capacities in the DPPH* assay. Also, all the samples showed same ranking orders in both DPPH* free radical scavenging assay and the reducing power assay. The reducing power was in the order of 100 μL/mL TOA > 100 μL/mL TOB > 10 mM BHT > 100 μL/mL RO (p<0.0001). These antioxidant tests demonstrated that both TOA and TOB were strong electron donors and could react with and convert free radicals to more stable products, and terminate the radical chain reactions (Yen, 1995).

Comparing anti-cancer activity of TOA and TOB

The anticarcinogenic properties of TOA and TOB were determined using the MTS assay. TOA and TOB inhibited Caco-2 and MCF-7 cancer cells proliferation in a dose dependent manner. At the concentration of 0.02 μL/mL, both TOA and TOB showed no or very little effect on the two cancer cell lines. The range of 0.2-20 μL/mL of TOA caused 23-69% cell inhibition against Caco-2 cells and 58-77% cell inhibition against MCF-7 cells, while the same concentration range of TOB caused 11-53% cell inhibition against Caco-2 cells and 10-80% cell inhibition against MCF-7 cells (**Figure 2.5 A** and **B**). The IC_{50} for Caco-2 cell and MCF-7 cell is 1.66 μL/mL (TOA), 21.5 μL/mL (TOB) and 0.122 μL/mL (TOA) and 1.219 μL/mL (TOB), respectively (**Table 2.2**). In case of Caco-2 cell line, at the range of 0.2-20 μL/mL, TOA exhibited higher inhibitive activity than TOB at the same concentration (p<0.0001) (**Figure 2.5 A**). In case of MCF-7, at the range of 0.2-2 μL/mL, TOA also exhibited higher inhibitive activity than TOB at the same concentration. **Figure 2.6** shows the inverted micrographs of the effect of TOA and TOB against MCF-7 cells at the concentration of 2 μL/mL. But at the

highest concentration tested of 20 μL/mL, there is no significant difference between TOA and TOB ($p<0.0001$) (**Figure 2.5 B**).

Discussion

Many researchers found that turmeric had strong antioxidant activity (Ruby, 1995; Rukkumani, 2004; Das, 2002; Zhou, 2000; Chatterjee, 1999) which was ascribed to curcumin or curcuminoids. However, HPLC analysis of our samples confirmed that there was absence of curcumin and curcuminoids. Therefore, we concluded that other compounds besides curcumin and curcuminoids were responsible for these activities.

As we now know, BHT, the synthesized antioxidant as food additive, was found to have carcinogenic side-effects (Ito, 1985) restricting its use (Mikova, 2001). The essential oil of rosemary (*Rosmarinus officinalis* L.) has both a desirable aromatic flavor and a strong antioxidant activity (Man, 2000). Because of these characters, rosemary oil has been used in many food products as a desirable flavoring ingredient and preservative (Özcan, 2003; Richheimer, 1996; Cuvelier, 1996; Man, 2000; Estévez, 2005; McCarthy, 2001; Sebranek, 2005). In this study, we found both TOA and TOB (100 μL/mL) possessed strong DPPH* free radical scavenging capacity and high reducing power when compared to the same concentration of rosemary oil and 10 mM BHT at the same reaction conditions.

Although both turmeric oils were extracted from turmeric rhizome, they had totally different chemical profiles. Sasikumar reported that turmeric oil obtained from turmeric powder by steam distillation contained about 60% turmerone, 25% zingiberene and small quantities of *d*-α-phellandrene, *d*-sabinene, cineole and forneol (Sasikumar, 2001). Other monoterpenes, sesquiterpenes, and their oxygenated derivatives were also

found in turmeric oil (Purseglove, 1981). Nevertheless, among turmeric oils from different sources, sesquiterpenes, ar-turmerone and turmerone usually comprise more than 50% of the oils. In TOA, ar-turmerone and turmerone accounts for about 40%, which is similar to the previous reports (Lawrence, 2003). But TOB contained more volatile chemicals such as 1-phellandrene (58%), cymene, 1,8-cineole, and α-terpinolene instead of less volatile chemicals like turmerone or ar-turmerone. Hu *et al*. studied the chemical constituents of turmeric rhizome essential oils of Chinese origin and found the major compounds were α-curcumene (34%), 1-(3-cyclopentylpropyl)-2,4-dimethyl-benzene (22%) and β-sesquiphellandrene (15%), but they could not detect ar-turmerone and turmerone (Hu, 1998). Gopalam *et al.* compared the constituents in turmeric oil from different cultivars and found there was significant quantitative variation in the main compounds among different cultivars (Gopalam, 1987). Cooray *et al*. examined the effect of maturity on the major components of the rhizome oil produced from a single turmeric cultivar grown in Sri Lanka, and found that sesquiterpenes (ar-turmerone and turmerone) increased while monoterpenes (1,8-cineole and α-phellandrene) decreased with maturity (Cooray, 1988). Chane-Ming *et al.* examined the essential oils from rhizomes, leaves and flowers of the same cultivar and found that major compounds in rhizomes were turmerones (37%), terpinolene (15.8%) and zingiberene (11.8%), but the leaves and flowers had similar chemical profile and were particularly rich in terpinolene with 76.8% (leaves) and 67.4% (flowers) (Chane-Ming, 2002). Taking into account of the above factors, the different compositions of our two tested turmeric oils (TOA and TOB) may be from different cultivars, or from the different plant parts, or from different maturity, or planted in different climate and soil, or extracted by different methods.

Although the chemical profiles of TOA and TOB are quite different, both of them have shown potent antioxidant capacities and strong growth inhibition against cancer cells. TOA showed a significantly higher antioxidant capacity than TOB in both *in vitro* bioassays within the tested concentration range. TOB contained some volatile flavors like α-terpinolene that was reported with a strong DPPH* free radical scavenging activity (Kim, 2004). In contrast, TOA contained less volatile chemicals including sesquiterpenes and oxygenated terpenics such as ar-turmerone, turmerone and curlone. These oxygenated compounds were reported with a potent antioxidant activity in the β-carotene-linoleate assay (Jayaprakasha, 2002). In addition, it was reported that ar-turmeorne had an insect repellent activity (Su, 1982), antibacterial activity (Negi, 1999), and antifungal activity (Jayaparakasha, 2001). Aratanechemuge *et al.* also reported that ar-turmerone could inhibit the growth of leukemia cells by inducing apoptosis (Aratanechemuge, 2002). Similarly in our study, oxygenated compounds-rich TOA also exhibited strong anticancer capacities against the Caco-2 and MCF-7 cancer cell lines, for which TOA at the concentration of 20 μL/mL showed inhibitive activity of 69% and 77%, respectively. In general, TOA showed a higher cell inhibition than TOB. The MCF-7 cell line seemed more sensitive to TOA than to TOB based on their IC_{50} values that were 0.122 μL/mL (TOA) and 1.219 μL/mL (TOB). However, due to the lack of chemical standards of ar-turmerone, turmerone and curlone, we could not determine their individual contribution and synergistic effects on their antioxidant activities in these two bioassays. Their inhibitive capacities against the Caco-2 and MCF-7 cancer cell lines and the molecular mechanism of how of ar-turmerone, turmerone and curlone inhibit cell growth will be further investigated by our research group.

Conclusion

Since many researches have found some chronic diseases are closely related to the existence of excessive free radicals (Grajek, 2005; Fridovich, 1999; McCord, 2000), intake of exogenous phytochemical antioxidants that act as scavengers of reactive oxygen species (ROS) and metal chelators may help to protect healthy human cells and reduce oxidative damages. In this study, both curcumin-free TOA and TOB have shown potent antioxidant and anticancer activities. In other words, such bioactive activities are contributed by the terpenes and oxygenated terpenes rather than the more extensively studied curcumin. In addition, since turmeric oil is considered non-toxic (Joshi, 2003) and has aromatic flavors, it can be an alternative natural antioxidant to BHT and rosemary oil for many food formulations, especially suitable for some ethnic foods.

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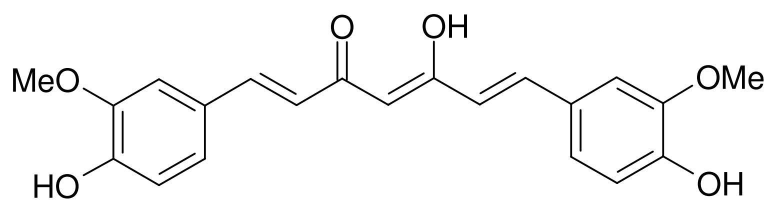
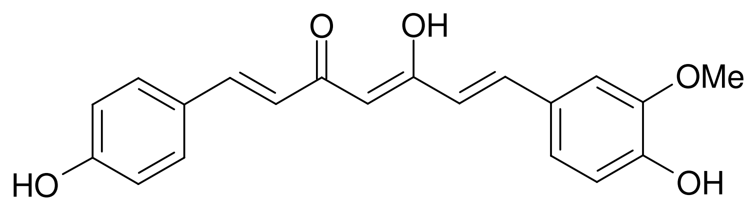
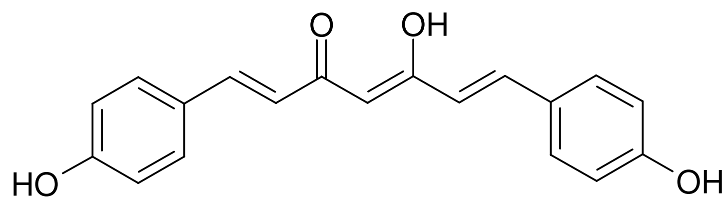


curcumin



demethoxycurcumin



bisdemethoxycurcumin

Figure 2.1 Structures of curcumin and curcuminoids.

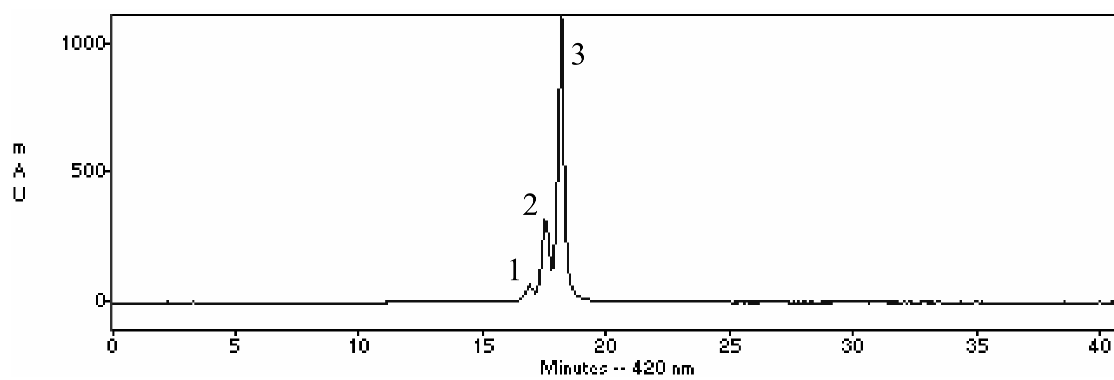


Figure 2.2 HPLC chromatogram of curcuminoids (420nm).

1. bisdemethoxycurcumin; 2. demethoxycurcumin; 3. curcumin.

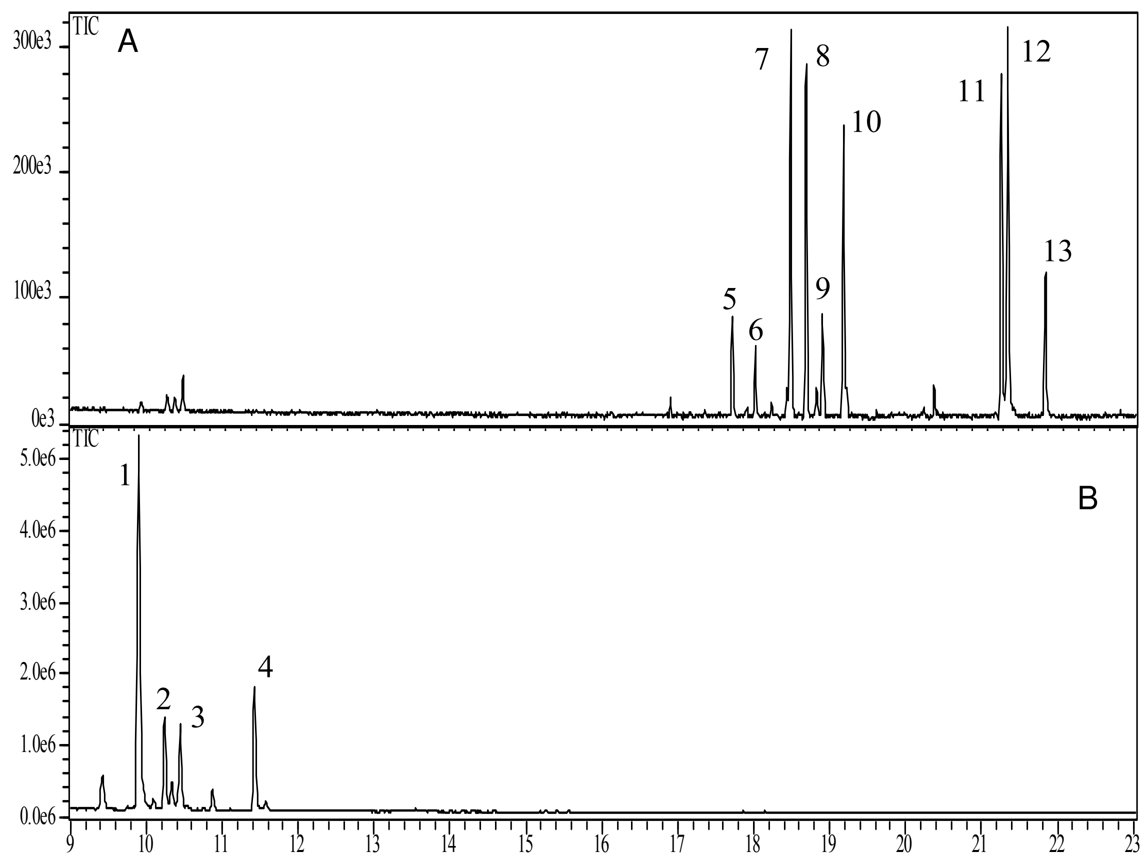


Figure 2.3 Gas chromatographic profiles of TOA and TOB.

A: TOA (250 ppm).

B: TOB (250 ppm).

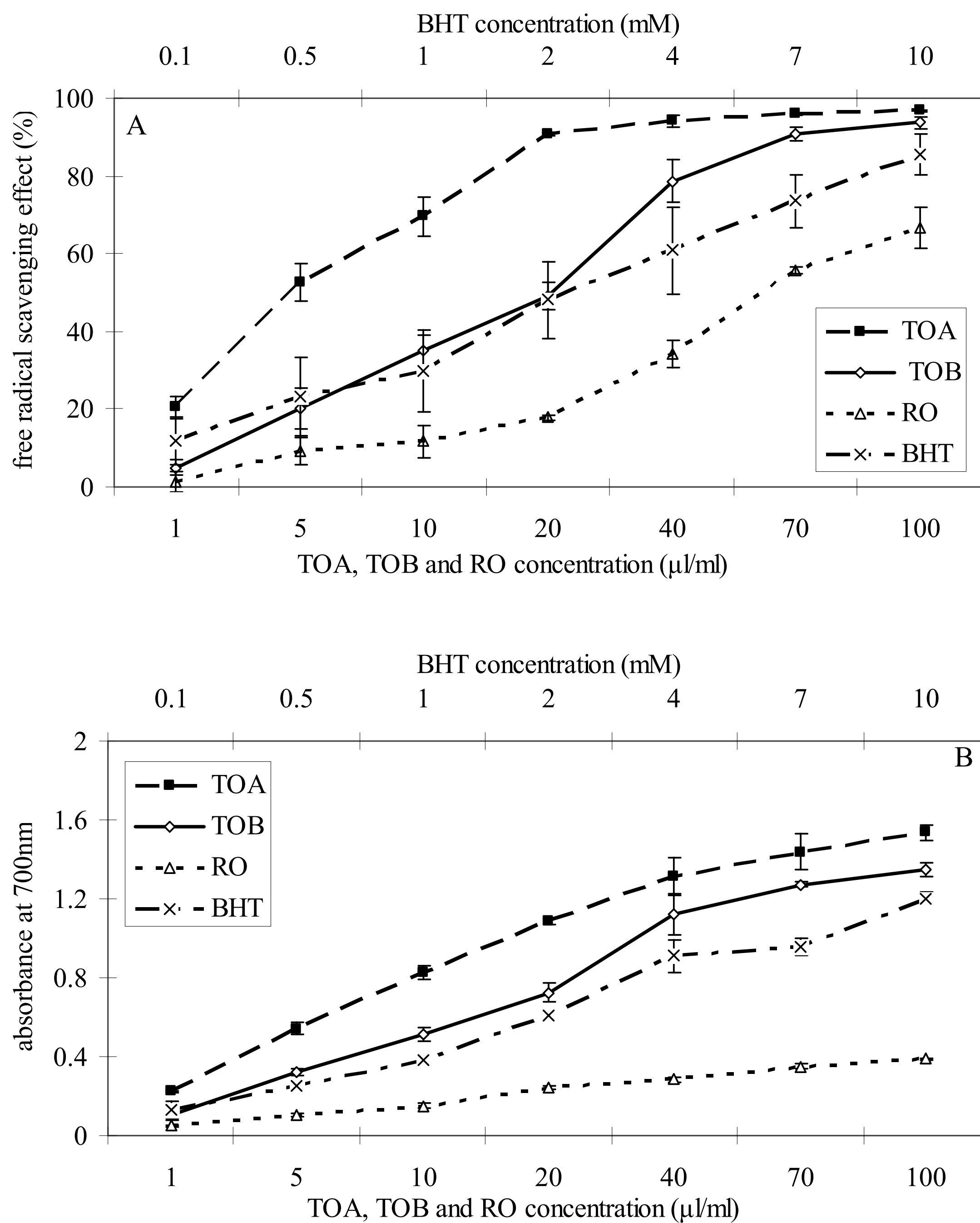


Figure 2.4 Antioxidative capacities of TOA, TOB and standards.

A: DPPH* assay, RO and BHT were used as standards.

B: Reducing power assay, RO and BHT were used as standards.

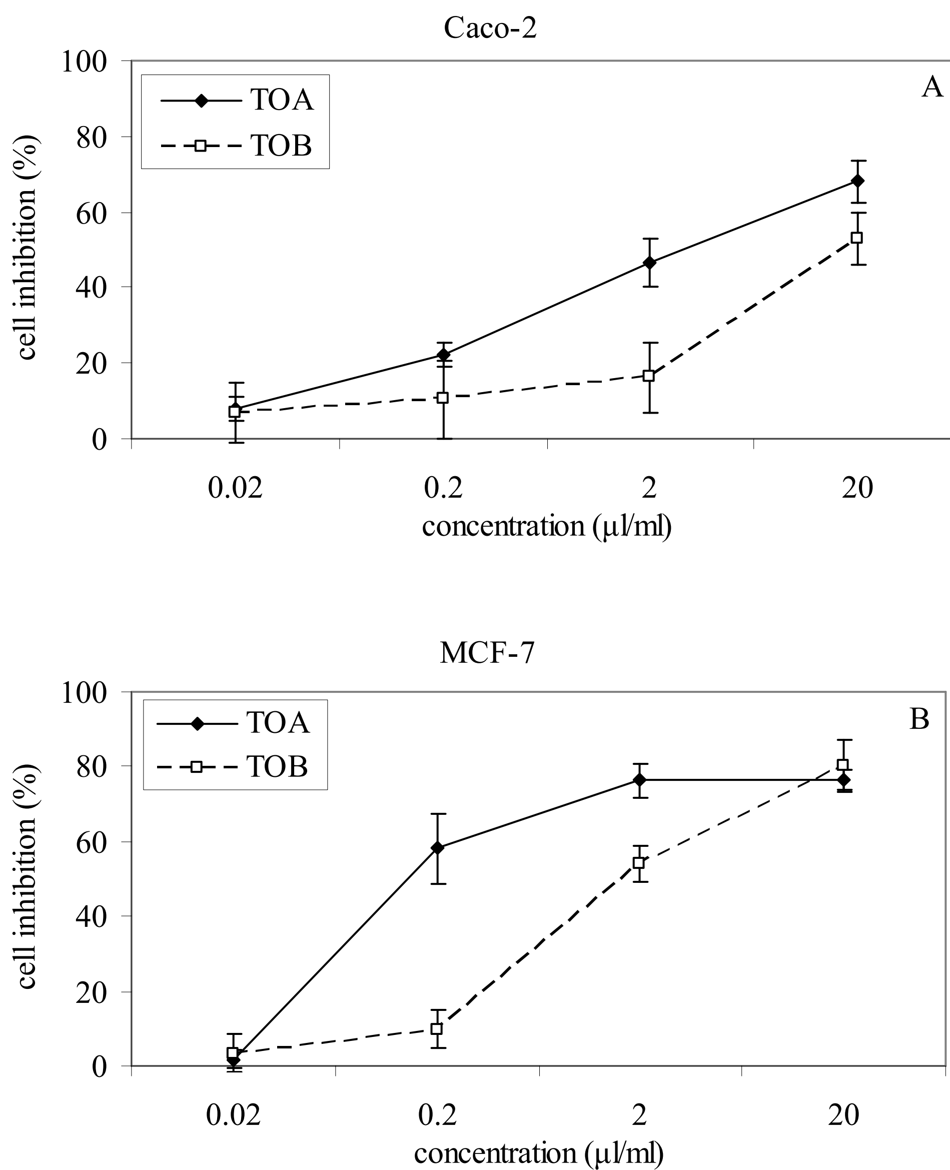


Figure 2.5 Anticancer ability of TOA and TOB.

A: Anti Caco-2 ability.

B: Anti MCF-7 ability.

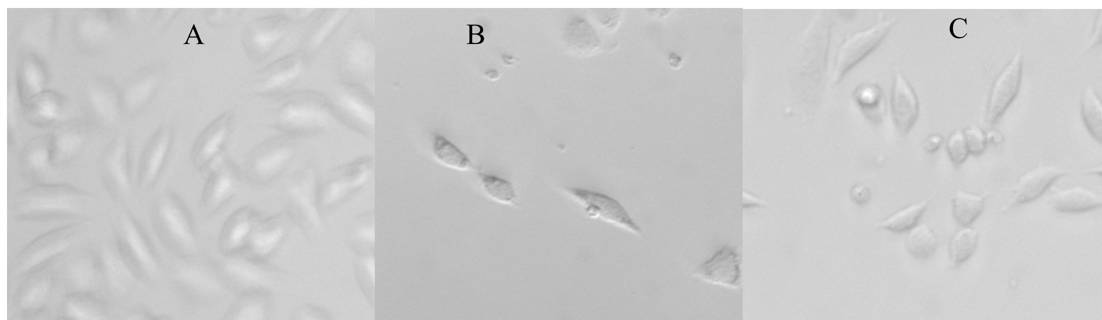


Figure 2.6 Inverted micrographs of TOA and TOB against MCF-7 cells.

A: Control.

B: Treated with 2 µL/mL TOA.

C: Treated with 2 µL/mL TOB.

Table 2.1 Chemical composition of TOB (1-4) and TOA (5-13).

Peak No.	Compound	RT (min)	RI[a]	Composition (%)	Identification method
1	1-Phellandrene	9.9	1014	58.07	Standard, MS, KI
2	Cymene	10.242	1032	12.23	MS, KI
3	1,8-Cineole	10.442	1043	15.07	Standard, MS, KI
4	α-Terpinolene	11.425	1092	14.63	Standard, MS, KI
	Subtotal amount of TOB			100	
5	Trans-caryophyllene	17.65	1444	4.58	MS, KI
6	Farnesene	17.958	1463	2.93	MS, KI
7	Ar-curcumene	18.417	1491	16.44	MS, KI
8	Zingiberene	18.617	1505	16.51	MS, KI
9	β-bisabolene	18.842	1518	4.82	MS, KI
10	β-sesquiphellandrene	19.117	1537	14.35	MS, KI
11	Ar-turmerone	21.183	1675	16.84	MS, KI
12	Turmerone	21.267	1680	16.45	MS, KI
13	Curlone	21.767	1715	7.08	MS, KI
	Subtotal amount of TOA			100	

[a] RI was calculated using a series of *n*-alkanes (C_8-C_{26}).

MS: Mass spectra.

KI: Kovats indices.

Table 2.2 IC_{50} values of TOA and TOB against Caco-2 and MCF-7 cell lines.

	IC_{50} in cancer cell line	
	Caco-2	MCF-7
TOA (µL/mL)	1.66	0.122
TOB (µL/mL)	21.5	1.219

CHAPTER 3

EVALUATION OF ANTIOXIDANT ACTIVITY OF CRCUMIN-FREE TURMERIC (*Curcuma longa* L.) OIL AND IDENTIFICATION OF ITS ANTIOXIDANT CONSTITUENTS

Abstract

Antioxidant capacity of curcumin-free turmeric (*Curcuma longa* L.) oil was evaluated by two different *in vitro* assays: the DPPH* free radical scavenging assay and reducing power assay. Results showed that the turmeric oil (TO) possessed strong free radical scavenging activity and reducing power when compared to standard antioxidants such as butylated hydroxytoluene (BHT) and α-tocopherol (VitE). An aliquot of 20 μL/mL TO showed 91% free radical scavenging activity in the DPPH* assay, which was comparable to 10 mM BHT (86%) and 10 mM VitE (96%) at the same condition. In the reducing power assay, the absorbance at 700 nm of 20 μL/mL TO was 1.085, which was comparable to 10 mM BHT (1.164). Higher concentration of TO at 100 μL/mL reached an absorbance at 1.537, which had no significant difference to 10 mM VitE (1.53). Among the complex constituents in the crude TO, ar-turmerone, turmerone, curlone and α-terpineol, were isolated and identified as the major components that have antioxidant effect by using various chromatographic techniques including silica gel open column chromatography, normal phase HPLC, and GC-MS. These results showed that TO and some of its inherent components can be potentially alternative natural antioxidants.

Introduction

There is an increasing interest in research of antioxidant activities of phytochemicals in diets due to an accumulative evidence that natural antioxidants can protect human bodies against excessive reactive oxygen species (ROS), which are considered the harmful by-products generated during normal cell aerobic respiration. Intake of exogenous antioxidants may help to maintain an adequate antioxidant status and the normal physiological function of a living system (Mimić-Oka, 1999). In food processing, many foods are subject to various factors that lead to the quality deterioration, i.e., lipid autoxidation. Therefore, natural and/or synthetic antioxidants are often added as food additives to improve or secure food quality related to texture, color, flavor and nutritional values, as well as the food shelf life. However, some synthetic antioxidants such as butyl hydroxyanisole (BHA) and butyl hydroxytoluene (BHT) are very effective, they are restricted in commercial applications because they may be harmful to human health (Ito, 1985). For this reason, it is important to explore some other naturally occurring non- or less-toxic antioxidants that can be used to prevent food oxidative deterioration. Natural antioxidants also have important usage as nutraceutical or cosmetic ingredients because they can form functional mixtures.

Turmeric (*Curcuma longa* L.) is a member of the *Zingiberaceae* family. It has been used as an important food ingredient in India for thousands of years because of its special aromatic flavors and attractive colors. Govindarajan (1980) reported that the dried rhizome of turmeric contained 3-5% essential oil and 0.02-2.0% yellow curcuminoids. Turmeric oil is usually obtained from turmeric powder by steam distillation for about 8-10h. It has a pale yellow color with a peppery and aromatic odor. The major components

in the oil include α-phellandrene, 1,8-cineol, zingiberene, ar-curcumene, turmerone, β-sesquiphellandrene, curlone and dehydrozingerone (Govindarajan, 1980). Turmeric is highly esteemed by the local people and considered a traditional medicine in the Ayurvedic system due to its medicinal properties. Many researches have also shown that the curcuminoids have various biological activities, such as antioxidant (Daniel, 2004; Ruby, 1995; Chatterjee, 1999; Rukkumani, 2004), anticancer (Nagai, 2005; Verma, 1997), and anti-arthritis (Funk, 2006) activities. Of the turmeric oil extracted from curcumin removed turmeric oleoresin (CRTO), a strong antioxidant activity was ascribed to the trace amounts of curcuminoids (Jayaprakasha, 2002). Despite numerous investigations on biological activities of curcumin, few have involved the curcumin-free turmeric oil that is considered a by-product with no commercial importance (Saju, 1998; Jayaprakasha, 2005). Therefore, there are strong economic reasons to explore new functionalities of other components in the curcumin-free turmeric oil. In this study, we investigated the antioxidant capacity of curcumin-free turmeric oil using two complimentary *in vitro* assays: 2, 2-diphenyl-1-picrylhydrazyl (DPPH*) free radical scavenging assay and reducing power assay. The antioxidant activity of curcumin-free TO was compared with that of commercial standard antioxidants, butylated hydroxytoluene (BHT) and α-tocopherol (VitE). Moreover, some TO components showing strong antioxidant activity were further separated from the crude TO by various chromatographic techniques such as open-column silica gel chromatography and normal phase HPLC, and identified by GC-MS.

Materials and methods

Materials and chemicals

The crude turmeric oil (TO) was purchased from Aromaland Company (Santa Fe, NM, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH*) free radical, butylated hydroxyl-toluene (BHT), α–tocopherol (VitE) and α-terpineol were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium ferricyanide and ferric chloride were obtained from J. T. Baker Chemical Co. (Phillipsburg. NJ). Trichloroacetic acid and all solvents were purchased from Fisher Scientific (Suwanee, GA).

HPLC profile of curcumin standard and crude TO

A Shimadzu LC-10AT HPLC system (Kyoto, Japan) was equipped with a Waters C_{18} reverse phase column (Xterra^{TM}, 4.6 x 150 mm, 5 µm) and a SPD-M10A photodiode-array (PDA) detector set at 420 nm. UV spectra were recorded in a region of 200-800 nm. Mobile phase was consisted of solvents: (A) water (0.25% acetic acid) and (B) acetonitrile, and run in a gradient profile in: 0-17 min, 40-60% B; 17-32 min, 60-100% B; 32-38 min, 100% B; 38-40 min, 100-40% B; 41 min, stop. Total flow rate of the mobile phase was 0.6 mL/min. Injection volume was 20 µL. Curcumin standard (dissolved in ethanol, 0.15 mg/mL) and TO [dissolved in DCM/methanol (v/v 1:2), 100 µL/mL] were analyzed by HPLC using the same aforementioned method.

Fractionation and Identification of Antioxidants from crude TO

Silica gel open column chromatography

A glass open column (25 x 2.5 cm) was packed with silica gel (70-230 mesh, 60Å). An aliquot of 1.5 mL crude TO was loaded and eluted by multistep solvent

gradients as follows: hexane, dichloromethane (DCM), ethyl acetate, acetonitrile, and methanol. The flow rate of mobile phase was 4 mL/min. Each collected fraction was 4 mL. After the open-column separation, those TO fractions possessing strong DPPH* free radical scavenging activities were pooled for further analyses.

Normal phase HPLC analysis

A Spherisorb silica column (250 x 4.6 mm, 5 μm; Waters, Milford, MA) was installed on a Shimadzu LC-10AT HPLC system (Kyoto, Japan) and equilibrated with DCM. To separate components in the DCM fraction obtained from the open-column chromatography, twenty microliters of the pooled fractions were further separated by the HPLC normal phase column and eluted at a flow rate of 1 mL/min by isocratic DCM. For separation of components in the ethyl acetate fraction, fifty microliters of pooled fractions were separated by the same column and eluted at a flow rate of 1 mL/min by linear gradient of methanol from 0 to 10% at 5-15 min to wash out polar compounds. The eluant was collected into 1 mL fractions after passing through the PDA detector for further analysis.

GC-MS identification

A Shimadzu's GC-MS system consisting of a GC-17A with a QP5050 Mass Spectrometer (Kyoto, Japan) was equipped with a DB-5 capillary column (60 × 0.25 mm, thickness 0.25 μm; J&W Scientific, Folsom, CA, USA) for all volatile chemicals' quantitative and qualitative analyses in this research. The oven temperature was programmed from 60 to 280˚C at a ramp rate of 8˚C/min and held at 280˚C for 30 min. The injector and ion source temperatures were set at 180 and 290˚C, respectively. The

detector voltage was 70 eV, and the scanning mass range was *m/z* 43-350. Helium was used as the carrier gas at a column flow rate of 1.2 mL/min. The sample injection volume was 2 μL with a split ratio of 1:1. Identification of compounds was based on comparison of their mass spectra and retention indices (RIs) with those of the authentic standards. Chemical RIs were calculated using series of *n*-alkanes (C_8-C_{26}). If standard compounds were not available, each unknown compound was tentatively identified by comparing the mass spectrum with that in the Wiley and NIST mass spectral databases and the previously published RIs obtained under the same conditions (Negi, 1999; Chatterjee, 2000; Adams, 2001).

Antioxidative capacity

<u>DPPH* free radical scavenging assay</u>

DPPH* free radical scavenging assay was adopted using the method described by Yamaguchi *et al.* (1998) with minor modification. An aliquot of 0.4 mL of sample dissolved in DCM was mixed with 0.4 mL of 0.25 mM DPPH* solution dissolved in DCM. The mixture was shaken vigorously and left in the dark at room temperature for 30 min. A control consisted of 0.4 mL solvent instead of sample. Because crude TO has color, so we also add 0.4 mL TO (at each concentration) to 0.4 mL solvent as blank, respectively. The absorbance of the resulting solution was measured spectro-photometrically at 517 nm. The scavenging effect of DPPH* free radical was calculated by the following formula:

$$\text{Scavenging effect (\%)} = \left(1 - \frac{\text{absorbance of sample at 517 nm} - \text{blank}}{\text{absorbance of control at 517 nm}}\right) \times 100 \qquad (1)$$

Reducing power assay

The reducing power of TO was determined by the method of Yen *et al*. (1995) and Chung *et al.* (2002) with minor modification. Turmeric oil was dissolved in acetone to prepare solutions in different concentrations. An aliquot of 0.5 mL of the sample was mixed with 1 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$], then the mixture was incubated at 50˚C for 20 min, followed by the addition of 1 mL of trichloroaceteic acid (10%) to the mixture and centrifugation at 3000 rpm for 10 min. The upper layer of solution (1 mL) was mixed with 1 mL distilled water and 0.2 mL $FeCL_3$ (0.1%). The absorbance of the mixture was measured at 700 nm. Higher absorbance of the reaction mixture indicated a higher reducing power.

Triplicates were performed for each concentration of the tested samples and standards in these two methods. The experiments were repeated three times on different days.

Statistical analysis

The data of the antioxidant activities of TO, BHT and VitE were subjected to the analysis of variance (ANOVA). Treatment means were separated by the least significant difference (LSD at $p<0.05$). Analyses were performed using the statistical software SAS 9.1 operated on the Windows system (SAS Institute Inc., Cary, NC).

Results and discussion

Confirming no curcumin or curcuminoids in crude TO

Standards of curcumin and its derivatives were analyzed by HPLC resulting in three peaks (**Figure 3.1**), including (1) bisdemethoxycurcumin; (2) demethoxycurcumin;

and (3) curcumin. These chemicals showed strong absorbance wavelength at 420-430 nm. Comparing the HPLC profile of the standards with that of TO, it was confirmed that our sample was the curcumin-free turmeric oil.

Determination of the antioxidant activity of crude TO

The antioxidant activity of crude TO was initially measured by the DPPH* free radical scavenging assay and reducing power assay, and compared with that of 10 mM BHT and 10 mM VitE. TO prepared in a serial concentrations (1, 5, 10, 20, 40, 70, 100 μL/mL) were tested by the two aforementioned assays. Results shown in **Figure 3.2 A** indicated that the DPPH* free radical scavenging activity of the crude TO increased with the increasing TO concentration. When 0.25 mM DPPH* solution was used, it could be saturated by the crude TO at the concentration of 20 μL/mL and above resulting in a scavenging activity of approximate 90%, which was comparable to the antioxidant ability of 10 mM BHT and 10 mM VitE at the same condition.

Figure 3.2 B shows the reducing powers of TO, BHT, and VitE. Similar to the DPPH* assay, the reducing power of crude TO also increased with the increasing concentration. The spectrometric absorbance of the reducing power of 20 μL/mL TO was 1.085, which was comparable to 10 mM BHT (1.164), but lower than 10 mM VitE (1.53) ($p<0.001$). When the concentration of TO increased to 100 μL/mL, its absorbance increased to 1.537, which had a close reducing power to 10 mM VitE without significant difference ($p<0.001$). These results demonstrated that the TO was electron donors, and could react with free radicals and convert them to more stable products (Yen, 1995).

Separation and identification of antioxidants in crude TO

Since the tested TO had shown strong antioxidant activities in two assays, its inherent antioxidants were then subject to a series of sequential separations with silica gel open column chromatography, and normal phase HPLC, and finally identified by GC-MS.

When the crude TO was separated by the silica gel open column chromatography using a stepwise solvent elution method with different solvents such as hexane, DCM, ethyl acetate, acetonitrile and methanol, a total of 235 fractions were collected. As shown in **Figure 3.3**, there were two strong antioxidant peaks corresponding to the DPPH* assay, namely, TO-I and TO-II, respectively. TO-I contained the fractions 85-95 that were eluted by DCM; while TO-II comprised the fractions 123-129 eluted by ethyl acetate. The strength of antioxidant activities of both TO-I and TO-II were similar, with the antioxidant activity values close to 85% in the DPPH* test. To further separate antioxidants in these fractions, all fractions within TO-I and TO-II were pooled respectively and separated by the Spherisorb silica HPLC column. HPLC separation chromatograms and online antioxidant determination by the DPPH* assay are shown in **Figure 3.4**. Major components of TO-I were eluted by DCM. TO-II was eluted by DCM and methanol. In **Figure 3.4 A**, there were 3 peaks, named TO-I-1, TO-I-2 and TO-I-3, respectively. However, only fraction TO-I-3 showed a high antioxidant activity (90%). **Figure 3.4 B** showed there were two chromatographic peaks, denoted as TO-II-1 and TO-II-2. The fraction TO-II-2 had a light yellow color and possessed a medium free radical scavenging activity (60%). Since GC-MS analysis indicated that the TO-II-2 fraction still contained more than 10 compounds (**Figure 3.5 C**), it was further

concentrated and separated by DCM/methanol (v/v 94:6) using the same column, which resulted into two peaks (**Figure 3.4 C**). However, only the second peak (TO-II-2-b) showd the antioxidant activity. Therefore, both the fraction TO-I-3 and the fraction TO-II-2-b were selected for further chemical identification by GC-MS.

Identification of antioxidants in TO-I and TO-II separated
by Spherisorb silica HPLC

Previous research (Raina, 2002) found that the crude TO contained nearly 100 compounds, including major chemicals such as ar-turmerone, turmerone, β-sesqui-phellandrene, curcumene, etc. In our sample, the major components were ar-curcumene (16.4%), zingiberene (16.5%), β-sesquiphellandrene (14.4%), ar-turmerone (16.8%) and turmerone (16.5%) (**Figure 3.5 A, Table 3.1**), which was similar to the previous report. Further chemical separation and identification of the compounds in the fractions TO-I-3 and TO-II-2-b revealed that there were only 3 compounds in the fraction TO-I-3 and some more compounds in the fraction TO-II-2-b. As profiled in **Figure 3.5**, ar-turmerone, turmerone, and curlone in the fraction TO-I-3 (**Figure 3.5 B**) and α-terpineol in the fraction TO-II-2-b (**Figure 3.5 D**) were identified after comparing their RIs and mass spectra with those in an essential oil library (Adams, 2001) and the Wiley and NIST mass spectral databases, as well as with the standard compounds under the same experimental conditions. **Figure 3.6** shows the structures of the four identified compounds. This result confirmed the previous suspect that the antioxidant activity of turmeric oil may be also attributed to the chemicals such as ar-turmerone, turmerone, and some other oxygenated compounds (Jayaprakasha, 2002). Although the mixture of ar-turmerone, turmerone and curlone showed high antioxidant activity in our preliminary screening, their individual

concentration-activity relationship could not be established due to the lack of available standards.

The fraction TO-II-2 was light yellow when concentrated. GC-MS analysis showed that the fraction contained more than 10 compounds and turmerol and α-terpineol were the major constituents (**Figure 3.5 C**). TO-II-2-b became colorless when the naturally yellow turmerol was removed. The concentrated fraction TO-II-2-b possessed 58% free radical scavenging activity when 0.1 mM DPPH* solution was used. Although α-terpineol (3-cyclohexene-1-methanol, α, α, 4-trimethyl) showed a moderate free radical scavenging activity during the concentration range of 10 to 100 μL/mL (**Figure 3.7**), it showed very weak reducing power.

Many studies have addressed the benefits of using turmeric because of its inherent bioactive chemicals such as curcumin and curcuminoids. However, there is a lack of information of the potential values of curcumin-free turmeric oil. Our present study revealed that ar-turmerone, turmerone, curlone and α-terpineol in the curcumin-free TO also possessed some strong antioxidant activities. Considering their other biological activities such as antifungal (Jayaprakasha, 2001), antibacterial (Negi, 1999), antivenom (Ferreira, 1992) and insect repellent (Su, 1982) activities, the curcumin-free TO that was considered a by-product without commercial value might be converted to a value-added product, for example, using TO as an alternative natural antioxidative food additive.

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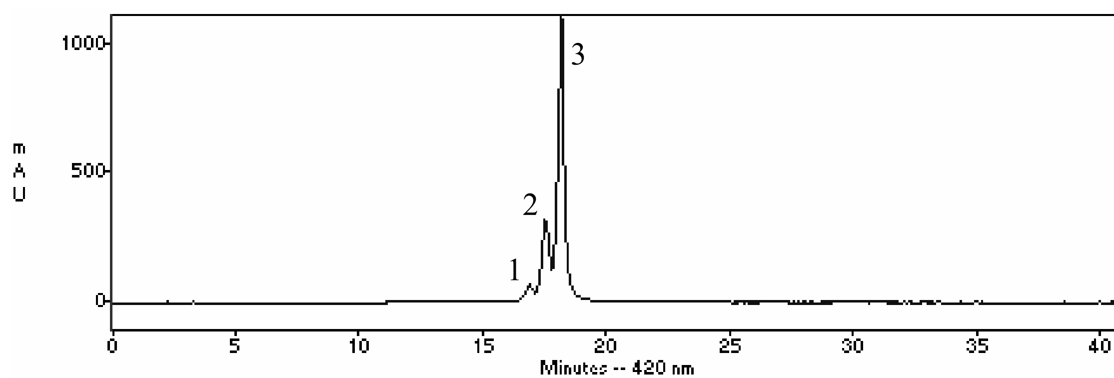

Figure 3.1 HPLC chromatogram of curcumin and curcuminoids in their absorbance at 420nm.

1. bisdemethoxycurcumin; 2. demethoxycurcumin; 3. curcumin.

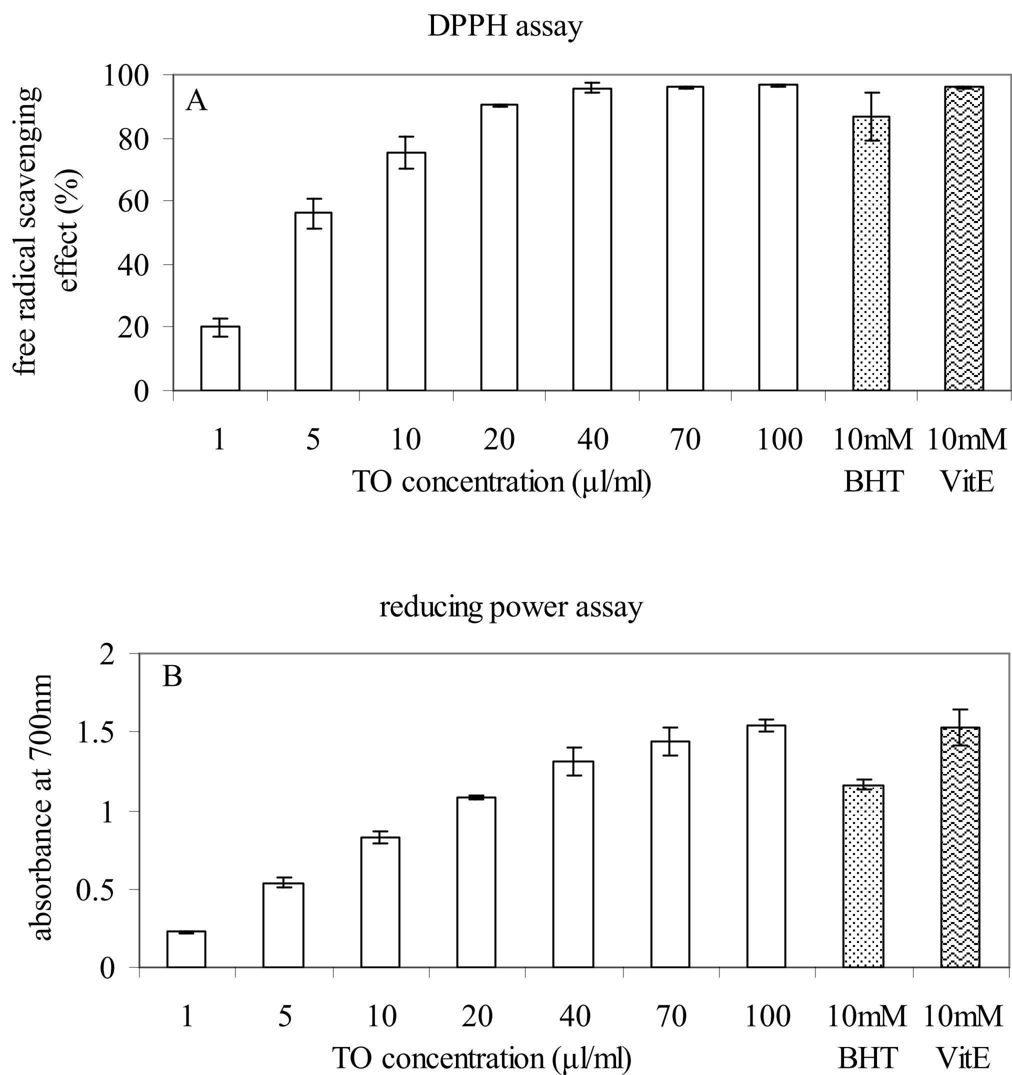

Figure 3.2 Antioxidative capacity of crude TO.

A: DPPH* assay, 10 mM and 10 mM VitE were used as standards.

B: Reducing power assay, 10 mM BHT and 10 mM VitE were used as standards.

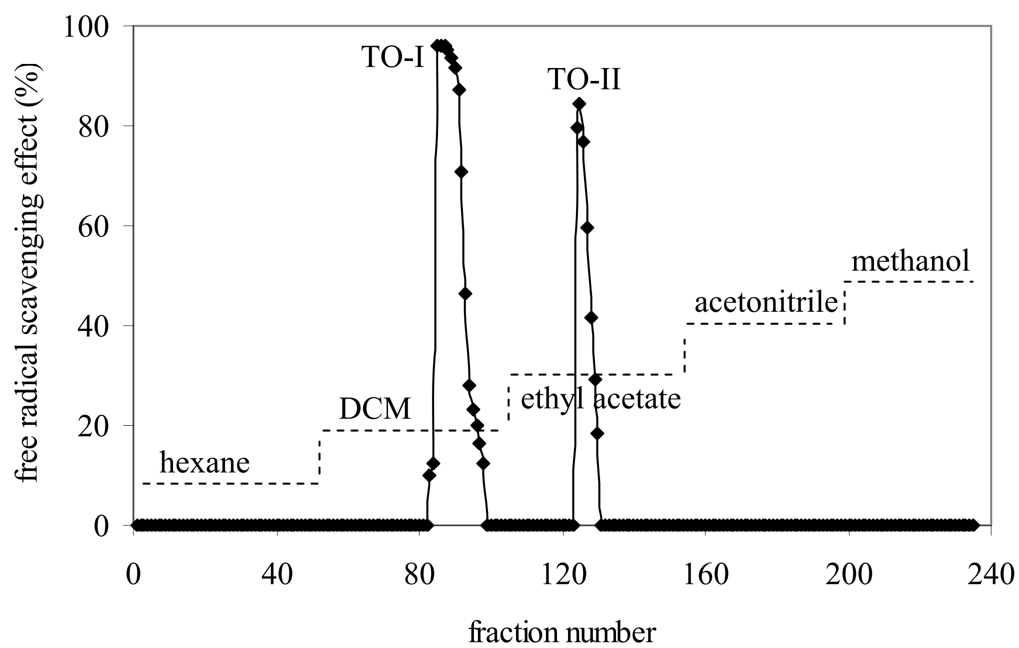

Figure 3.3 Free radical scavenging activity of fractions of crude TO separated by silica gel open column chromatography.

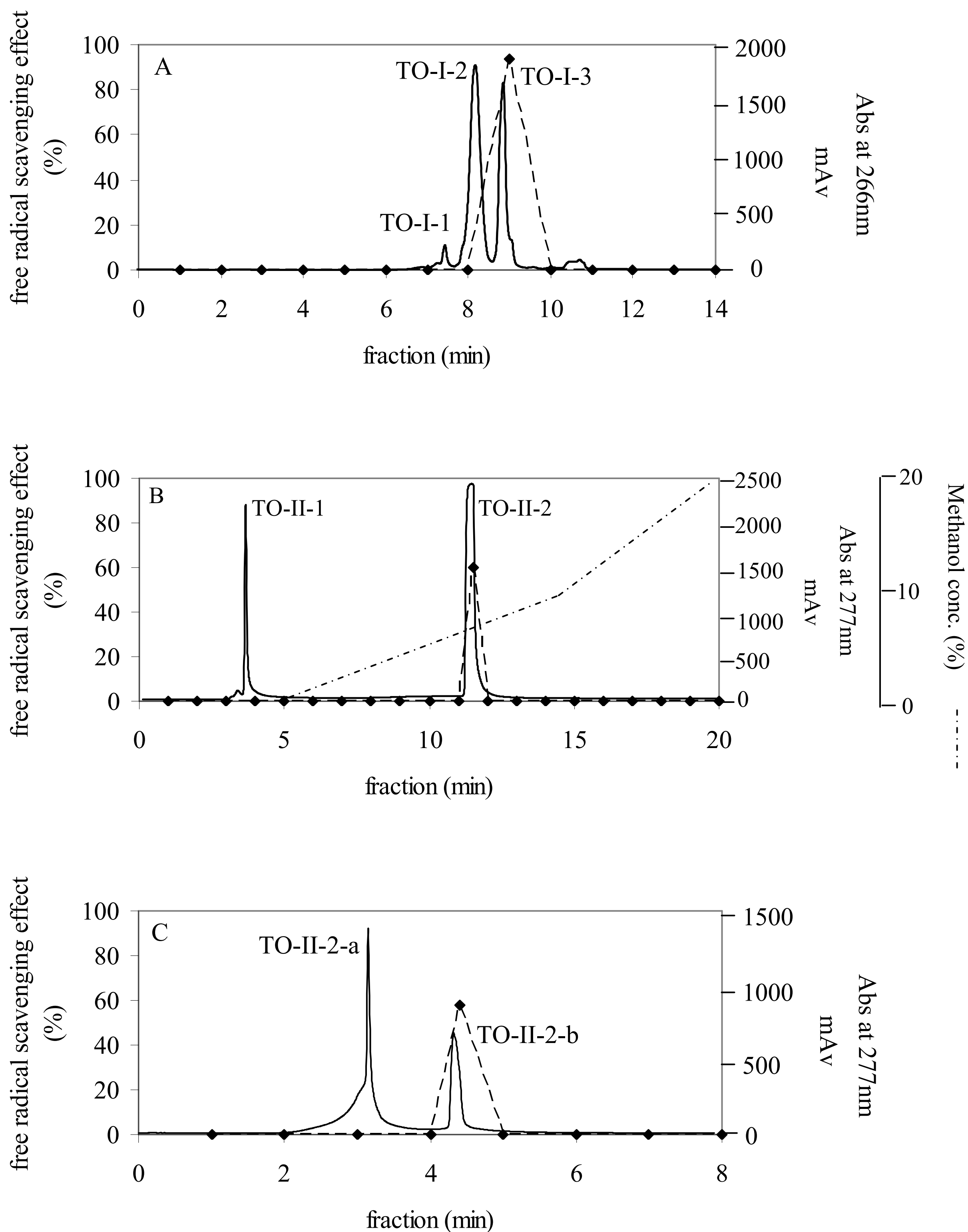

Figure 3.4

Figure 3.4 (continued) Chromatograms of TO-I and TO-II and their free radical scavenging activities.

A: TO-I separated by Spherisorb silica HPLC, isocratic method, mobile phase: DCM.

B: TO-II separated by Spherisorb silica HPLC, gradient method, mobile phase: a. DCM; b. methanol.

C: TO-II-2 separated by Spherisorb silica HPLC, gradient method, mobile phase: DCM /methanol (v/v = 94:6).

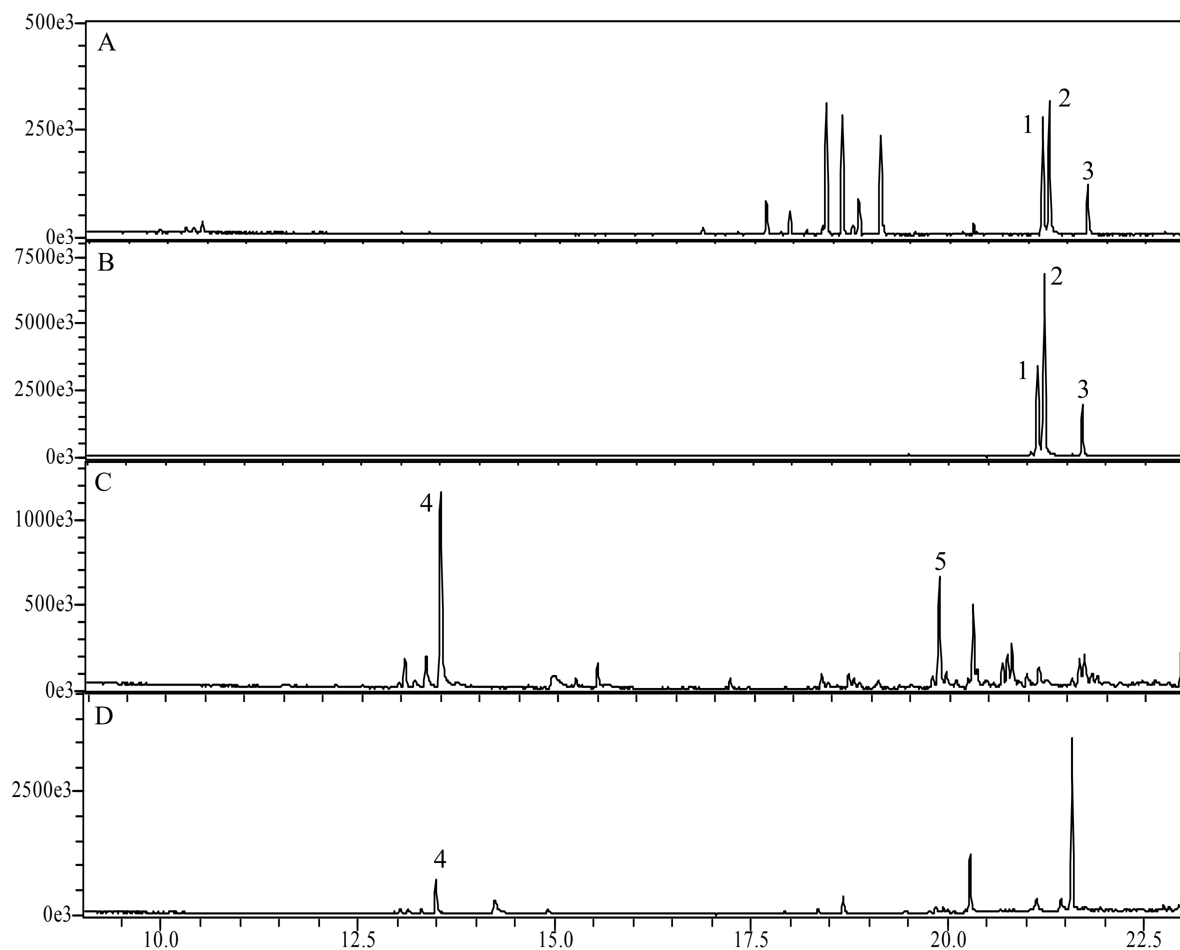

Figure 3.5 Gas chromatographic profiles of crude TO and its fractions.

A: Crude turmeric oil.

B: Fraction TO-I-3.

C: Fraction TO-II-2.

D: Fraction TO-II-2-b.

1. ar-turmerone; 2. turmerone; 3. curlone; 4. α-terpineol; 5. turmerol.

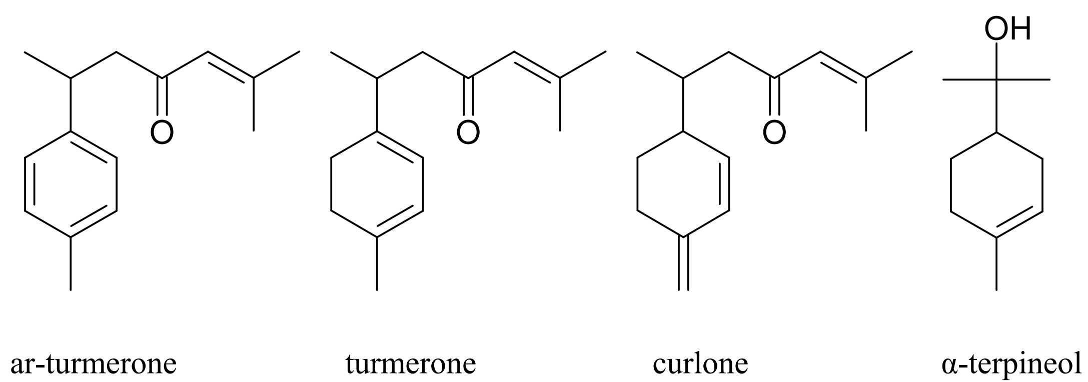

Figure 3.6 Structure of turmeric components that have antioxidant activities.

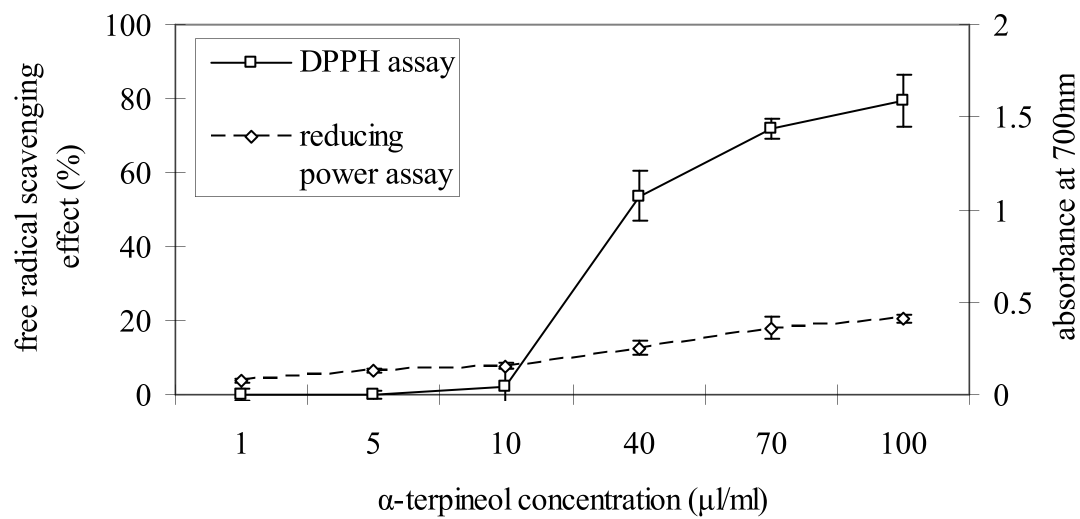

Figure 3.7 Free radical scavenging assay and reducing power assay of α–terpineol.

Table 3.1 Composition of crude TO.

Retention time (min)	Compound	RI[a]	Composition (%)	Identification method
17.650	Trans-caryophyllene	1444	4.58	MS, KI
17.958	Farnesene	1463	2.93	MS, KI
18.417	Ar-curcumene	1491	16.44	MS, KI
18.617	Zingiberene	1505	16.51	MS, KI
18.842	β-bisabolene	1518	4.82	MS, KI
19.117	β-sesquiphellandrene	1537	14.35	MS, KI
21.183	Ar-turmerone	1675	16.84	MS, KI
21.267	Turmerone	1680	16.45	MS, KI
21.767	Curlone	1715	7.08	MS, KI

[a] RI was calculated using a series of *n*-alkanes (C_8-C_{26}).

MS: Mass spectra.

KI: Kovats indices.

APPENDICES

Appendix A

DPPH* assay

DPPH* (2,2-diphenyl-1-picrylhydrazyl, MW: 394.22) is a stable free radical and shows a characteristic absorption at 517 nm due to its odd electron. It is purple color. As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, or a free radical scavenging antioxidant, the absorption strength decreased resulting in decolorization that is stoichiometric with respect to the number of electrons captured (**Figure 4.1**). Although DPPH* is considered as a stable free radical, its solutions slowly deteriorate. The solution need to be stored in glass container under nitrogen atmosphere, and covered with aluminum foil. Under these conditions a standard solution may be prepared and stored with a loss of free radical activity not exceeding 2-4% per week (Blois, 1958).

Sample can be diluted to a series of concentration in solvents, then use the same solvent to dissolve DPPH* and get the last concentration of 250 μM. Take one aliquot of sample and add the same volume DPPH* to make the DPPH* concentration to 125 μM. Then shake the mixture vigorously and left in the dark at room temperature for 30 min. Then measure the absorbance at 517 nm using spectrometer. If the sample has color, blank control should be set. The scavenging effect of DPPH* free radical was calculated by this formula:

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

Regarding the solvent to be used, the method seems to work equally well with methanol, ethanol and DCM, any of which seems not to interfere with the reaction. The

use of other solvent systems, such as almost neat extracts in water or acetone, seems to give low values for the extent of reduction (Molyneux, 2004).

Appendix B

Reducing power assay

The mechanism of reducing power assay is the presence of reductants (antioxidants) would result in reducing Fe^{3+}/ferricyanide complex to the ferrous form. The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Yen, 1995; Chung, 2002). To accurately measure the total reducing power, the following conditions must be met. (1) All, and only, antioxidants can reduce Fe^{3+} / ferricyanide complex under the reaction conditions. (2) The reaction rate must be sufficiently fast enough that the reaction can be completed in a short assay time. (3) The oxidized antioxidant and its secondary reaction products should have no absorption at 700 nm, the maximum absorption of Perl's Prussian blue (Qu, 2002).

In this assay, sample needed to be diluted in solvent (solvent should be miscible with water) in a series of concentration. 0.5 mL of sample mixed with 1 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$, dissolved in distilled water], and the mixtures were incubated at 50˚C for 20 min. Then 1mL of 10% trichloroaceteic acid (dissolved in distilled water) was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of solution (1mL) was mixed with distilled water (1mL) and 0.2 mL $FeCL_3$ (0.1%). The mixture was shaken vigorously and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power.

Appendix C

Packaging silica gel open column

Preparing silica gel

Put suitable amount of silica gel into a beaker. Add some hexane into the beaker and swirl gently to make a “slurry”. Remove air bubbles by sonication or vacuum pump.

Preparing open column

Clamp column securely and straightly to a ring stand, leaving enough space at the bottom to allow for switching of the flasks or beakers you will use to collect the eluted solvent. Attach a funnel to the top of the column and place a beaker underneath.

Packing open column

Pour the “slurry” into the column via the funnel and let it settle in the column. Open the stopcock and let some of the solvent drip through. As the solvent collects in the waste beaker below you can use it to resuspend the remaining silica that is stuck in your “slurry” beaker and reapply to the top of the column. Repeat as necessary until almost all of the silica has been transferred to the column and has settled down to form an even, tightly packed cylinder, saturated with solvent. Close the stopcock when the column seems to have packed down to about 2-3 cm from the top of the column. The solvent level should just cover the top of the column. If the top surface of the packed silica appears crooked, tap gently on the side of the column until it flattens out. Do not ever let the column run dry. Avoid little cracks and channels. If your column runs dry or forms channels, you need to pack it again.

Loading sample

If your sample is solid, you need to dissolve it first. The solvent you used is as little as possible. Using a glass pipette, start to add your dissolved sample to the top of the column gently. When you add the sample to the column, you want the amount of solvent at the top of the column to be quite small. When there is a couple of millimeters of solvent above top of saturated silica gel, open the stopcock and allow the sample to run into the column, adding drops of the dissolved sample slowly and gently to the top via the pipette so that the column will not run dry.

Separation

Once the sample is loaded onto the column, you can gently add 10 mL of fresh solvent (you want to use it as mobile phase) to the column and start the separation. Add more solvent or connect the column to the solvent pump. At all times make sure that there is no danger of running dry.

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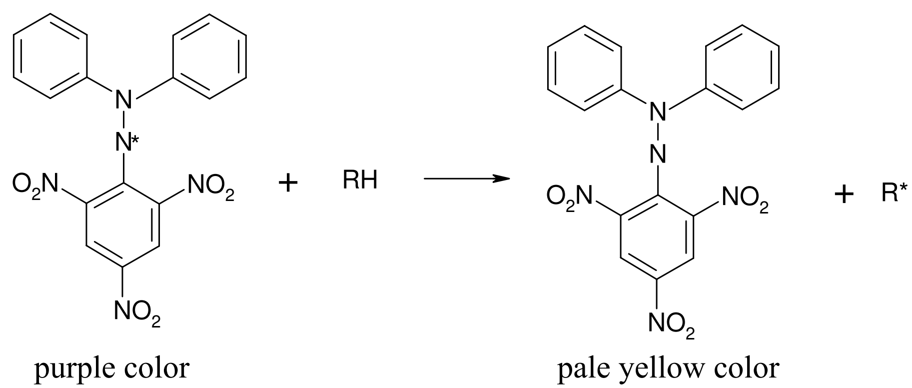

Figure 4.1 DPPH* structure.