

SAPPANWOOD WATER EXTRACT:
EVALUATION OF COLOR PROPERTIES, FUNCTIONAL PROPERTIES, AND
TOXICITY

By

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To my mother, Pimprapai Kunchavalee Sinsawasdi, and my father, Dr. Narong
Sinsawasdi

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LIST OF ABBREVIATIONS

CFU	Colony forming unit
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
ET	Electron transfer reaction
GAE	Gallic acid equivalent
GM	Glucose minimum
HAT	Hydrogen atom transfer reaction
MIC	Minimum inhibition concentration
MRSA	Methicillin-sensitive strain of <i>Staphylococcus aureus</i>
ORAC	Oxygen radical absorbance capacity
SPW	Sappanwood or <i>C. sappan</i> heartwood
RF5	Water extraction of sappanwood using a 5-hour reflux
RF24	Water extraction of sappanwood using a 24-hour reflux
RT	Water extraction of sappanwood by shaking at room temperature for 6 hours
TEAC	Trolox equivalent antioxidant capacity
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TP	Total polyphenol
VRE	Vancomycin-resistant enterococci

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SAPPANWOOD WATER EXTRACT: EVALUATION OF COLOR PROPERTIES,
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The red color from water extracts of sappanwood (heartwood of *Caesalpinia sappan* L.) has been used in Thai and Indonesian beverages for hundreds of years. The colorant may have potential as an alternative for synthetic red dyes. However, scientific data on this red colorant is limited.

Water extracts of *Caesalpinia sappan* prepared from different methods (reflux at 5h (RF 5); 24h (RF 24); room temperature extract at 6 h (RT)) were evaluated for color, functional properties, and toxicity. Color properties were described by CIE L*a*b* measurements, hue angle, chroma, and UV-vis spectra. Total phenolic (TP) content and antioxidant activities were evaluated by the Folin-Ciocalteu colorimetric method, and by the oxygen radical absorbance capacity (ORAC) and 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assays, respectively. Mutagenicity was determined by the Ames assay (with and without metabolic activation) while antimicrobial activity was determined by disc diffusion method.

In contrast to other naturally derived red pigments such as anthocyanins, the color of *C. sappan* pigments is red at pH 8 - 12, and appears yellow at lower pH. Brazilein and brazilin, the compounds responsible for giving red color to the extract, were

detected and identified by a high performance liquid chromatography and mass spectroscopy (LC-MS). Total phenolic content and antioxidant activities in RF 24 were not significantly higher than RF5, and thus RF24 was excluded from further evaluation. The extracts (RF5 and RT) showed no mutagenicity. The extracts exhibited antimicrobial activity against common spoilage bacteria including *Alcaligenes faecali*, *Bacillus coagulans*, and *Pseudomonas puida* at a concentration of 50 mg/mL. The stability of color to heat, fluorescent light and storage temperature at different pH levels was examined. The hue of the extract was affected by concentration (0.005 – 0.4000 mg/mL) but the visual color remained in pink to red shade for pH 8 and 9. Results indicated that the color from sappanwood extract was more stable at higher pH (9) than at lower pH (7).

Overall, results from this current study show the potential for developing sappanwood water extract into a naturally derived red color additive for non-acidic food products.

CHAPTER 1 INTRODUCTION

Justification

A naturally-derived red colorant that is safe for human consumption is in demand by the food industry. Recent clinical research, which is known as “the Southampton Study”, has brought attention from several news media as well as advocate groups on the use of artificial food colors (McCann and others 2007; Institute of Food Technologists 2011). The relationship between hyperactivity in children and consumption of synthetic colors was considered inconclusive by experts such as Institute of Food Technologists and the U.S. FDA (Larsen 2008; Gravani 2011). However, the U.K. Food Standards Agency, which commissioned the Southampton Study, has mandated that food manufacturers across European Union to put a health warning “may have an adverse effect on activity and attention in children” on food and drink products containing synthetic dyes starting from 20 July 2010 (Harris 2011; U.K. Food Standards Agency 2010).

Though not mandated, food manufacturers outside the EU have shown increasing interest toward replacing synthetic dyes with more natural alternatives. General Mills, one of the biggest food companies in the world with annual sales of more than fifteen billion dollars, has long stated the need for color innovation to replace the synthetic color Red 40 (Allura red) on its company website (General Mills 2011). Warner-Jenkinson Co., which is a major food color manufacturer, also acknowledges the consumer demand for natural food coloring (Burrows 2009).

Natural colorants have certain limitations such as being dull in color, sensitive to pH, and low color strength, thus requiring higher concentrations for food application.

Certain natural colorants such as anthocyanins are susceptible to degradation especially with light and heat, their use is limited to low pH food; also they have a low extraction yield. Furthermore, since they are extracted from plant products such as seeds, skin, or roots, other problems include seasonal dependency, off-flavor, and contamination of insecticides, herbicides, and bacteria can occur (Wrolstad 2004; Griffiths 2005; Castañeda-Ovando and others 2009). Exotic sources of natural colorant such as carmine from cochineal insects can be subjected to price variables and public acceptance (Nachay 2009).

Exploring alternative natural colorants with different origins and properties is needed to help food manufacturers meet consumer demands for natural products. Sappanwood or *Caesalpinia sappan* L. is a redwood grown in many parts of Asia. Its history of use as a red colorant for beverages dates back to hundreds of years (Detanand 1975). However, the information on color properties for the *C. sappan* pigments is currently very limited. Therefore, this study will evaluate the potential of *C. sappan* water extract by determining the chromaticity and stability of its pigments, and other benefits such as its antioxidant and antimicrobial activities, and preliminary toxicity.

Hypothesis

The water extract of *C. sappan* heartwood can be developed into a color additive. As it is of plant origin, the pigments obtained may be sensitive to pH, light, heat, and oxygen. The extract may also contain phytochemicals which not only yield red colors, but also have other functional properties such as antimicrobial and antioxidant activities, with low toxicity.

Specific Objectives

1. To review previous published studies and literature on the topics of the *C. sappan* and its potential benefits.
2. To examine color properties of *C. sappan* water extracts.
3. To evaluate antioxidant activities of *C. sappan* water extracts.
4. To obtain preliminary evaluation of toxicity of the *C. sappan* water extracts in terms of mutagenicity.
5. To evaluate antimicrobial activities of the *C. sappan* water extracts toward food spoilage bacteria.
6. To understand the stability of pigments from *C. sappan* water extracts to pH, light, and heat.

CHAPTER 2 LITERATURE REVIEW

Caesalpinia Sappan

Caesalpinia sappan is known as East Indian red wood or sappanwood. Although the *C. sappan* is considered a native plant of India, Malaya (Malay Peninsula) and Sri Lanka, it has been found in many other parts of Asia such as China, Indonesia, Viet Nam, and Thailand. The heartwood of sappanwood was historically valued for both its red pigments and for its medicinal properties throughout Asia. The medicinal properties of sappanwood were documented in the Indian Ayurveda (Kennedy and others 2004; Kennedy and others 2008), Chinese traditional herbs (Efferth and others 2008), Japanese pharmacopoeia (Na and others 2001), medicinal Plants in Vietnam (WHO, Institute of Materia Medica 1990) and medicinal Thai plants (Det-anand 1975). Documented pharmacological benefits include activating blood circulation as well as antitumor, antimicrobial, and immunostimulant properties. Sappanwood was listed as a natural source of red dye in several scientific references (Defilipps 1998; Vankar 2000; Ferreira and others 2004; Whitney and others 2006; Petroviciu and others 2010; Rosenberg 2008; Cooksey 2009). However, the chromaticity of the extract along with the CIE color measurements has not yet been reported.

History of Consumption

Sappanwood water extract has been used as an ingredient of beverages to quench thirst in Thailand (Det-anand 1975) and in Indonesia (Roesnadi and others 1977; Batubara and others 2009). In Thailand, a red concoction known as “Ya U-tai” supposedly contains sappanwood extract. This concoction has been marketed in Thailand under a trade name “Utairasamee” for more than 80 years (Utaitip 2011).

Typically, a few drops of “Ya U-tai” are added to a glass of water prior to drinking or consumption. In Indonesia, the sappanwood extract is incorporated in several beverages such as “Bir Pletok”, “Wedang uwuh”, “Wedang Secang” (Secang drink), and “Jamu”. Bir Pletok originated from the Batavian region of Indonesia, and it has a distinctive pink color derived from *C. sappan* heartwood extract (Batubara and others 2010; Hulupi 2003), as well as other ingredients such as ginger and lemongrass. Sappanwood is added to Wedang uwuh drink mainly to impart a red color (Susanto 2010), while other drinks derived from sappanwood are simply called Wedang Secang or Secang drink (Wedang means drink, Secang means sappanwood in Indonesian language). Many recipes for the drinks can be found on World Wide Web search engines such as Google.com. On the other hand, Jamu is a traditional medicinal drink in Indonesia for health maintenance purposes (Roesnadi and others 1977), and of the many commercially available Jamus, those containing sappanwood include post partum herbs, which are claimed to help relieve stomach pain after birthing, stimulating blood circulation, and promoting health. Another Jamu called tonic tea was promoted as being able to slow the aging process, improve blood circulation, and increase energy.

The other well-known species of the genus *Caesalpinia* is *C. echinata* and it is native to South America. *C. echinata* is named brazilwood because it also contains red pigment brazilin and the country where brazilwood was discovered was later named “Brazil” by the Portuguese. Since discovering the red dye from brazilwood (*C. echinata*) in 1500, it has replaced sappanwood (*C. sappan*) as a cheaper source of the red dye. In the middle ages, the heartwood of sappanwood was imported into Europe as a source of red dye (brazilin), which was used to dye fabric and as an ink (Irish and Irish

1996; Armstrong 1992; Kennedy and others 2004; Berger and Sicker 2009). The primary focus of the current research work is on *Caesalpinia sappan* or brazilin extracted from *C. sappan* heartwood.

Although the sappanwood (*C. sappan*) extracts have been used in many cultures, limited information on its health effects and epidemiological data are available. The objective of this review is to examine the current literature from 1975 to August 2011 with the goal of covering the medicinal uses as well as potential benefits (antioxidative, antimicrobial), and detrimental effects of the extract. Furthermore, the review will summarize current knowledge related to the chemical composition and current use of the extracts as a food additive with specific focus on the *C. sappan* heartwood.

Color Properties of Sappanwood Extracts

Berger and Sicker (2009) proposed that two chromophores exist on a molecule of brazilin (ring A and ring B). When those two rings are separated, the molecule does not absorb much light in the visible region; hence, brazilin appears pale yellow or colorless. When the molecule is oxidized, ring B which has a quinone structure, becomes conjugated with ring A, which absorbs light in the higher visible wavelength (Figure 2-1). The oxidized brazilin is called brazilein. It is the quinone structure attached to the aromatic ring of brazilein that acts as a chromophore. A shift in absorbance maxima (bathochromic shift) of brazilein in ethanol from 445 nm to 525 nm occurred when 0.1 N NaOH was added to the solution. Since the color in NaOH solution is red, this color transition is called a red shift. The researchers proposed that the changes in molecular structure of the brazilein molecule were due to deprotonation at the OH group of C3 as illustrated in Figure 2-2.

Because brazilein is a natural dye, it is susceptible to photooxidation, which affects its chromophore. For the purpose of improving speed for fabric dyeing and minimizing photooxidation, technology such as complexation with transition metals was used. The transition metal or mordants used with the red dye from *C. sappan* was alum ($KAl(SO_4)_2 \cdot 12H_2O$). Historically, not only mordant helps stabilize the color of fabric, it also helps to make the dye water insoluble. For fabric, less water solubility is desirable since it improves color fastness, thus better preserving its original color (Vankar 2000).

Wongsooksin and others (2008) investigated the properties of mordant of sappanwood extract and alum, which has been used to dye silk to red color in Thailand. Alum yields Al(III) ion the complex, and the researchers proposed a molecular structure of the complex as $Al(brazilein)_2$; they reported the absorption spectra of brazilein as well as the Al-brazilein complex. In the experiment, the pH of brazilein solution was controlled at 4.5 without the pH adjustment for the mordant dye solution. The difference that was found in the three major absorption bands suggested that the peak with a maximum absorption at 446 nm was associated with the B-ring on brazilein, and the wavelength maxima at 276 nm was associated with the A-ring (Figure 2-1). Adding higher concentrations of alum decreases the absorbance at 446 nm while it increases absorbance at 509 nm.

Antioxidant Activities of Sappanwood Extracts

High antioxidant activities of sappanwood extracts from different geographical locations and from using various solvents including methanol, water, petroleum ether, chloroform, and ethyl acetate (Sasaki and others 2007; Pan and others 2004; Badami and others 2003; Safitri and others 2003; Na and others 2001) is summarized in Table 2-1.

Using DPPH radical scavenging, Na and others (2001) reported that methanol extraction of sappanwood (100 µg/ml) had 80% higher antioxidative activity (in lipid peroxidation) than that of a control (α -tocopherol) which ranged from 60 to 80%; they also tested methanol extract of 138 other Korean herbal medicines. Yingming and others (2004) compared the protective effect of ethyl acetate extracts of sappanwood with BHT against lipid oxidation in peanut oil kept at 60 °C for 20 days. Results showed that 0.20% (w/w) of the dried sappanwood extract is more efficient than BHT. Badami and others (2003) examined the antioxidative activity of sappanwood extracts from India by both *in vivo* (Nitric oxide) and *in vitro* assay (DPPH method). The authors used various solvents to extract the *C. sappan* heartwood, including petroleum ether, chloroform, ethyl acetate, methanol, methanol: water (50:50), and water. The high antioxidant activities in terms of low IC₅₀ were reported for ethyl acetate extract, methanol extract, methanol: water extract, and water extract. Petroleum ether extract and chloroform extract, however, showed no antioxidative properties. Palasuwan and others (2005) tested antioxidative activity of water extract of *C. sappan* heartwood from Thailand by ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6sulphonic acid) radical cation decolorization assay. The water extract prepared by boiling in water for 10 minutes (1:20 w/v) showed total antioxidant activity of 9.53 mM/g.

The compounds responsible for the antioxidative properties in the aqueous extracts of sappanwood were isolated by Safitri and Ratu (2003) and were identified as 1',4'-dihydro-spiro[benzofuran-3(2H),3'-[3H-2]benzopyran]-1',6',6',7'-tetrol (Figure 2-3) and 3-[[4,5-dihydroxy-2(hydroxymethyl) phenyl]-methyl]-2,3-dihydro-3,6-benzofurandiol (Figure 2-4). Both compounds are considered flavonoids.

Table 2-1. Summary of antioxidant activities and benefits of various *C. sappan* heartwood extracts in foods

Author	Country of origin	Purpose	Extraction Method	Application medium	Effective concentration	Antioxidant activities	Benefits in food products
Han and Rhee 2005	Republic of Korea	n/a	Extracted 3x w/ 95% MeOH (1:4) by stirring on a hot plate at 40°C for 3 h, filtered. All filtrate combined and dried under vac. Stored dried extract in desiccator 12 h, then in air-tight glass jar at -20°C.	Goat meat and beef patties	0.01% or 0.05% (w/w)	Not quantified	Antioxidants, retain red color in beef patties
Palasuwan and others 2005	Thailand	Screening of Thai medicinal plants for antioxidant activities	Boiled in water at a ratio of 1:20 (w/v) for 10 min. Cooled, then centrifuged at 2,500 rpm for 15 minutes, then filtered.	n/a	1:20 of extract dilution	9.53 mM/g using radical cation decolorization assay	n/a
Saraya and others 2009	Thailand	Antioxidant, antimicrobial, and toxicity of sappan water extract	Freeze-dried, drum dry, and water bath dried of water extraction	Thai chili paste	2, 4, 8 times of the selected max. bacteria MIC	DPPH: IC50 at 63.4mcg/ml	After 3 mos., Total Aerobic Counts were 41.9%, 40.9%, and 44.6%, respectively
Sasaki and others 2007	Japan	Antioxidant and anti-inflammatory tests on brazilin, brazilein, sappanchalcone, protosappanins A, B and C	Isolated brazilin, brazilein, sappanchalcone, protosappanin A, protosappanin B, and protosappanin C from methanolic extraction of <i>C. sappan</i>	n/a	Brazilin completely suppress iNOS gene expression at 100 µM	Brazilin, brazilein and sappanchalcone significantly inhibit lipopolysaccharide (LPS)-induced NO production by J774.1 cell line	n/a

Table 2-1. Continued

Author	Country of origin	Purpose	Extraction Method	Application medium	Effective concentration	Antioxidant activities	Benefits in food products
Badami and others 2003	India	In vitro and in vivo antioxidant activity of extracts of <i>C. sappan</i> heartwood	10g heartwood powder + 400 DI water, reflux 2 h, cooled, filtered, adjusted final vol. to 400 mL	HPTLC	chloroform: acetone: glacial acetic acid: water = 10:3:2:2	DPPH, IC ₅₀ = 6.94 ± 0.96 mcg/mL	n/a
			10g heartwood powder + 400 DI water, microwaved at 540 W for 20 min			DPPH, IC ₅₀ = 7.4 ± 0.47 mcg/mL	n/a
Yingming and others 2004	China	Screening of antioxidant properties as PV in peanut oil against BHT	50g chips in 500 mL, Soxhlet for 24 h Use rotary evaporator to dry at 60 degree C. Then vacuum-dried at 30 °C, 0.07 Mpa.	Peanuts oil	0.20% w/w of extract	PV = 24.08, compared to PV with BHT, which was 26.52	n/a
Safitri and others 2003	Indonesia	Antioxidant activities of isolated compounds from sappanwood extract	Methanol fraction of the intitial water extract was further separated by column chromatography with chloroform, ethyl acetate, methanol, and water. Two aromatic compounds were obtained.	n/a	IC ₅₀ of less than 300 µg/ml for xanthine oxidase inhibition, superoxide anion radical scavenging, and hydroxyl radical scavenging	Inhibitory effect on xanthine oxidase activity, scavenging effect on superoxide anion and hydroxyl radicals. Both compounds were more effective than α-tocopherol, β-carotene, and BHT when IC ₅₀ values were compared.	n/a

Antimicrobial and Probiotic Activities of Sappanwood Extracts

Antimicrobial activities of *C. sappan* heartwood extracts and their isolated compounds (active compounds) have been reported by many researchers. Kim and others (2004) examined the antimicrobial activity of *C. sappan* heartwood extracts using various solvents (chloroform, n-butanol, methanol, and hot water) against the methicillin-sensitive strain of *Staphylococcus aureus* (MRSA). They found that methanolic extract exhibited the biggest inhibition zone on agar plates, followed by the extracts from n-butanol, chloroform, and aqueous extracts, respectively. Another study revealed that brazilin was the responsible compound in a methanol extraction of *C. sappan* heartwood that inhibits the growth of MRSA and vancomycin-resistant enterococci (VRE). This same methanolic extract also showed inhibitory activity against *Streptococcus mutans* and *Prevotella intermedia* which were responsible for dental carries (Xu and Lee 2004).

Lim and others (2007) also evaluated *C. sappan* extracts as a probiotic factor to inhibit harmful bacteria in the human gut and thereby contribute to the growth of desirable gut microflora (or probiotic bacteria). The researchers took the methanol extract of the *C. sappan* heartwood and further extracted it with methanol, hexane, chloroform, ethyl acetate, butanol, and water. Each of the secondary extracted fractions were then tested for growth inhibitory responses against both desirable probiotics, i.e. *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Lactobacillus casei*, and harmful bacteria, i.e. *Clostridium perfringens* and *Escherichia coli*. The methanol fraction shows the most preferable activities as it supported the growth of desirable bacteria while suppressed those of the undesirable ones. The methanolic fraction was further separated, and the active compound was identified as 5-hydroxy-1,4-naphthoquinone.

The antimicrobial and probiotic activities of *C. sappan* heartwood extracts shown in these studies revealed its future potential as an antimicrobial agent for medicinal use.

Medicinal Benefits of Sappanwood Extracts

Medicinal benefits of *C. sappan* include anti-acne (Batubara and others 2010), anti-inflammatory (Washiyama and others 2009), anti-allergenicity (Yodsaoue and others 2009), anti-tumorigenesis (Efferth and others 2008), analgesic (Hemalatha and others 2007), protection against oxidation-induced cell injury (Bae and others 2005; Choi and others 1997; Sasaki and others 2007; Shen and others 2007), cardioprotective effects (Zhao and others 2006), diabetes prevention (Yang and others 2000), and apoptosis induction (Ye and others 2006). Many of these studies were conducted to identify active compounds in *C. sappan* heartwood purified extracts. The potential active compounds were brazilin, brazilein, hematoxylin, and sappanchalcone.

It was reported that a methanol extraction of *C. sappan* heartwood yields Brazilin, which is also a natural red dye. Batubara and others (2010) isolated brazilin from a methanol extract of *C. Sappan* and evaluated its antimicrobial, antioxidative, and lipase inhibition activities. They found that brazilin (Figure 2-1) as well as other *C. sappan* compounds such as protosappanin A (Figure 2-5) were effective as antimicrobial agents against *Propionibacterium acnes* bacteria compared to the common acne medicine tetracycline. The authors concluded that brazilin has the potential to be an anti-acne agent.

A group of researchers from Japan studied seven compounds isolated from a methanolic extraction of sappanwood (Washiyama and others 2009). Each compound was tested by an *in vitro* assay for inhibition of chemical mediators of inflammation, using the J774.1 cell line and reported results as IC₅₀ values (concentration of the

isolated compounds effective to reduce these undesired mediators by 50%). The mediators of inflammation induced the measurement of NO and PGE2 inhibition, as well as the ability to suppress mRNA expression of TNF- α , IL-6, COX-2, and iNOS. This study also included an *in vivo* assay for anti-inflammatory effect on carrageenan-induced mouse paw edema as % paw edema. The NO synthesized through iNOS gene induces tissue injury. The PGE2 synthesized through the COX-2 gene accelerates inflammation. The TNF- α is an early stage inflammation mediator, inducing the synthesis of IL-6 or serotonin, resulting in the activation of T cells and inflammation related cells. Thus, the compounds that suppress iNOS, COX-2, TNF- α , and IL-6 gene expression are expected to show an anti-inflammatory effect. From the test mentioned, it was found that brazilin inhibited NO production, but almost no inhibition in PGE2. As for sappanchalcone, protosappanin, and protosappanin E, they inhibited both NO and PGE2 production, and suppressed TNF- α , IL-6, COX-2, and iNOS mRNA expression. When tested *in vivo* in mice, a crude methanolic extract showed stronger activity than brazilin. Thus, the methanolic extraction of sappanwood contained active compounds other than brazilin for the inhibitory effect on mouse paw edema. Structures of the brazilin, protosappanin A, B, C, D, E, and sappanchalcone, are shown in Figures 2-1, 2-5, 2-6, 2-7, 2-8, and 2-9.

In another study, *C. sappan* heartwood and roots from Thailand were extracted with dichloromethane and tested for anti-allergic activity (Yodsaoue and others 2009). Allergy or hypersensitivity type I is an immune dysfunction. An allergic reaction is an immunoglobulin E (IgE)-mediated immune response, resulting in a histamine secretion from mast cells and blood basophils. When granules in mast cells or basophils

degranulate, an enzyme β -hexosaminidase is more convenient and has been used instead of detection of histamine for an anti-allergy assay. The inhibitory effects were performed on the RBL-2H3 cells and it was found that sappanchalcone possessed the most potent antiallergic activity against antigen-induced cell degranulation. The sappanchalcone has an inhibitory concentration (IC_{50}) value as low as 7.6 μ M, which is 6-fold higher than that of ketotifen fumarate, a clinical drug with IC_{50} of 47.5 μ M. The other potent compound identified was the 3-deoxysappanchalcone, which has an IC_{50} of 15.3 μ M.

Efferth and others (2008) systematically screened the bioactivity of natural products that were derived from medicinal plants used in traditional Chinese medicine. Extracts from 76 medicinal plants were evaluated for their abilities to inhibit the growth of tumor cells. Of the 253 extracts, 23 extracts from 18 plant species that could reduce cell growth of human CCRF-CEM leukemia cells below 20% of untreated cells (controls) at a test concentration of 10 μ g/ml, and *C. sappan* was among the 18 species identified as having this potential. In the same year, another group of researchers studied the herb medicine formula “Yang Wei Kang Liu” (YWKLF) containing sappanwood on the survival rate of late stage (stage IV) gastric cancer patients, and the apoptosis of human gastric cancer cell line (Li and others 2008). They reported that the combination of chemotherapy with oral administration of YWKLF significantly increased the survival of the late stage gastric cancer patients. In addition to sappanwood extract, the formula contains five more components including Panax ginseng.

Another study on the analgesic activity of an ethanol extract of sappanwood, with sappanwood from India was reported on Swiss albino mice (Hemalatha and others

2007). The ethanol extract was further fractionated with petroleum ether, diethyl ether, and ethyl acetate for a total of 4 extracts in this study. The test was carried out on Swiss albino mice, which orally received either the extracts or the control for 30 minutes before being induced into pain. The 0.6% v/v solution of acetic acid were introduced intraperitoneally (i.p.), and resulted in pain which was represented by the writhing of the mice. The number of writhing in 20 minutes was recorded, and it was found that both concentrations of 200 and 400 mg/ml significantly inhibited acetic acid-induced writhing compared to the effects of aspirin.

In the period of 2005-2008, many studies were published on the protective activity of sappanwood and its extracts on oxidation-induced injury to cells. Sasaki and others (2007) reported constituents, including brazilin, brazilein, sappanchalcone, and 3 types of protosappanin on this oxidation, and found that brazilin and sappanchalcone showed higher inhibitions of NO production than other compounds. Choi and others (2007) demonstrated the protective effects of brazilin and sappanwood extracts against tert-butylhydroperoxide-induced cell death in House Ear Institute-Organ of Corti1 cells. Similar studies on sappanwood and its extracts were also published in other journals (Choi and Kim 2008; Bae and others 2005; Palasuwan and others 2005).

Cardioprotective effects of sappanwood extract were reported. Zhao and others (2006) found brazilein to have cardioactive effects (increased contractility) on hearts isolated from guinea pigs. The authors suggested that brazilein had the potential to be developed into inotropic drugs to modulate the heart condition (strength of heart beats). Xie and others (2000) reported the evaluation of methanolic crude extract of *C. sappan* along with its isolated compounds Brazilin and hematoxylin on a rat's aortic ring to

evaluate their relaxant effects. All three extracts were found to be effective in relaxing the aortic ring which could be supporting evidence to the folk medicine claim that sappanwood promotes blood circulation.

Brazilin was also tested for its ability to enhance cellular immune responses in type I diabetic mice. It was found that brazilin increased the responsiveness of immune cells, and the authors suggested that brazilin might have the potential to prevent type I diabetes that is induced by bacterial or fungal infections (Yang and others 2000).

The oxidized form of brazilin is called brazilein, this compound also has been reported to have some pharmacologic potential such as increasing immunity, improving cardiovascular health, acting as an anti-inflammatory, as well as being a relaxant. Ye and others (2006) reported that methanolic extract of *C. sappan* heartwood has immunocompetence effects including the property of inducing apoptosis of mice spleen lymphocytes and the ability to inhibit lymphocyte proliferation. The authors suggested that the active compound in this extract was brazilein. Brazilein was found to have anti-inflammatory effect and thus, may provide some protection to the brain against ischemia/reperfusion injury (Shen and others 2007). In a study on rat thoracic aorta, effectiveness of 100 μ M of brazilein was reported as 116% of 20 mM caffeine in inducing the contraction (Shen and others 2008).

Chemical Compounds Isolated from *C. sappan* Heartwood

Chemical compounds in sappanwood have been identified and reported by many authors. Techniques used for separation and identification include solvent extraction, X-Ray, NMR, and MS.

A group of Japanese researchers led by Toshihiro Nohara published several reports in the 1980's. Saitoh and others (1986) examined the methanol extraction of *C.*

sappan heartwood for antihypercholesteremic activity and identified 6 aromatic compounds including 3-benzylchroman derivatives, proposed to be precursors of brazilin biosynthesis. The complete molecular structures of these compounds were not determined. Shimokawa and others (1985) used spectroscopy to identify compounds in sappanwood and they reported two aromatic compounds which they named caesalpins J and P. In another study, Miyahara and others (1986) used X-ray analysis to identify an interesting compound from a methanolic extract, and which was called “caesalpin J”. It was thought to be derived from the 3-benzylchroman derivatives identified previously by Saitoh and others (1986). Fuke and others (1985) detected the responsible compounds for hypercholesteremia activity of *C. sappan* heartwood extract and identified 2 aromatic compounds. They simply called them compound 2 and 3, with compound 1 being brazilin.

Nagai and others (1984, 1986) reported the isolation of protosappanins A (I, II, and III), B, and C (Figure 2-5 and 2-6). Protosappanin A (I) was also identified by Batubara and others (2010) and Washiyama and others (2009). Protosappanin C was also identified by Washiyama and others (2009)

Namikoshi and others (1987) worked with several other researchers to identify compounds from sappanwood extracts. They published 5 research articles in 1987 and identified a total of 7 new compounds from sappanwood along with other 15 known compounds that were separated for the first time. This group reported structures and categorized a novel class of homoisoflavonoids which contain a 3,4-dihydroxy-homoisoflavan structure as illustrated in Figures 2-10.

Additional reports on the separation and identification of sappanwood extract were mostly after 2000. Yang and others (2002) used ethanol extraction of sappanwood to isolate a new compound. The ethanol extraction that is soluble in ethyl acetate was further separated by repeated column chromatography over silica gel. The compound isolated was named lactone brazillide A (Figure 2-11). The molecule is highly oxygenated and is suspected to be a product of autoxidation for brazilin.

Zhao and others (2008) worked on an ethanolic extract of sappanwood and reported a new homoisoflavan, which has a brown color. The compound was identified by spectroscopic method as 7,3',4'-trihydroxy-3-benzyl-2H-chromene (Figure 2-12).

Fu and others (2008) reported 3 new compounds, together with those previously identified. These compounds are 3-benzylchroman derivatives. According to their report, the phenolic compounds in sappanwood extract are grouped into four subtypes by structure, i.e. brazilin, chalcone, protosappanin, and homisoflavonoid.

Sappanwood Extract as a Food Additive

This section summarizes reports on applications of sappanwood extract as a food additive, benefits include using it as a red colorant, food antioxidant, and food preservative. Indrayan and Guleria (2001) reported the physical appearance and yields of dyes extracted from sappanwood and proposed its use as food colorants. Depending on solvents, i.e. water, methanol, ethanol, aqueous NaOH, colors ranged from orange-red, orange, to red-violet with yields ranging from 0.838% to 5.025% (Guleria and others 1997); however, this study did not identify the active compounds. Another study by Badami and others (2007) examined the effectiveness of a red dye extracted from sappanwood using water and microwave heating. The researchers found that the extraction assisted by microwave heating at 540 watt for 20 minutes increased the yield

of the red dye by almost 14%, but they did not identify the chemical compound(s) responsible for the red color. However, they confirmed that the compounds from microwave-assisted extraction were the same as the traditional hot-water extraction since they both had the same UV spectrum, R_f on TLC, and peak area on HPTLC. In addition to the red color, the microwave-assisted water extract also exhibited the same antioxidant activities as demonstrated using DPPH and nitric oxide methods.

To date, only three articles have been published regarding the application of sappanwood extract in food, one is on antioxidative while the other two are for antimicrobial applications. The antioxidative properties of the ethanolic extract of sappanwood in beef patties were studied by Han and Rhee (2005). Their results show that at a concentration as low as 0.01%, the sappanwood ethanolic extract was effective in preventing lipid oxidation in beef patties. Saraya and others (2009) reported the highest antimicrobial activity in a freeze-dried water extract of sappanwood compared to oven-drying and water bath-drying methods. The freeze-dried extract was effective against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* Typhimurium as determined by the Minimum Inhibitory Concentration (MIC) below a 500 µg/mL. When applying to chili paste, a popular local product, the sappanwood extract showed protection against bacterial growth for up to 6 months. The extract had no major influence on fungal growth. In another study regarding using sappanwood extract for food preservation, the antimicrobial activity was evaluated using an agar-well diffusion method against common spoilage bacteria and yeast in coconut milk. The sappanwood extract showed protection based on zone of inhibition (mm) against several *Bacillus licheniformis* and *Klebsiella pneumoniae* strains, and *Trichosporon mucoides*. The

extract, however, showed no activity against yeast (Phattayakorn and Wanchaitanawong 2009).

Conclusion

Extracts of *C. sappan* heartwood, or the so-called sappanwood, have been used in many cultures. *C. sappan* heartwood extracts from various solvents have been documented to have potential as natural food additives, as a health promoting beverage, and also as medicinal drugs. The food additive potential of these extracts includes use as red food colorants, antioxidants, and antimicrobials to help prolong shelf life and promote food safety in various food products. Brazilin is an important compound as it is responsible for many of the functional properties of the sappanwood extract, as well as being a precursor of the red colorant, brazilein. A few of the many possible active chemical compounds in sappanwood extracts promoting these applications have been identified.



Figure 2-1. Molecular structures of brazilin (left) and brazilein (right).

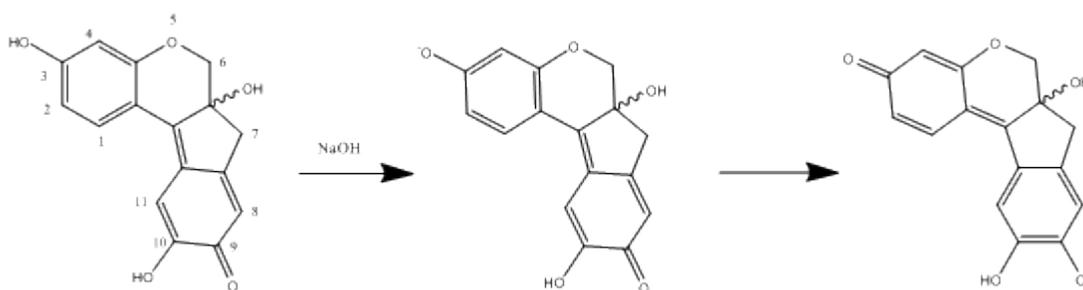


Figure 2-2. Proposed mechanism of red shift of brazilein when 0.1N NaOH was added

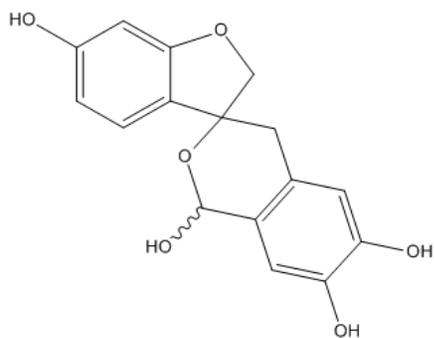


Figure 2-3. 1',4'-dihydro-spiro[benzofuran-3(2H),3'-[3H-2]benzopyran]-1',6',6',7'-tetrol

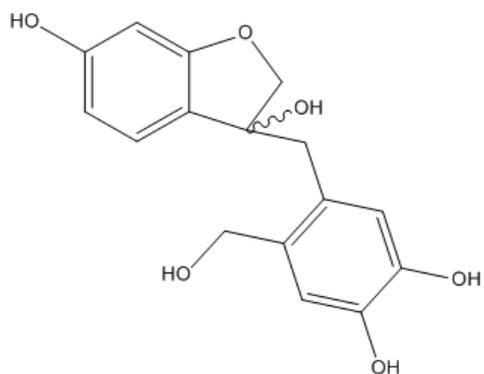
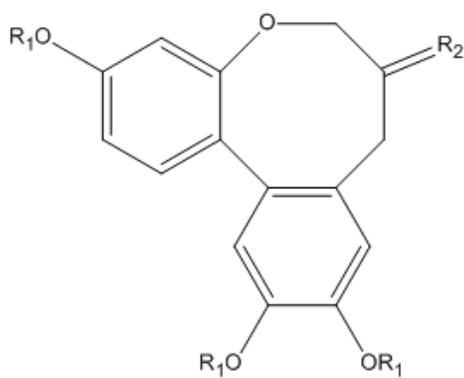
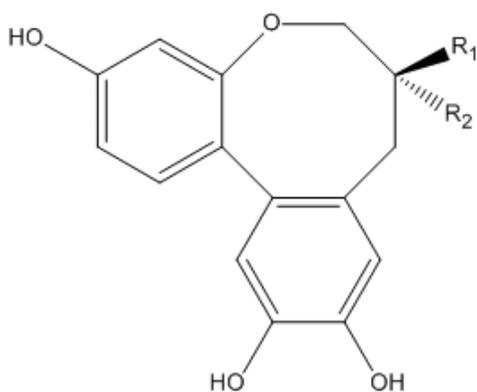


Figure 2-4. 3-[[4,5-dihydroxy-2(hydroxymethyl) phenyl]-methyl]-2,3-dihydro-3,6-benzofurandiol



Protosappan A (I)	$R_1 = H$	$R_2 = O$
Protosappan A (II)	$R_1 = CH_3$	$R_2 = O$
Protosappan A (III)	$R_1 = H$	$R_2 = H, OH$

Figure 2-5. Protosappanin A



Protosappanin B,	$R_1 = CH_2OH$	$R_2 = OH$
Protosappanin C,	$R_1 = CHO$	$R_2 = OH$

Figure 2-6. Protosappanin B and protosappanin C

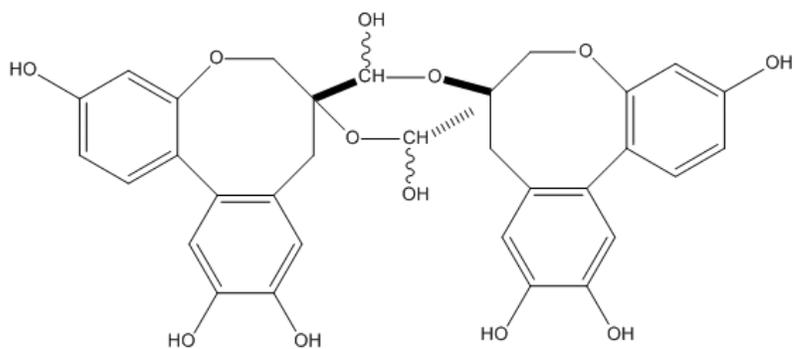


Figure 2-7. Protosappanin D

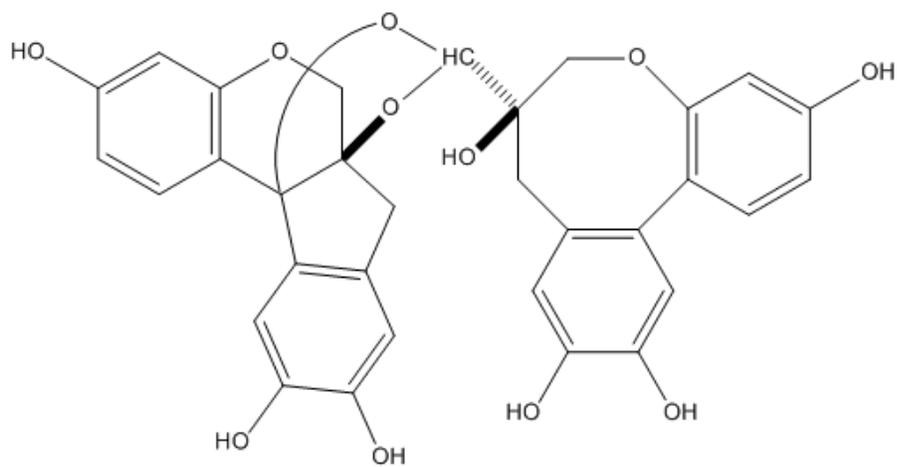


Figure 2-8. Protosappanin E

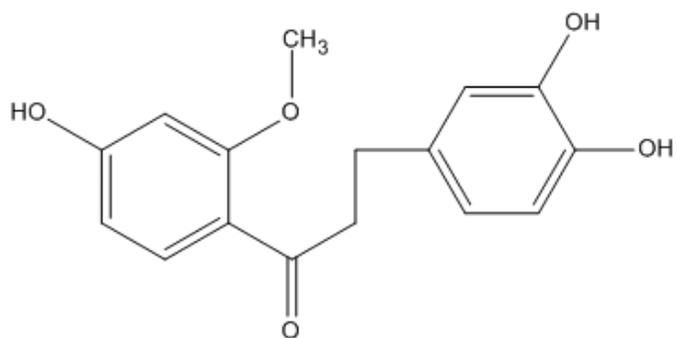


Figure 2-9. Sappanchalcone

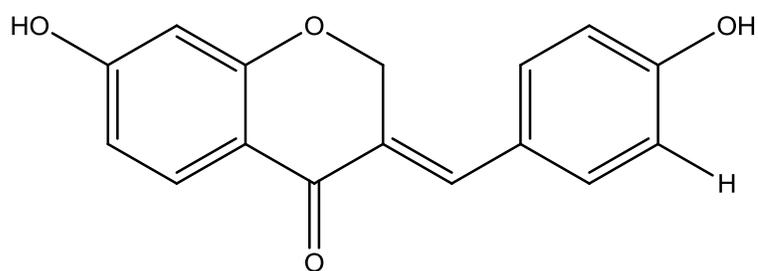


Figure 2-10. 7-hydroxy-3-(4'-hydroxybenzylidene)-chroman-4-one

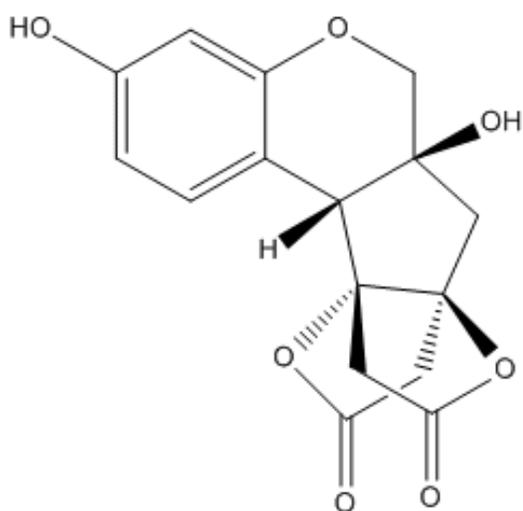


Figure 2-11. Brazilide A

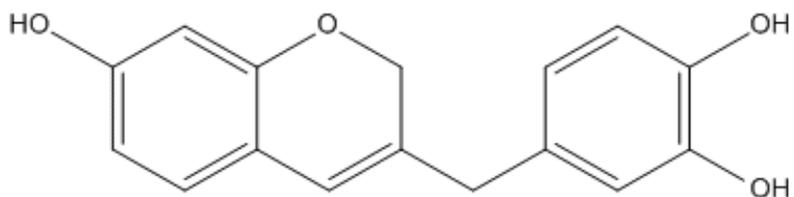


Figure 2-12. 7,3',4'-trihydroxy-3-benzyl-2H-chromene

CHAPTER 3 ANTIOXIDANT CAPACITIES AND COLOR CHARACTERISTICS OF SAPPANWOOD WATER EXTRACTS

Background Overview

The solvation extraction involves a partition process in which chemical compounds in the solid matrix are transferred to the extractant. The process begins with the sorption and capillarity of the solvent causing sample particles to swell, and then any soluble components diffusing from the solids into the liquid. The factors that contribute to efficacy of the solid/liquid extraction process include type and volume of solvent, pH, temperature, particle size of solid, and number of extraction steps (Self 2005).

For a complex sample such as heartwood, extraction at high temperatures such as with a boiling solvent, can be less desirable as it contributes to non-active compounds being forced into solution (Berger and Sicker 2009). Boiling has been used for the extraction of color compounds from sappanwood for food use (Det-anand 1975). This extraction method is considered a solid/liquid extraction type with sappanwood as a solid and hot water as a solvent (liquid).

Color is very important for foods and beverages because it is the first attribute that consumers see when they look at a food product. Color instruments can be used to measure color as opposed to a visual inspection, which can be subjective. Of the many color measurement standards, the CIE $L^*a^*b^*$ system (International Commission on Illumination, Vienna) is widely used in the U.S. food industry (Wrolstad and others 2005; Wallace and Giusti 2008). The CIE established a standard for the light source used in color measurements, such as D_{65} for the average daylight and A for the incandescent.

Color can be represented in terms of hue (color), lightness (brightness or value), and chroma (saturation). Color as we name it is represented by hue, which can be

calculated from redness (a^* value) and yellowness (b^* value) as arctan of b^*/a^* . Hue is represented by the angle from 0° to 360° , with 0° as red, 90° as yellow, 180° as green and 270° as blue. Color can be bright, midtone, or dark and can be represented by L^* value (lightness), with 100 means absolute white and 0 means absolute black. Apart from hue and lightness, another value independent to neither hue or L^* is Chroma or saturation. High chroma value indicates higher saturation of color (Wrolstad and others 2005).

Antioxidant polyphenolic compounds from plants, in particular, fruits and vegetables, have been shown to have these health promoting properties. Antioxidant activities and total phenolic acid content may be used as one of the criteria for screening and selecting the right extractions of sappanwood water extract in addition to measuring color intensity.

Most antioxidant activity measuring methods share the same principle, i.e. generating free radical species, detecting an end point, and then comparing it to the protection of the sample (Rice-Evans and others 1996). Depending on mechanisms, antioxidant activities are varied (Tsao and Deng 2004). Many *in vitro* and *in vivo* models have been used to evaluate the antioxidant activities of polyphenols. The oxygen radical absorption capacity (ORAC) method and Trolox equivalent antioxidant capacity (TEAC) are based on the ability of the antioxidant to neutralize peroxy radical. According to Huang and others (2005), the ORAC mechanism is based on the hydrogen atom transfer (HAT) reaction. The other type of antioxidant capacity measuring assay is based on the ability of antioxidants in to reduce oxidant through electron transfer (ET) reactions. The assays that are based on ET reaction include 2,2-di(4-tert-octylphenyl)-1-

picrylhydrazyl (DPPH) and the total phenols assay by Folin-Ciocalteu reagent. DPPH can be used to evaluate the antioxidant capacity of fruit and vegetable juices or extracts. The ORAC is a common assay for antioxidant activity evaluation of naturally occurring phytochemicals, including botanical extracts (Büyükbalci and El 2008; Fernandez-Panchon and others 2008; Talegawkar and others 2009).

The objectives of this study are to evaluate total polyphenol content, antioxidants activities and chromaticity of sappanwood water extracts, both at boiling (reflux) and at room temperatures (shaking).

Materials and Methods

Raw Materials and Chemicals

Sappanwood was purchased from Jao-Krom-Poe, the oldest and most reputable Thai herb pharmacy in Bangkok, Thailand (Usuparatana 1997). Methanol, Folin-Ciocalteu Reagent, gallic acid, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic dehydrate, and sodium carbonate were purchased from Fisher Scientific Co., (Pittsburgh, PA, U.S.A.). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and fluorescein (free acid) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). 2,2' – Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA, U.S.A.)

Extracts Preparation

Size reduction of the heartwood chips

The pieces of sappan heartwood chips were reduced to 2 mm or less with Wiley Mill as shown in Figure 3-1 and Figure 3-2 (Thomas-Wiley Lab Mill Model 4; Swedesboro, NJ, U.S.A.). Size reduction of the sappanwood chips to sappanwood

powder is illustrated in Figure 3-3. The sappanwood powder was manually shaken in large plastic bag for 10 minutes to homogenize, then transferred to an airtight container and kept at -10 °C in a freezer.

Water extraction of sappanwood

The water extraction of sappanwood was done with a hot water extraction and cold water extraction, and each extraction was performed in triplicate. The first treatment was a reflux of 5.0 g of sappanwood powder in 200 mL of deionized water for 24 hours (RF24). The second treatment was similar to the first one, but the reflux was only 5 hours (RF5).

For the cold water extraction (RT), 3.0 g of sappanwood powder was added to the Erlenmeyer flask with 100 mL of deionized water and shaken for 6 hours on an Excella E1 Platform shaker (New Brunswick Scientific: Edison, NJ, U.S.A.).

Note: because there was no previous study on the cold water extraction of sappanwood, a preliminary study in house was conducted at extraction times of 2, 4, 5, 6, 7 and 8 hours in triplicate. The highest absorbance at 445 nm was observed at 6 hours of extraction by shaking with 3.00 grams of sappanwood in 100 mL of water at room temperature. Additional details of the study can be found in Appendix A.

After extraction, extracts were centrifuged with Beckman Coulter, Allegra X-15R (Brea, CA, U.S.A.) for 5 minutes at 1738 x g (4000 rpm) then filtered through Whatman #1 filter paper. The filtered extracts were freeze-dried in a freeze-dryer (Freeze-dryer 5, Labconco, Kansas City, MO, U.S.A.). The freeze-dried extracts were identified as RF24, RF5, and RT, and were stored at -20 °C until subsequent analyses on total phenolic content, antioxidant capacities, chromaticity, and brazilin/ brazilin were analyzed.

Folin-Ciocalteu Assay for Total Phenolic Content

Freeze-dried extracts (0.01 g) were reconstituted with 1.0 mL of methanol. Gallic acid in methanol at concentrations of 0, 75, 150, 300, 450, and 600 mg/L were used as standards for the calibration curve. Each test tube contained 1 mL of 10% of 2 N Folin-Ciocalteu solution in water, 100 μ L of sample solutions; and 1.0 mL of 15% sodium carbonate. After vortexing, tubes were incubated for 30 minutes at room temperature. A microplate reader (Molecular Devices, SPECTRA max 190, with softmax pro software; Sunnyvale, CA, U.S.A.) was used to read absorbance at 765 nm and compare results against calibration curves of gallic acid. Results are reported as mg/L gallic acid equivalent. The assay was done in triplicate.

Antioxidant Activity

DPPH Radical Scavenging Activity

Sample solutions were prepared by dissolving 0.01 g of freeze-dried extracts in 1 mL of methanol and the final volume was adjusted to 10 mL in volumetric flask with deionized water. Trolox in methanol with concentrations of 0, 200, 400, 600, 800, and 1000 μ M were used as standard solution used as calibration standards. A working solution of DPPH was prepared by diluting DPPH with methanol to the initial absorbance of 0.9 – 1.0 at 515 nm. The assay was performed by adding 950 μ L of DPPH solution and 50 μ L of an aliquot of samples or standards, then vortexed to mix. After incubation at room temperature for 1 hour, absorbance at 515 nm was measured by a microplate reader (Molecular Devices, SPECTRA max 190, with softmax pro software; Sunnyvale, CA, U.S.A.). The assay was done in triplicate and results are reported as μ M of Trolox equivalent.

Oxygen Radical Absorbance Capacity (ORAC)

Solutions of Trolox in phosphate buffer pH 7.4 were prepared at concentrations of 0, 5, 10, 15, 20, and 25 μM to be used as the standard references for the calibration purposes. The freeze-dried sappanwood water extracts were reconstituted with 10% methanol in deionized water, at a dilution of 1:100 and 1:1000. The experiment was performed in a 96-well plate with a microplate reader (SPECTRA max Gemini XPS, Molecular Devices, Sunnyvale, CA, U.S.A.). In each well, 50 μL of Trolox or sample solutions were added; for a blank, phosphate buffer was used in place of the sample. Then, 100 μL of 20 nM Fluorescein was added into each well, and the plate was mixed for 3 minutes and incubated for 7 minutes at 37°C. To start the reaction, 50 μL of 140 mM AAPH in the phosphate buffer was added into each well immediately using an 8-well multi-channel pipet. Fluorescence of each well was measured every minute for 40 minutes, then the differences of areas under the curve of fluorescence and time was calculated by the software (SoftmaxPro5, Molecular Devices; Sunnyvale, CA, U.S.A.) and expressed as μmole Trolox equivalents per mg of the freeze-dried samples.

Chromaticity of Sappanwood Water Extract

Two of the freeze-dried hot water extractions, i.e. the 24-hour reflux (RF24) and 5-hour reflux (RF5) were weighed to 0.10g and diluted with deionized water 100 mL in 100 x 16 mm test tubes. Then, 3.0 mL of the solution was added to 12.0 mL of phosphate buffer pH 2-12. For the cold water extraction (RT), similar preparation was performed but only 0.0400 g of the freeze-dried SPWE was used. This concentration is to accommodate similar absorbance max at pH = 9. Details on the buffer preparation can be found in Table 3-1.

Brazilein Analysis (HPLC-DAD-ESI-MSⁿ Analysis)

Brazilein was analyzed by HPLC using an Agilent 1200 SERIES HPLC system (Agilent, Palo Alto, CA, U.S.A.), equipped with an autosampler/ injector and diode array detector. Compounds were then separated on a SB-C₁₈ Zorbax Stablebond Analytical column (4.6 mm x 250 mm, 5 μM, Agilent Technologies; Rising Sun, MD, U.S.A.). The mobile phases consisted of 0.5% formic acid in H₂O (phase A) and methanol (phase B). The flow rate was 1 mL/min. The UV-vis spectra were scanned from 220 to 650 nm on a diode array detector with detection wavelengths of 445 and 556 nm. A linear gradient was used as follows: 0% to 5% B from 0 to 2 min, 5% to 30% B from 2 to 10 min, 30% to 40% B from 10 to 50 min, 40% to 85% B from 50 to 60 min, 85% to 95% B from 65 to 70 min, and 95% to 0% B from 70 to 75 min; followed by a re-equilibration of the column for 5 min before the next sample analysis. Then an Electrospray ionization mass spectrometry (ESI-MS) interfaced with the HPLC system was performed with an HCT ion trap mass spectrometer (Bruker Daltonics; Billerica, MA, U.S.A.). An electrospray ionization was performed at both positive and negative modes during the same run using a nebulizer 45 psi, drying gas at 11.0 L/min, a dry temperature of 350 °C, and an ion trap for scanning ranging from *m/z* 100.0 to 2200.0.

Statistical Analysis

Each treatment condition was repeated in triplicate. An analysis of variance (ANOVA) and mean separation using Tukey's Studentized (HSD) Range test ($p < 0.05$) were performed in order to evaluate the differences in total phenolic content and antioxidant activities between extraction times, temperatures, and treatments; SAS 9.2 statistical software was used (SAS Institute Inc., Cary, NC, U.S.A.). An example of the SAS output is shown in Appendix B.

Results and Discussion

Total Phenolic Content and Antioxidant Capacities

Total phenolic content as measured by the Folin-Ciocalteu assay from different extraction methods are in Table 3-2, i.e. RF24, RF5, and RT. Total polyphenol content in RF24 was 17.15 GAE/g of SPW (mg gallic acid equivalent per g of sappanwood). The amount is significantly lower than that which resulted from less extraction time (5 hour extraction, RF5), which was 27.92 GAE/g of SPW.

The antioxidant capacity of RF 24, as measured by the DPPH method, was also significantly lower than the antioxidant capacity of RF5. However, the antioxidant capacities determined by the ORAC assays of both samples were not significantly different. This may be because the prolonged heat exposure, such as the 24-hour reflux used with RF24, caused more degradation of certain antioxidative compounds than shorter extraction times. Since the DPPH assay has a different antioxidant principle than the ORAC assay, it is likely that the antioxidative compounds that were active in the hydrogen atom transfer reaction and detected in the ORAC assay were not as sensitive to heat as the antioxidants that based their activity on an electron transfer—a type of transfer which is detected by the DPPH assay (Huang and others 2005).

The cold water extract (RT) was found to have the highest total polyphenol content compared to the hot water extractions (RF5 and RF24). However, the antioxidant capacities of RT, as measured by the DPPH and ORAC assays, were not significantly different from the antioxidant capacities of RF5. A possible reason why the polyphenol content was high when the antioxidant capacity remained the same is because although there is a higher content of heat sensitive polyphenols in RT, these heat sensitive compounds are not antioxidants. Moreover, heat applied during the extraction could

contribute to more of the compounds with no antioxidative properties leaching out to the extract. Since the antioxidant capacities in this experiment were calculated against the dried weight of the extract, it is possible that the weight proportion of the antioxidative compounds compared to the non-active compounds of the heated extracts are less than the weight proportion to the non-heat-treated extracts. Thus, the room temperature extraction may be more desirable in terms of antioxidative properties, as less energy is required for the comparable amount of antioxidative compounds obtained.

The higher antioxidative capacity of sappanwood extracts may be a potential indicator of potential health benefits. An antioxidant activity can serve as another criterion for choosing the method of extraction when using sappanwood as a food additive.

Chromaticity of Sappanwood Water Extract

Brazilein, the compound that is responsible for the red color in sappanwood extract, was detected but not quantified in all samples (RF24, RF5, RT). Then, mass spectrum in positive ion mode of brazilein is shown in Figure 3-4. The spectrum displays the ion $[M+H]^+$ with $m/z = 285$. In addition, further fragmentation also yields $m/z = 175$ which agreed with the fragmentation suggested earlier by Hulme and others (2005).

Freeze-dried sappanwood water extracts were reconstituted by diluting them with an aqueous pH 9 buffer to achieve a chroma value (color intensity) of approximately 30. As shown in Table 3-3, RF24 and RF5 at the same concentration of 0.22 mg/mL contribute to a chroma value of at least 29 while RT at 0.08 mg/mL had a chroma value of more than 34, suggesting that higher amounts of colorant compounds exist in RT compared to RF24 and RF5 at the same pH (pH = 9). The visual colors of the extracts

are shown in Figure 3-5. The pH of 9 was chosen because of the highest a^* , chroma values and highest absorbance at λ_{\max} compared to others, in the range of 2 – 12.

The higher color saturation at lower concentrations of the extract is desirable as less colorant is required to achieve the same result, thus RT is also preferable on this criteria. The result agreed with the earlier analysis, which suggests that higher heat applied during extraction did not contribute to higher antioxidant activity. Thus, RF5 along with RT were chosen for further evaluation.

The color measurement was performed using CIE in term of redness (a^*) and hue angle, and it was found that the redness (a^*) increased sharply when the pH was 6 and higher (Figure 3-6). This trend was applied to all extracts (RF24, RF5, and RT). Since color is three dimensional, the hue angle is taken into consideration as plotted in Figure 3-7. The plot shows that at lower pH levels, extracts were more yellow while solutions at pH levels of 7 and over were red to red-orange. This property of changing to red color at higher pH is the opposite of the well-known natural red pigment, anthocyanins, which change to blue at higher pH levels (Wrolstad 2004). Thus, this property provides a possibility of applying a natural colorant to food products with higher pH values. To date, no other natural colorants provide red color to low acidic food products.

Therefore, sappanwood extracts may offer a possibility to replace synthetic coloring in these food products.

The UV-vis spectra of the RT at pH 2 -12 are shown in Figure 3-8. A shift of spectral band (bathochromic effect) is found at pH 7 and above. The absorbance maxima was found at 539 nm; this is different from the previous report of 525 nm by Berger and Sicker (2009). However the plot of the absorbance at λ_{\max} shows a similar

trend, i.e. higher absorption in the visible region when the pH of the solution is 7 and over (Figure 3-9).

Conclusion

It was found that the hot water extraction at shorter reflux time (RF5) along with the cold water extraction (RT) were chosen for subsequent analysis due to their high antioxidant activity and high color saturation. Bathochromic shift was found on UV-Vis spectra to the absorption maxima of 539 at pH 7 and over, which agreed with the increase in redness and hue angle toward red color when the pH was higher. This property can offer an advantage over the popular natural red colorant, anthocyanins, the application of which is limited to only low acid foods.



Figure 3-1. Wiley Mill (Thomas-Wiley Lab Mill Model 4).

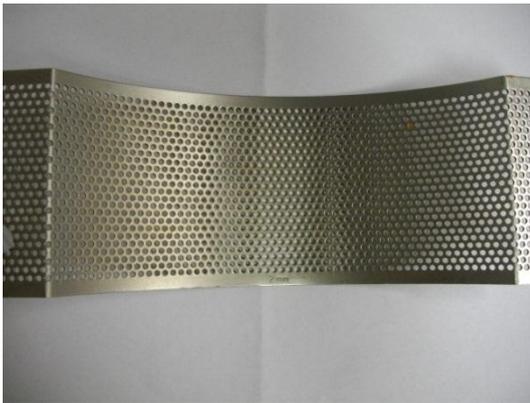


Figure 3-2. Metal screen with 2 mm diameter attached to the Wiley Mill (Thomas-Wiley Lab Mill Model 4).



Figure 3-3. Sappanwood powder ground from sappanwood chips.

Table 3-1 Buffer preparation for chromaticity testing

pH	Volume (mL)									Final volume	Reference
	0.2 M KCl	0.2 M HCl	0.1 M Citric acid	0.2 M dibasic sodium phosphate	0.05 M Disodium hydrogen phosphate	0.2 M monobasic sodium phosphate	0.2 M Glycine	0.1 M NaOH	0.2 M NaOH		
2	50	6.5								100	Haynes 2011
3			39.8	10.2						100	
4			30.7	19.3						100	
5			24.3	25.7						100	
6			17.9	32.1						100	Stoll and Blanchard 2009
7			6.5	43.6						100	
8				94.7		5.3				200	
9							50		8.8	200	
10							50		32	200	
11					50				4.1	100	Haynes 2011
12					50				26.9	100	

Table 3-2. Total polyphenol content and antioxidant activities of sappanwood water extracts (RF5, RF24, RT)

Extraction method	Total Polyphenol (mg GAE/ g of SPW chips)	DPPH (mM Trolox equivalent/g of SPW chips)	ORAC (mcM Trolox/g of SPW chips)
Reflux 5 hrs	27.92 ^b ± 1.68	64.23 ^a ± 6.59	12.46 ^a ± 0.90
Reflux 24 hrs	17.15 ^c ± 1.06	33.88 ^b ± 2.24	13.81 ^a ± 0.74
Shake 6 hrs at room temp.	49.91 ^a ± 0.42	76.12 ^a ± 10.61	14.21 ^a ± 0.48

Values expressed are mean ± SD of three experiments. Data represents the mean of n=9. Values with similar letters within columns are not significantly different (Tukey's HSD, p > 0.05)

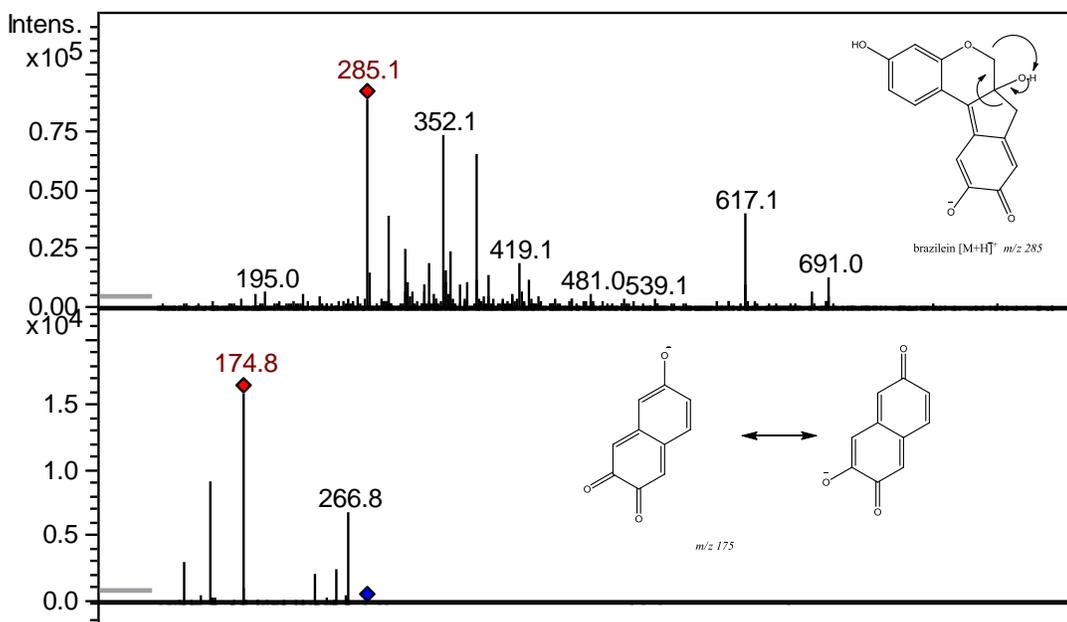


Figure 3-4. Positive ion electrospray product ion mass spectra of brazilein. Brazilein is detected in the extract of a sappanwood sample using HPLC-MS², showing ion with $m/z = 285$ in positive ion mode, further fragmentation also yields $m/z = 175$. Molecular structures of fragments agree with the proposed by Hulme and others (2005).

Table 3-3. Chroma of sappanwood water extracts at pH 9.

Extracts	Concentration (mg/mL)	Chroma at pH = 9
RF5	0.22	30.49
RF24	0.22	29.55
RT	0.08	34.22

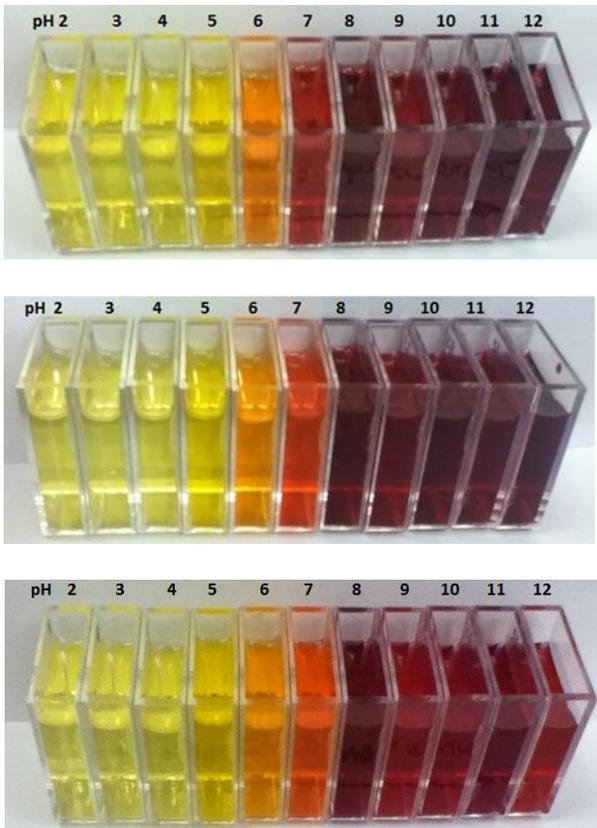


Figure 3-5. Visual colors of aqueous solution of sapanwood extracts (RF5, RF24, RT) at pH 9. Photos from top to bottom are RF5, RF24, and RT.

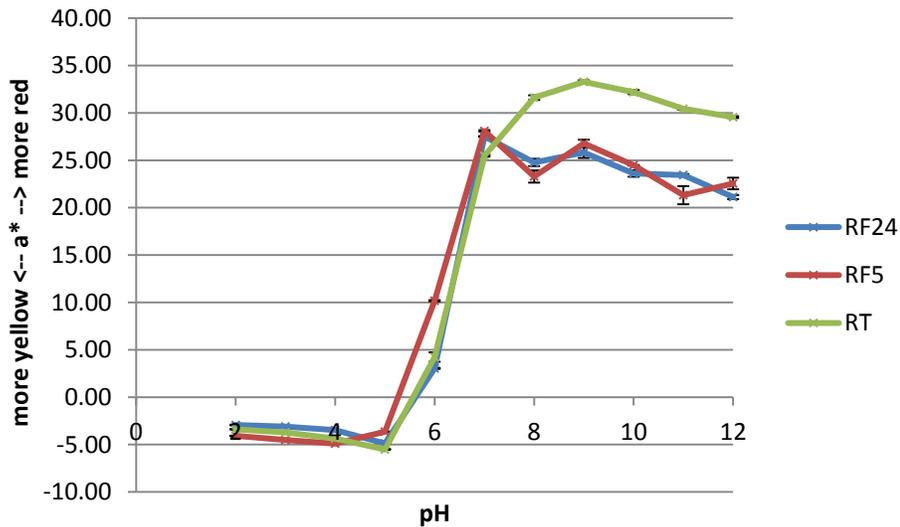


Figure 3-6. Redness of sapanwood water extracts represented by mean a* value (n = 3).

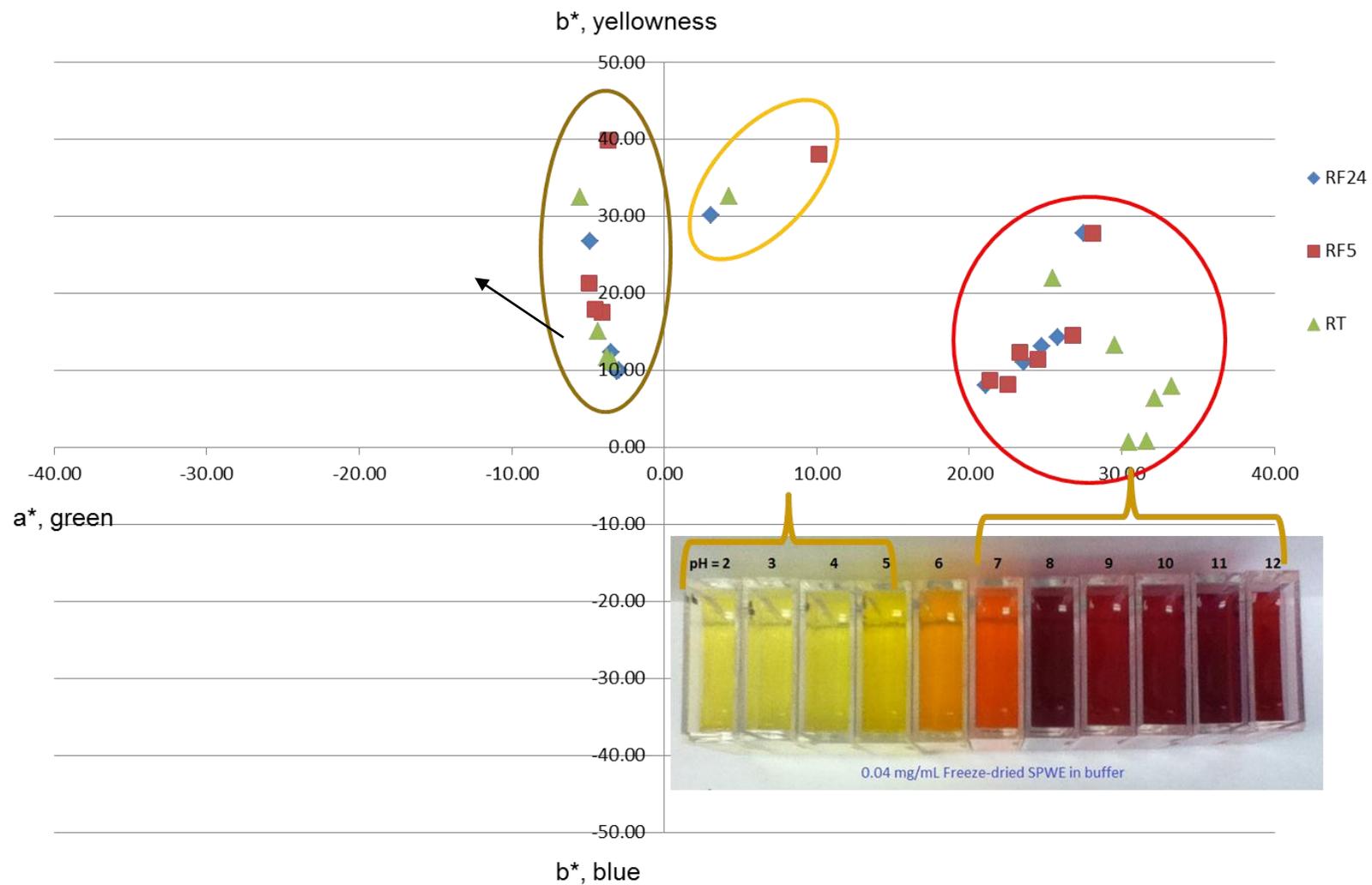


Figure 3-7. Chromaticity as a^* , b^* and hue angles of SPWE. The hue angles of SPWE pH 2-5 were low in a^* but high in b^* (data points are in the brown circle). The hue angles of SPWE pH 7-12 were high in a^* (data points are in the red circle). The hue angles of SPWE at pH 6 are in the yellow circle.

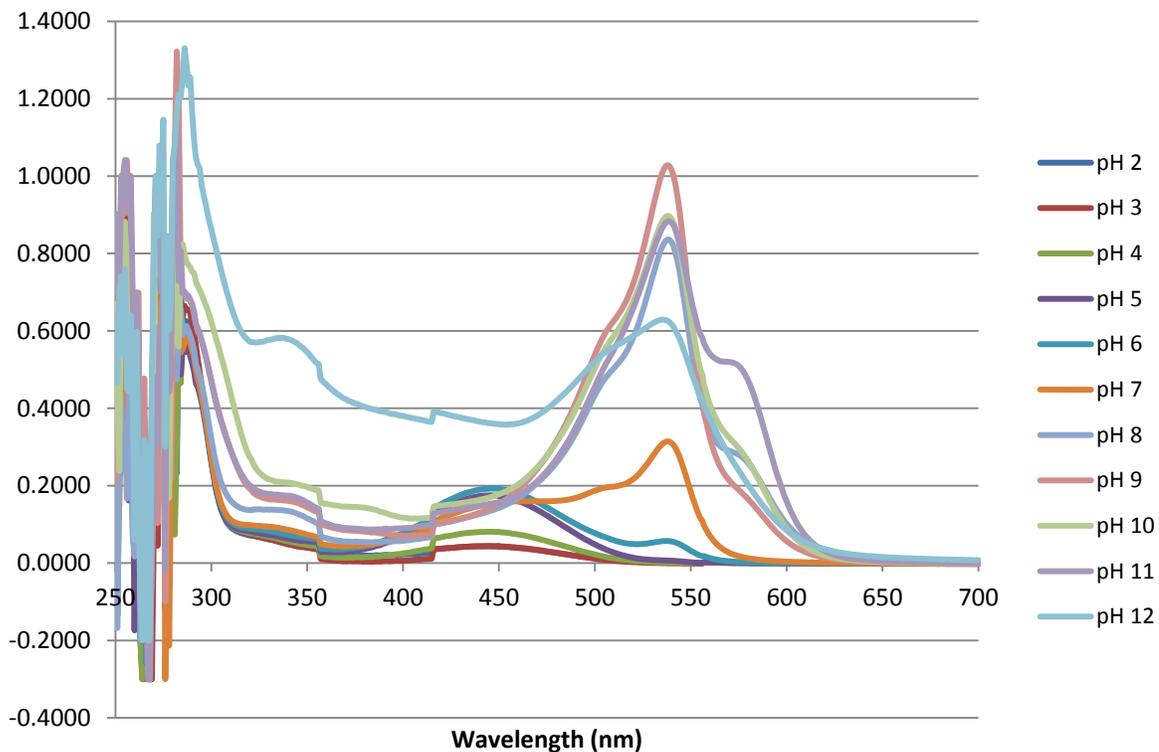


Figure 3-8. UV-vis spectra of sappanwood (RT) extract showing bathochromic shift at pH 7 and higher.

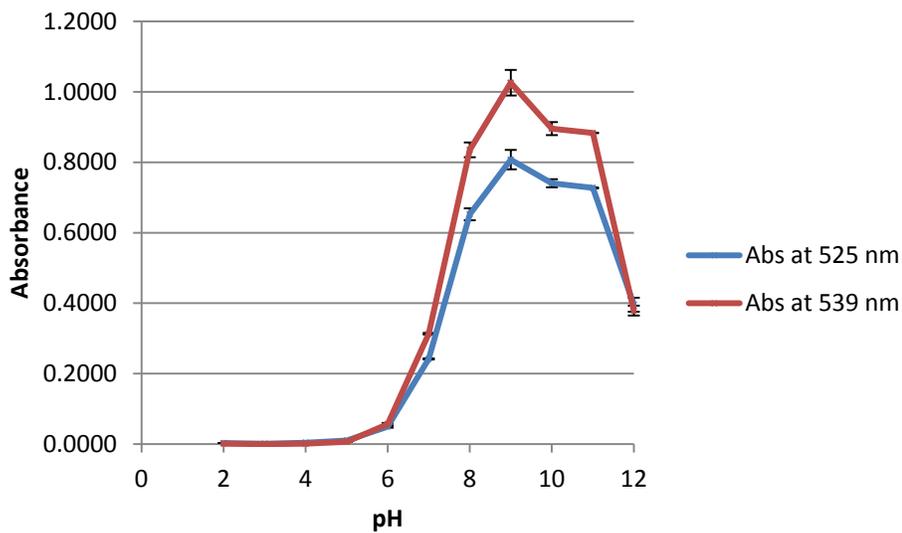


Figure 3-9. Absorbance of sappanwood extracts RT at 525 and 535 nm in buffer solutions pH 2 – 12.

CHAPTER 4 AMES MUTAGENICITY ASSAY AND ANTIMICROBIAL ACTIVITY OF SAPPANWOOD WATER EXTRACTS

Background Overview

In order to assess the potential of developing sappanwood water extract as a food additive, toxicological testing is essential. The Office of Food Additive Safety, U.S. FDA, has recommended several toxicological tests as a guidance for the food industry (Center for Food Safety and Applied Nutrition/ U.S. Department of Health and Human Services 2006). Genetic toxicity tests, such as the bacterial reverse mutation test are common tests recommended for all chemicals, including those with low, intermediate, and high concern levels.

The bacterial reverse mutation test or Ames mutagenicity assay uses different strains of *Salmonella* Typhimurium which were mutated for different sensitivity towards different types of DNA-damaging chemical mutagens. Unlike regular *Salmonella*, these mutants cannot synthesize biotin from histidine. Because biotin is essential for their growth, these *Salmonella* strains used for the Ames test cannot grow in an environment with limited availability of biotin unless they undergo a reverse mutation. The reverse mutation is caused by an exposure to certain mutagens which can restore their ability to synthesize biotin from histidine (Maron and Ames 1983). In the assay, a glucose minimal agar plate with top agar containing a minute amount of histidine was used to create the environment with limited histidine and biotin for the Ames mutagenicity test.

Since sappanwood extract may contain xenobiotics (organic compounds not normally produced in the body), their metabolites too should be analyzed because they may be toxic while the original compound is not. In order to enable the bacterial assay to metabolize the test chemicals via cytochrome P450, the same way as mammals, a

metabolic activation system such as rat liver homogenate or S-9 microsomal fraction containing cytochrome P450 is added to the test system (Maron and Ames 1983; Mortelmans and Zeiger 2000). The S-9 fraction used in the Ames test is a supernatant of liver homogenate of rat centrifuged at 9000 x *g* for 20 minutes, which contains cytochrome P450. The cytochrome P450 is a group of major enzymes involved in the metabolism of xenobiotics in mammals (Guengerich 2007).

For general screening, a tier approach is recommended. In this approach, *S. Typhimurium* strains TA98 and TA 100, both with and without metabolic activation, were recommended to be used in the initial step. Results are represented as mean of revertant colonies per plate \pm standard deviation. While there are many approaches on how to determine mutagenicity, the setting of fold increase as a cut-off point is widely used; and usually when there is a 2 - 3 fold increase (in the numbers of colonies) from negative control, the extract is considered mutagenic (Mortelmans and Zeiger 2000).

The antimicrobial property of sappanwood extract was investigated in several studies. The antimicrobial susceptibility spectrum includes those pathogens that are of clinical importance, and those food spoilage bacteria and fungi. For its medicinal use, (Xu and Lee 2004) studied the principle antibacterial properties of sappanwood, which included the identification of responsible compound, the spectrum of antibacterial activity as well as the mode of action. In this study, the researchers first evaluated different fractions of sappanwood extract against methicillin-resistant *Staphylococcus aureus* (MRSA) 595445 and vancomycin-resistant enterococci (VRE) and found that the methanolic extract was slightly more effective than the aqueous extract. When the methanolic extract was further fractionated with other solvents, such as hexane, ether,

butanol and water, the ether fraction was shown to have the highest antimicrobial activity. This fraction was then further separated with silica gel column chromatography and thin layer silica gel chromatography. The responsible compound in the ether fraction was identified as brasilin (with the same molecular structure as brazilin on this current report) by $^1\text{H-NMR}$ spectrum. When it was tested against fourteen bacterial pathogens, it was found that brazilin had high antimicrobial activity against *Streptococcus pyogenes* (Group A Strep) M1 and *Streptococcus agalactiae* (Group B Strep) A909 with the minimal inhibitory concentration (MIC) as low as $4\ \mu\text{g/mL}$. In the attempt to understand the mechanism of antibacterial action, a radiolabel incorporation assay was used to evaluate the effect of brazilin on DNA and protein synthesis by MRSA using dimethyl sulfoxide (DMSO) as a negative control. The low level of incorporated radiolabel thymidine and serine of brazilin samples showed that the antibacterial effect resulted from the inhibition of DNA and protein synthesis. In addition, brazilin did not show cytotoxicity to Vero cells up to $1\ \text{mg/mL}$. Thus, these researchers concluded that brazilin has the potential to be developed into an antibiotic.

Another group of researchers from Korea (Kim and others 2004) also investigated the antimicrobial activity of sappanwood extract against several strains of MRSA using a disc diffusion method and the researchers also determined the MICs of the extracts. Similar to the study by Xu and Lee (2004), the methanolic extract of sappanwood was found to have higher antimicrobial activity, but active compounds were not identified in the study.

Lim and others (2007) tested the antimicrobial properties of four isolated compounds from sappanwood against intestinal bacteria. The intestinal bacterial

strains tested were pathogens as well as probiotics, i.e. *Bifidobacterium bifidum* ATCC 29521, *Bifidobacterium breve* ATCC 15700, *Clostridium perfringens* ATCC 13124, *Escherichia coli* ATCC 11775, and *Lactobacillus casei* ATCC 27216. It was concluded that the 5-hydroxy-1,4-naphthoquinone (Figure 4-1) had an inhibitory effect selectively on pathogens such as *C. perfringens* and less inhibitory effects on probiotic bacteria.

For food preservation using sappanwood extract, two studies were documented. First, Phattayakorn and Wanchaitanawong (2009) used a disc diffusion method to test 25 Thai herb extracts against 11 strains of coconut milk spoilage microorganisms. They found that methanolic extract of sappanwood had an antimicrobial effect on some bacteria such as *Bacillus licheniformis*, *Klebsiella pneumonia*, and *Trichosporon mucoides*, but they did not identify active antimicrobial compounds.

The second study evaluated the antimicrobial activity of sappanwood extract against *E. coli*, *S. aureus*, *S. Typhimurium* and *C. albicans* (Saraya and others 2009). In this study, the investigators found that freeze-dried powder of sappanwood extract showed higher antimicrobial activity than drum- or water bath drying methods. The freeze-dried powder of the sappanwood was selected for its high antimicrobial activity for further evaluation. When the freeze-dried extract was applied as a preservative to chili paste, a popular Thai food ingredient, there was no inhibitory effect against fungal growth, but the extract did show inhibition against microbial growth (total aerobic plate count) for up to 6 months.

The objectives of this study are to obtain preliminary toxicity evaluation of sappanwood water extracts (both at boiling-RF5, and at room temperature-RT) in term of mutagenicity, as well as antimicrobial activities against spoilage bacteria. The Ames

mutagenicity assay will be used for the toxicity evaluation, and a disc diffusion method will be used to evaluate the antimicrobial activity. The antimicrobial results obtained can help to determine additional functional benefits of sappanwood as a food preservative, while the Ames mutagenicity test can serve as additional evidence of its toxicity.

Materials and Methods

Sappanwood Extracts Preparation

Two sappanwood extracts, RF5 and RT were used in Ames mutagenicity and antimicrobial assays. The extraction method was described in detail earlier in Chapter 3. Briefly, RF5 was a hot water extraction of sappanwood using a reflux apparatus for 5 hours, and RT was an extraction at room temperature by shaking for 6 hours. Each of the extracts was diluted to 500, 5000, 25000, 33330, and 50000 µg/mL, which is the weight of the freeze-dried extract to the volume of sterile water, with sterile deionized water. These concentrations contribute to the concentration per plate of 50 to 5000 µg per plate. The highest concentration and range of concentrations of test chemical per plate were those recommended by Mortelmans and Zeiger (2000).

Chemicals and Microbiological Media

Tryptic soy agar (TSA) was purchased from Bacto (Becton, Dickinson and Company, Sparks, MD, U.S.A.). Glucose was purchased from Difco (Houston, TX, U.S.A.). Agar powder was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Sodium azide, 9-aminoacridine and 2-Aminoanthracene were purchased from MP Biomedicals, LLC (Solon, OH, U.S.A.). Daunomycin, top agar supplemented with 0.6% L-Histidine and D-biotin, Vogel-Bonner E salts (VB salts 50x), post mitochondrial supernatant (S-9), rat (Aprague Dawley) liver, Aroclor 1254-induced, and Moltox NADPH generator (Regensys A and Regensys B) were purchased from Molecular

Toxicology (Boone, NC, U.S.A.). Dulbecco sodium phosphate buffer, 0.1 mM, pH 7.4 was purchased from Sigma (Saint Louis, MO, U.S.A.).

Bacteria Strains

For Ames mutagenicity assay, cultures of *Salmonella* Typhimurium (TA 98 and TA 100) were purchased from Molecular Toxicology, Inc. (Boone, NC, U.S.A.).

For the antimicrobial activity assay by disc diffusion method, cultures of *Alcaligenes faecali* ATCC 8750, *Pseudomonas putida* ATCC 12633, and *Bacillus coagulans* ATCC 7050 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, U.S.A.).

Ames Mutagenicity Assay

The mutagenicity was evaluated on two strains of *Salmonella* Typhimurium (TA 98 and TA 100). The assay was performed as described by Mortelmans (2000) with some modifications. Glucose minimal agar plates (GM agar plates) were prepared by aseptically adding 50 mL of sterile glucose solution (10% w/v), 20 mL sterile VB salt solution and 930 mL of sterile agar at 65 °C, and then mixing well with a magnetic stirrer. It was aseptically poured (25 mL of the agar medium) into each of 100x15 mm petri dishes (Fisher Scientific, Pittsburgh, PA, U.S.A.).

The bacterial cultures were grown by picking 5 colonies of the bacteria from TSA, inoculating in 25 mL of TSB broth in a flask, and then shaking 12 – 16 hours at 110 rpm in a water bath at 37°C (Gyrotory Water Bath Shaker, Model G76, New Brunswick Scientific Co. INC.; Enfield, CT, U.S.A.). The desired density of overnight culture was $1-2 \times 10^9$ colony forming unit (CFU)/mL at an absorbance of 1.2 – 1.4 at 660 nm.

After autoclaving, the top agar (2 mL) with 0.6% histidine and biotin were transferred to a sterile glass tube (100 x 13 mm) and kept at 48 °C in a water bath until use.

For the samples with a metabolic activation system, the S9 mix was added to NADPH generating solution (Regensys A and Regensys B). The system was prepared by rehydrating the S9 with 2.1 mL ice cold sterile deionized water and mixing well. Then 1.6 mL of rehydrated S9 was added to the ice cold Regensys A bottle, mixed well and kept on ice until used. When ready to use, the Regensys B (NADP) was added to the Regensys A bottle and mixed thoroughly.

The Ames assay was performed by aseptically pipetting 0.50 mL of 0.1 mM sodium phosphate buffer pH 7.4, 0.1 mL of sappanwood extracts, and 0.10 mL of overnight *Salmonella* culture (about $1-2 \times 10^8$ cfu/mL) into top agar (48 °C), and then mixing well with a vortex. The mixture was immediately poured onto the surface of GM agar plates and swirled quickly for even distribution of the top agar mixture. Once the top agar was solidified, plates were inverted and incubated at 37 °C for 48 hours. For samples where metabolic activation was required, the phosphate buffer was replaced by the S9 mix (same volume of 0.1 mL).

Negative (sterile deionized water) and positive (daunomycin) controls were included in every assay in the same manner as the test sample (0.1 mL per plate). Sterile deionized water was used as a negative control for all experiments. Daunomycin (60 µg/mL) was used as positive control for TA 98 assay, while for TA 100, the positive control was sodium azide (50 µg/mL). The experiments were done in triplicate for each concentration, and for positive and negative controls.

Results were recorded by counting the number of colonies after 48 hours of incubation, and the background of each sample dish was also compared to the negative control in the absence of the background (thinning).

Antimicrobial Activity by Standard Agar Disc Diffusion Method

The standard agar disc diffusion method was performed as described in the Manual of Clinical Microbiology (Baron and Murray 2003). Nutrient agar plates were aseptically prepared as directed by the manufacturer (23 g nutrient agar per 1 L of deionized water). Each bacterial culture was prepared by inoculating 5 colonies of the bacteria into a glass tube containing 7 mL of sterilized TSB. The bacterial suspensions were incubated at 37 °C until the cell density of 1×10^8 cfu/mL was reached. The density was determined by turbidity according to McFarland standard (absorbance of approximately 0.1 at 600 nm).

Freeze-dried cold water extracts of sappanwood were reconstituted with sterile deionized water to achieve the concentrations of 0.5, 5, and 50 mg/mL. Then 30 μ L of the extracts at each concentration was impregnated onto a sterile blank disc (6 mm diameter) and dried aseptically. Then the discs were aseptically placed on the surface of the nutrient agar plate, one disc each for the three concentrations was placed on the same petri dish along with the negative control (sterile deionized water). The experiment was repeated in triplicate. The plates were incubated for 24 hours at 37 °C, except for those inoculated with *A. faecalis* which were incubated for 48 hours. The antimicrobial property was determined by measuring the size of inhibition zone (area where no growth of bacteria was observed) in millimeters. The experiments were done in triplicate. Extracts with inhibition zones of higher than 6.0 mm are considered effective.

Statistical Analysis

The number of revertant colonies data from the antimutagenicity assay, inhibition zone data, and the antimicrobial activity test, were reported as means and standards deviation. Zone inhibition data was analyzed by an analysis of variance (ANOVA) and mean separations were performed by Tukey's studentized range test ($p < 0.05$) in order to evaluate the difference in inhibition zone between the type and concentrations of SPW extracts. SAS 9.2 statistical software was used (SAS Institute Inc., Cary, NC, U.S.A.). An example of the SAS output is shown in Appendix B.

Results and Discussion

Ames Mutagenicity Assay

A mutagenicity assessment was performed on 2 types of sappanwood extracts, i.e. reflux with water for 5 hours (RF5) and shaking for 6 hours at room temperature (RT). The mutagenicity tested on *S. Typhimurium* TA 98 and TA 100 of the extracts as determined by number of revertant colonies grown on the glucose minimum agar (GM) plate with histidine limited top agar is shown in Tables 4.1 and 4.2, for RF5 and RT, respectively.

The RF5 was tested at five different concentrations ranging from 50 to 5000 μg per plate and the number of revertant colonies of all 5 concentrations is shown in Table 4-1. The RF5 results were not higher than that of the sterile deionized water (negative control), and many folds lower than the positive control. Because numbers of revertant colonies were not at least 2 fold higher than that of the negative control, it can be concluded that the RF5 did not have any mutagenicity. RF5 with the metabolic activation system (samples with S9 fraction of rat liver added) also showed similar

results of low revertants. This result suggested that, similar to the original compounds, metabolites of RF5 also had no mutagenicity.

Table 4-2 shows the number of revertants for *S. Typhimurium* TA 98 and TA 100 in the presence of RT, both with and without metabolic activation system mutagenicity. Similar to results for RF5, RT results also suggest that cold water extracts of sappanwood do not contribute to mutagenicity.

According to Ames assay principles as published by Maron and Ames (1983), and by Mortelmans and Zeiger (2000), repeatable results from at least 2 independent laboratories are needed in order to confirm that the extracts are non-mutagenic as determined by *S. Typhimurium* TA 98 and TA 100. Also, as a tiered approach was used, further tests on different strains of *S. Typhimurium* are recommended. The absence of mutagenicity of sappanwood water extract from the current study agrees with the absence of toxicity of sappanwood extract evaluated in rats as well as the lack of cytotoxicity against mammalian cells (Xu and Lee 2004; Sireeratawong and others 2010).

The cytotoxicity was conducted on Vero cells with brazilin concentrations ranging from 100 to 1000 µg/mL. When compared with the control (no brazilin), the number of viable cells were not different (Xu and Lee 2004). In the toxicity on rats, the hot water extraction of sappanwood from Thailand was administered to five Wistar rats per gender at a dose of 5,000 mg/kg body weight. The acute toxicity was determined by general behavior, mortality, and changes in gross appearance of internal organs during the 15-day experiment. The subacute toxicity was studied for 30 days by measuring body weight, organ weight, hematological, and blood chemicals. The authors concluded that

the sappanwood extract did not exhibit either acute or subacute toxicity in rats (Sireeratawong and others 2010).

Results from these two previous studies on the absence of toxicity for sappanwood extract and brazilin support the data generated in this study on the absence of mutagenicity for sappanwood extract. These results also serve as scientific evidence that sappanwood extract and brazilin are not toxic to humans. In addition, the final concentration in food products of the sappanwood extract is self-limiting because at higher concentrations the color of the product will be too intense for consumption.

According to the Office of Food Additive Safety, other recommended toxicological tests include, but are not limited to, subchronic toxicity with rodents and non-rodents and one-year toxicity studies with non-rodents. Human studies including epidemiology studies may be needed if the levels considered in foods is thought to be high for certain substances (Center for Food Safety and Applied Nutrition/ U.S. Department of Health and Human Services 2006). Nevertheless, the current results support the data presented from other relevant toxicity studies that the sappanwood extracts are neither toxic nor mutagenic.

Antimicrobial Activity Evaluated by Disc Diffusion Method

Antimicrobial activity using the disc diffusion method enables the comparison of antimicrobial activity among sappanwood extracts toward the tested bacterial strains. After reconstitution in deionized water, concentrations of the extracts were 0.5, 5.0 and 50.0 mg/mL (of freeze-dried sappanwood extract); the absolute weight concentrations after the impregnation were 15, 150, and 1500 µg per disc, respectively. Samples exhibiting clear zones of inhibition beyond the perimeter of the discs (6 mm) were

determined as having antimicrobial activity (or effective) against the test bacterial strains, thus the diameters of the inhibition zones can be measured in mm.

From the results (Table 4-3), at 15 µg per disc, no antimicrobial activity was observed in any sappanwood extracts against the test bacteria. *Bacillus coagulan* was the most sensitive bacterial strain to the sappanwood water extracts (RF5 and RT). At 150 µg/discs, the antimicrobial activity was detected only on *Bacillus coagulan* with inhibition zones of 18 and 19 mm for RF5 and RT respectively. *Pseudomonas putida* was the least responsive to the antimicrobial activity of sappanwood extracts, even at 1500 µg per disc, the inhibition zone was about 9 mm, which is smaller compared to that of *Alcaligenes faecalis* and *B. coagulans*, which had an inhibition zone of 24 – 26 mm and 33 mm respectively.

No significant difference was found for the same amount of extract per disc for the same bacterial strain when comparing the hot and cold water extraction (RF5 and RT). This indicates the possibility that both hot water extraction (RF5) and cold water extraction (RT) may have nearly the same type and quantity of antimicrobial compounds. It is possible that excess heat used in the 5 hours reflux extract did not contribute to the extractable amount of antimicrobial compounds.

Results obtained and discussed from this study as described above suggest the potential of sappanwood water extract as a food additive, and similar results were found from previous studies (Kim and others 2004; Xu and Lee 2004; Lim and others 2007; Phattayakorn and Wanchaitanawong 2009; Saraya and others 2009). Although results from the current study as well as others demonstrated the antimicrobial activity of the sappanwood extract against various types of bacteria, the mechanisms or mode of

reaction are not fully understood. Further identification, isolation, and quantification of active compounds responsible for antimicrobial activity, and understanding their mode of action is needed. This type of information will be beneficial to the development of sappanwood extract into a food preservative (additive), or food colorant with antimicrobial activity. Since each food system has unique properties, application of the aqueous sappanwood extract on a specific model is needed to determine the actual shelf-life of each food product.

Conclusion

Sappanwood water extracts (RF5 and RT) did not exhibit mutagenicity as evaluated by Ames mutagenicity assay. Moreover, the extracts exhibited some antimicrobial activity against the growth of selected spoilage bacteria (*A. faecalis*, *B. coagulans*, and *P. putida*). Based on these results, sappanwood water extracts may have potential to be applied into food products both as a red colorant and as a food preservative.

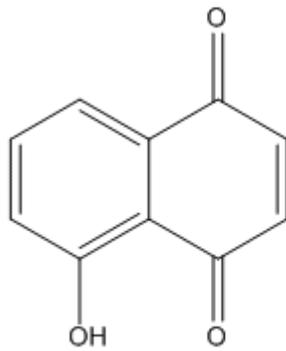


Figure 4-1. 5-hydroxy-1,4-naphthoquinone

Table 4-1. Mutagenic dose response of RF5 sappanwood water extract to Salmonella Typhimurium (TA98 and TA100) as represented by the mean number of revertant colonies (CFU/plate) +/- standard deviation (n=3)

Dose level (µg/plate)	Number of revertant colonies (CFU/plate)	
	TA 98	TA 100
<i>Without metabolic activation</i>		
Sterile deionized water (NC)	27.0 ± 5.0	11.0 ± 5.0
Daunomycin (60µg/plate; PC)	316.7 ± 25.4	-
Sodium azide (50µg/plate; PC)	-	1118.7 ± 200.2
5000	0.0 ± 0.0	0.0 ± 0.0
3333	3.3 ± 2.1	4.7 ± 5.0
2500	18.7 ± 8.0	2.7 ± 0.6
500	22.3 ± 2.5	7.7 ± 4.9
50	24.0 ± 6.9	12.0 ± 3.5
<i>With metabolic activation (S-9)</i>		
Sterile deionized water (NC)	39.0 ± 8.0	12.0 ± 1.0
Daunomycin (60µg/plate; PC)	1980.0 ± 404.2	-
Sodium azide (50µg/plate; PC)	-	133.7 ± 13.6
5000	10.3 ± 3.5	1.7 ± 1.2
3333	16.3 ± 7.6	6.7 ± 1.2
2500	17.0 ± 4.4	5.7 ± 1.5
500	28.0 ± 8.2	11.0 ± 5.0
50	27.0 ± 1.0	14.0 ± 1.0

Values expressed are mean ± SD of three experiments. Two fold or more of number of revertant colonies is an indicator of mutagenicity; NC = negative control; PC = positive control.

Table 4-2. Mutagenic dose response of RT sappanwood water extract to *Salmonella* Typhimurium (TA98 and TA100) as represented by the mean number of revertant colonies +/- standard deviation (n=3)

Dose level (µg/plate)	Number of revertant colonies (CFU/plate)	
	TA 98	TA 100
<i>Without metabolic activation</i>		
Sterile deionized water (NC)	26.0 ± 4.0	8.0 ± 4.0
Daunomycin (60µg/plate; PC)	315.0 ± 27.1	-
Sodium azide (50µg/plate; PC)	-	1205.3 ± 237.5
5000	2.7 ± 3.1	2.0 ± 1.7
3333	12.0 ± 2.6	6.3 ± 3.5
2500	22.7 ± 4.9	6.3 ± 2.1
500	19.3 ± 0.6	10.7 ± 2.5
50	27.3 ± 6.1	10.7 ± 3.8
<i>With metabolic activation (S-9)</i>		
Sterile deionized water (NC)	33.0 ± 4.0	18.0 ± 9.0
Daunomycin (60µg/plate; PC)	1556.0 ± 256.1	-
Sodium azide (50µg/plate; PC)	-	195.0 ± 45.3
5000	15.7 ± 2.5	5.7 ± 1.2
3333	19.7 ± 7.8	9.3 ± 4.0
2500	21.0 ± 1.0	10.3 ± 3.8
500	33.0 ± 2.6	8.3 ± 2.9
50	31.7 ± 2.3	12.0 ± 2.0

Values expressed are mean ± SD of three experiments. Two fold or more of number of revertant colonies is an indicator of mutagenicity; NC = negative control; PC = positive control.

Table 4-3. Antibacterial activities of sappanwood water extracts against three strains of food spoilage bacteria.

Wt. of freeze-dried extract per disc (μg)	Inhibition zone (mm)	
	RF5	RT
<i>A. faecalis</i>		
Sterile deionized water	0.00 \pm 0.00e	0.00 \pm 0.00e
1500	A26.33 \pm 0.58b	A24.33 \pm 1.53b
150	0.00 \pm 0.00e	0.00 \pm 0.00e
15	0.00 \pm 0.00e	0.00 \pm 0.00e
<i>B. coagulans</i>		
Sterile deionized water	0.00 \pm 0.00e	0.00 \pm 0.00e
1500	A33.33 \pm 0.58a	A32.66 \pm 0.58a
150	A19.33 \pm 1.15c	A18 \pm 1.73c
15	0.00 \pm 0.00e	0.00 \pm 0.00e
<i>P. putida</i>		
Sterile deionized water	0.00 \pm 0.00e	0.00 \pm 0.00e
1500	A8.67 \pm 1.15d	A8.67 \pm 0.58d
150	0.00 \pm 0.00e	0.00 \pm 0.00e
15	0.00 \pm 0.00e	0.00 \pm 0.00e

Values expressed are mean \pm SD of three experiments. Extracts with inhibition zone higher than 6.0 mm are considered effective. Different upper case letters in front of inhibition zone values in the same rows and different lower case letters in columns represent values that are significantly different ($p < 0.5$).

CHAPTER 5 STABILITY OF RED COLOR FROM SAPPANWOOD COLD WATER EXTRACT IN AQUEOUS SOLUTIONS

Background Overview

Ideal properties of food color additives include stability in a wide range of colorant concentrations, the pH of products, heat treatment, sulfur dioxide exposure, and storage conditions (such as temperature and exposure to air and light) that are common in processed food products. The color of the finished products should be vibrant with no off-flavor. The raw materials should be inexpensive, free of insecticides, herbicides or microbial contamination, and they should not be seasonal-dependent. The yield of the extraction should be high and the colorants obtained should have high color strength. Moreover, raw materials of plant origins may be preferred as they can be qualified as vegetarian, and kosher approved (Downham and Collins 2000; Kilcast and Subramaniam 2000; Wrolstad 2004; Griffiths 2005; Castañeda-Ovando and others 2009; Nachay 2009).

The stability over a wide range of concentration needs to be evaluated because it can be unpredictable. Normally, as concentration increases, colorant solutions will show higher color saturation (higher chroma value), while the color shade (hue) is less affected by concentration. Exceptions are, for example, cyanidin solution which is red at high concentrations, but changes to purple hues at low concentrations (Hutchings 1999).

Susceptibility to degradation under heat and light exposure is a common characteristic of plant-derived colorants such as anthocyanins, which are available commercially as red colorants from various plant materials. Another important factor that causes instability of color is pH. Most anthocyanins, for example, change color and

become instable at pH levels above 4. These factors are important as they are major limitations to the application of colorants in food (Wrolstad 2004).

The molecular structure of brazilin, a compound responsible for the red color of sappanwood cold water extract, is considered a homoisoflavonoid, which is a subgroup of flavonoid. Brazilin is a reduced form, and it is the form that naturally exists in sappanwood. When exposed to oxygen and light, it is oxidized to brazilein, thereby changing color from colorless (brazilin) to red (brazilein) (Vankar 2000; Ferreira and others 2004; Wongsooksin and others 2008; Berger and Sicker 2009; Petroviciu and others 2010).

It should be noted that prior to this current study, there was no study on the stability of brazilein or sappanwood extract. The objective of the present study is to investigate the stability of aqueous sappanwood extract as affected by concentration, heat and light. The results obtained can be useful in furthering the development of sappanwood extract as a color additive in food products.

Materials and Methods

Raw Materials and Chemicals

Sappanwood was purchased from Jao-Krom-Poe, the oldest and most reputable Thai herb pharmacy in Bangkok, Thailand (Usuparatana 1997). Potassium chloride, hydrochloric acid, citric acid, dibasic sodium phosphate, dibasic hydrogen phosphate, monobasic sodium phosphate, and glycine were purchased from Fisher Scientific Co., (Pittsburgh, PA, U.S.A.). The preparation of pH 7, 8 and 9 buffers was completed as described in Table 3-1.

Sappanwood Extracts Preparation

Preparation of the extract was described in Chapter 3. The freeze-dried sappanwood water extract (RT) was used to evaluate concentration effects. However, for heat stability and storage stability evaluations, the cold water extracts were used without further freeze-drying step. After the extraction and filtration as described in Chapter 3, extracts were blanched by heating the extracts to 212 °F for 10 minutes, and then cooled down immediately on ice water (Cevallos-Casals and Cisneros-Zevallos 2004). The concentration of the extract was standardized when the temperature of the extract reached 25 °C by diluting it with deionized water until an absorbance of 1.0 at 539 nm was reached for the solution of the extract at pH 9 (extract : buffer pH 9 = 1:6). The absorbance was obtained by a spectrophotometer (Beckman Coulter DU 640; Indianapolis, IN, U.S.A.). The standardized extracts were kept refrigerated (4 °C) then used within 24 hours after preparation.

Color Measurements

Color characteristics as recommended by The International Commission on Illumination (CIE) were measured as L*, a*, b*, hue, and chroma using a Minolta CT-310 colorimeter (Minolta Corporation, Ramsey, NJ, U.S.A.). The solution of the extract and buffer was placed in a 10 mm plastic cell (Minolta CM-A131), and put in the Minolta cell holder (CR-400). The colorimeter was set up using the light source D65. The CIE L*, a*, b*, hue, and chroma values of each sample obtained were averages of the duplicate measurements and were read directly from the colorimeter.

The change of color was quantified as the metric distance between two colors, represented as the overall color difference or ΔE . The ΔE was calculated from the

reflectance data: L^* , a^* , b^* used the following formula, where L_0 , a_0 , and b_0 are the reference values at time 0 (Medeni 2001).

$$\Delta E = \sqrt{(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2}$$

Spectrophotometric Analysis

The absorbance of solutions of sappanwood extract in buffer from the wavelength of 250 to 700 nm was measured by a UV-vis spectrophotometer (Beckman Coulter DU 640; Indianapolis, IN, U.S.A.) using disposable cuvettes (1.5 mL purchased from Fisher Scientific, Pittsburgh, PA, U.S.A.). Percent color retention at λ max was calculated using the following formula, where Abs_0 is the absorbance at time 0.

$$\% \text{ color retention at } \lambda \text{ max} = [(Abs - Abs_0) / Abs_0] \times 100$$

Color Stability Study of Sappanwood Extracts Under Different Conditions

Thermostability

Thermostability (at 80°C) of the sappanwood water extract (at pH 7, 9, and 12) was evaluated under three different conditions (light and air; no light and air; no light and no air). The temperature control (80 - 82 °C) was accomplished by submerging sample tubes (glass) in a water bath shaker (Gyrotory, Model G76, New Brunswick Scientific Co., INC.; Enfield, CT, U.S.A.). In each glass tube (16 x 100 mm), 2.0 mL of standardized sappanwood extract was added to 12.0 mL of buffer pH 7, 9, and 12. Sample tubes for each treatment condition were prepared in duplicate. After tightly closing with a screw cap and mixing well with a vortex, all tubes were then immediately placed in the water bath shaker. Samples were pulled out at 0, 0.5, 1.0, 2.0, 3.0, 5.0 and 7.0 hours, cooled immediately in ice water, and then analyzed for color and UV-vis

spectra. No air condition is defined as flushed with nitrogen, and no light means it was performed in the dark.

Effects of concentrations on chromaticity

The freeze-dried powder from the cold water extraction of sappanwood (RT) as described in chapter 3 was dissolved in warm deionized water (approximately 60 °C) to achieve concentrations of 5, 10, 20, 40, 50, 100, and 200 µg/mL. The dilution scheme was described in Appendix C. Each concentration was prepared in triplicate (3 test tubes per concentration). All samples were analyzed for color measurements.

Storage stability

To evaluate the stability of red color from sappanwood extract during storage, sappanwood extract at the same concentrations (pH 7, 8, and 9) were stored under 3 different conditions (room temperature, with light; room temperature, no light; and at 4 °C, no light). In each glass tube (16 x 100 mm), 2.0 mL of standardized sappanwood extract was added to 12.0 mL of buffers pH 7, 8, and 9. Sample tubes for each treatment condition were prepared in triplicate (54 tubes were prepared for each pH). After tightly closing with a screw cap and mixing well with a vortex, samples tubes were placed in the 3 different conditions. Samples were pulled out at days 0, 3, 7, 14, 21, and 35, then analyzed for color and UV-vis spectra. Light condition was defined as 24 hour exposure to fluorescent light per day, and no light means it was performed in the dark.

Buffer pH 7, 8, and 9 was prepared as described in Chapter 3. Screw cap test tubes (16 x 100 mm) containing 12 mL buffer were autoclaved at 121 °C for 30 minutes then kept at room temperature until ready to use.

Statistical Analysis

Redness (a^* value), % color retention, chroma, hue angle, and color difference data were reported as mean and standard deviation. Color difference data was analyzed by analysis of variance (ANOVA) and mean separations were performed by Tukey's studentized range test ($p < 0.05$) in order to evaluate the difference in the color difference between storage time and storage conditions. SAS 9.2 statistical software was used (SAS Institute Inc., Cary, NC, U.S.A.). An example of the SAS output is shown in Appendix B.

Results and Discussion

Thermostability

The thermal stability (80 °C) of the extracts was evaluated by exposing the sappanwood extracts (pH 7, 9, and 12) for a total of 7 hours in different experimental conditions (light and air; no light and air; no light and no air). As sappanwood extract is a product of plant origin, blanching of the extract was performed to deactivate any polyphenoloxidase enzymes that may be presented as described by Cevallos-Casals and Cisneros-Zevallos (2004) for 100% deactivation of the enzymes in other plant materials.

The effects of heat exposure at different pH levels was evaluated by the CIE color system (a^* , hue, and chroma), and spectrophotometric analysis expressed as % color retention at λ max (539 nm). Figures 5-1 showed changes in a^* value (redness) during heating of the sappanwood extracts. The redness for all extracts (pH 7, 9, and 12) was reduced with longer heating duration. However, redness of sappanwood solution at pH 12 was reduced to less than 0.00 after only 0.5 hours of heating time while solutions at

pH 7 and 9 were higher than 15.00 after 2 hours of heating. This result indicates that the heat stability of sappanwood extract at pH 12 is very low.

Similar to the previous report in chapter 3, UV-vis spectra of the sappanwood extracts at the various pH values showed maximum absorption in the visible region at wavelength of 539 nm ($\lambda_{\max} = 539$ nm). Comparison of UV-vis spectra of sappanwood extracts at pH 9 showed that the absorbance at 539 nm was reduced with the duration of heating (Figure 5-2). A comparison of the color retention at 539 nm of sappanwood extract pH 9 was shown in Figure 5-3, with best color retention demonstrated when samples were heated without exposure to light and air (approximately 70% after 7 hours of heating at 80 °C). When air is present, the % color retention at 539 nm was reduced to less than 20% regardless of light exposure.

Sappanwood extract at pH 12 was excluded in the subsequent experiment as its thermal stability is very low. In addition, the pH of food is unlikely to be higher than 9. Thus, pH of 7, 8, and 9 were selected for further stability studies.

Concentration Effects on Color of Sappanwood Extracts

Aqueous solutions of sappanwood extract at pH 7, 8, and 9 were prepared at concentrations of 5 – 200 $\mu\text{g}/\text{mL}$. Saturation of color or color intensity is represented by chroma, which was calculated as $(a^{*2} + b^{*2})^{1/2}$. As illustrated in Figure 5-4, intensity of color in term of chroma values of sappanwood extracts at pH 8 and 9 was increased as the concentration of the freeze-dried sappanwood in the solution increased from 5 – 40 $\mu\text{L}/\text{mL}$, and then was reduced. For SPW extract at pH 7, the chroma values continued to increase with the increase of the concentration to the concentration of 200 $\mu\text{L}/\text{mL}$, which was the highest concentration tested. For colorants, it is desirable to have color

intensity increase with the concentration of color. Thus, this experiment showed that sappanwood extracts met this required quality up to a chroma value of approximately 34 – 36 for pH 8 and 9, and up to a chroma value of 40 for pH 7.

The next important parameter is the visual color itself; thus, actual photos for visual color comparison are shown in Figure 5-5. The visual colors agreed with the chroma values measured, i.e. colors were in the same shade, that is light orange to dark orange in pH 7, and light pinkish red to intense red for pH 8 and 9. Color saturation was higher as concentrations increased, until the maximum was reached for pH 8 and 9 (chroma values of 34 – 36), while color saturation of extract at pH 7 was highest at the highest concentration (200 µg/mL).

Hue angle was calculated as $\arctan b^*/a^*$ and expressed as degree on a 360° grid. Close to 0° is bluish-red, and as the value approaches 90°, a more yellowish to yellow color results. Hue angle should remain the same while chroma increases as concentration of colorant increases (Wrolstad and others 2005). The hue angle of all samples increased with the concentration of sappanwood extract in the buffer solution (Table 5-1). The range of hue angle suggested that the direction of bluish-red toward orange red as colorant concentration increased for the pH 9 solution. Solutions at a pH 9 also had the most stability (least degree of change) toward concentration changes because, of all 7 concentrations tested, there were only 4 groups of mean hue angles that were significantly different (Tukey's HSD test, $p < 0.05$), followed by a total of 5 groups of means in pH 7, and 7 groups of means in pH 8.

Storage Stability

Visual colors of sappanwood water extract solutions at pH 7, 8, and 9 were shown in Figure 5-6. For comparison in absolute values, color difference (ΔE) was used to determine the effect of pH, storage temperature, and light exposure during storage of the sappanwood extract (Table 5-2).

Storage at room temperature, both with and without light significantly increased color difference (ΔE) for all sappanwood extracts. Storage at 4°C in the dark contribute to significantly less color difference for all extracts after 35 days of storage for all pH levels (7, 8, and 9). The only exception is for pH 9 which, after 35 days showed no significantly difference in ΔE was observed between storage at room temperature and storage at refrigerator temperature when stored in the dark. Thus, eliminating light exposure, even when stored at room temperature, can significantly prolong the color of sappanwood extract at pH 9. For the extract at pH 7 and 8, storage at refrigerator temperature without light exposure had significantly less ΔE than the other two conditions (room temperature with and without light exposure). From these results, sappanwood solution at pH 9 had better storage stability when compared to the sappanwood solutions at pH 7 and 8.

When comparing the ΔE (color difference) of sappanwood extract at pH 9 over storage time in different experimental conditions, it can be observed that there was no significant difference in ΔE between Day 0 and Day 14. There was also no significant difference between ΔE on day 14 of all 3 experimental conditions. This could be an indication that, at a pH of 9, the sappanwood solution can be stored in either room or

refrigerator temperature, either with or without light exposure, for up to 14 days with no significant changes in color of solution based on color difference values.

Conclusion

Stability of sappanwood extracts at various pH values was tested. The solution of sappanwood extract at pH 9 showed the highest stability to heat, light, and oxygen. A higher concentration did not dramatically change the shade of color, which is a desirable property for a color additive. Results obtained from this study suggest the factors that contribute to the higher stability of red color are controlling the pH to pH 9, limiting light and oxygen exposure, and low temperature storage. Further investigation on the degradation mechanisms of brazilein and how to retard these reactions could lead to sappanwood as a potential color food additive.

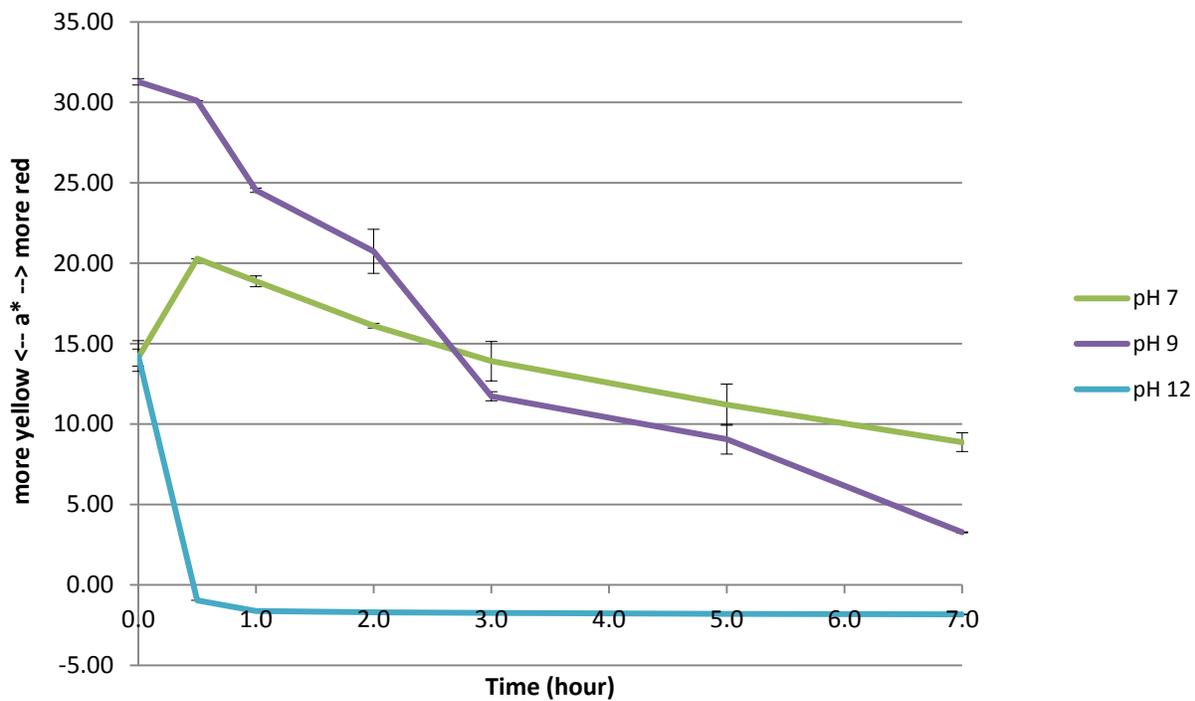


Figure 5-1. Effects of pH (2, 5, 7, 9, and 12) on heat stability of sapanwood extracts in terms of a* value (redness) with light and air exposure

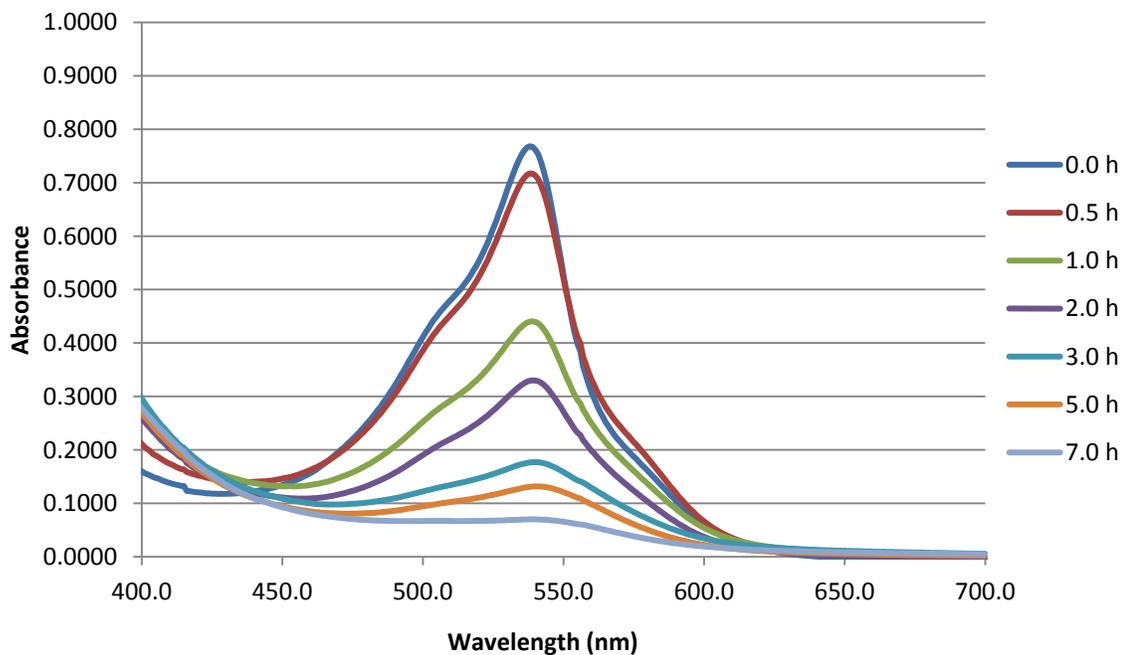


Figure 5-2. UV-vis spectra of sapanwood extract at pH 9 during heating (80 °C) with light and air exposure.

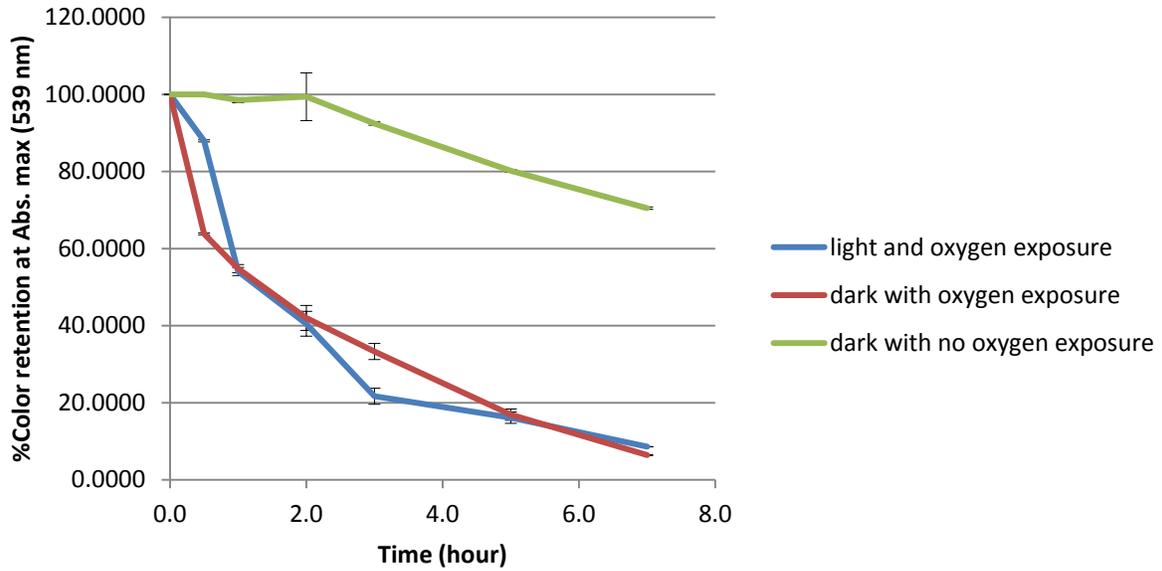


Figure 5-3. Effects of heat on % color retention of sappanwood extracts (at 539 nm) at pH 9 under different environments. The blue line represents light and air exposure; the red line represents air exposure but no light; and the green line represents no heat and no air exposure.

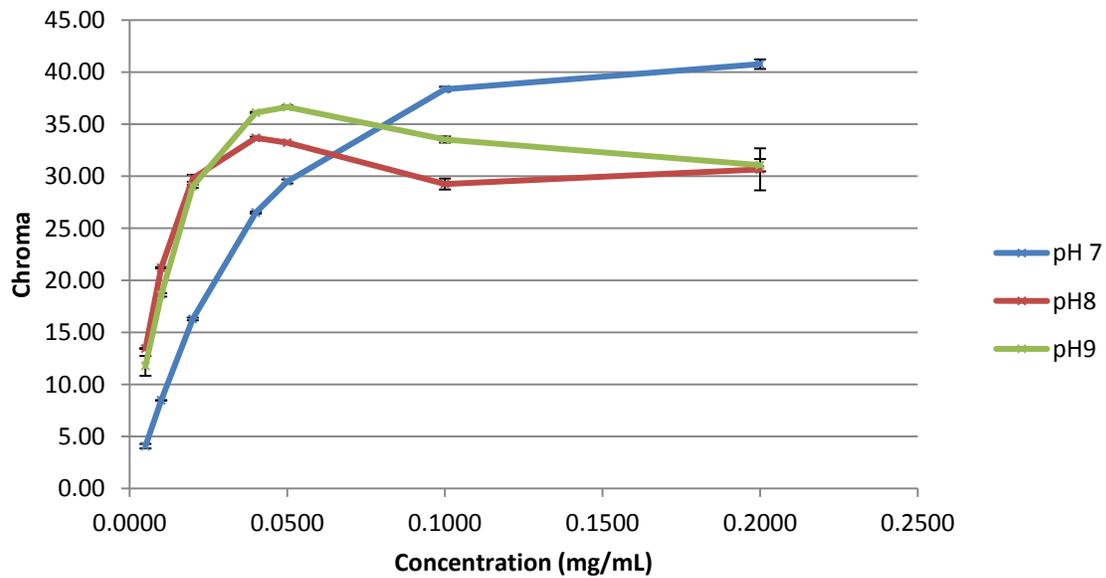
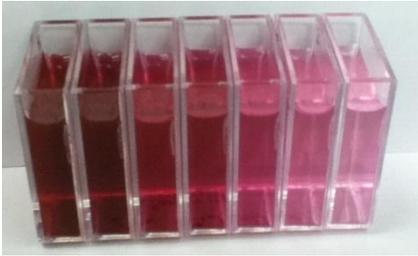


Figure 5-4. Effects of sappanwood extract concentrations on color saturation (chroma) at pH 7, 8 and 9.



pH = 7



pH = 8



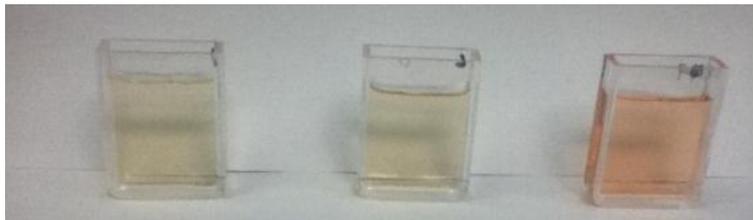
pH = 9

Figure 5-5. Visual colors of different sapanwood extract concentrations at pH 7, 8, and 9 (high to low from left to right).

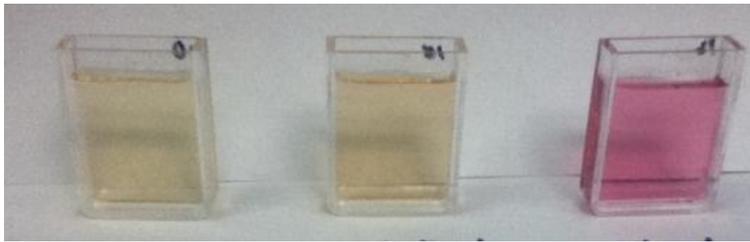
Table 5-1. Effects of various concentrations of sapanwood water extracts on hue angle at pH 7, 8, and 9

Concentration ($\mu\text{g/mL}$)	Hue angle								
	pH 7			pH 8			pH 9		
200.0	45.83	^a \pm 0.51		389.94	^a \pm 1.07		377.63	^{cd} \pm 0.84	
100.0	47.05	^a \pm 0.09		376.58	^b \pm 1.42		360.10	^{cd} \pm 0.54	
50.0	39.15	^a \pm 0.22		360.81	^c \pm 1.90		344.75	^{cd} \pm 0.10	
40.0	34.61	^c \pm 0.47		354.97	^d \pm 0.26		341.76	^c \pm 0.24	
20.0	23.05	^d \pm 0.39		347.84	^e \pm 0.28		342.71	^c \pm 11.48	
10.0	11.92	^e \pm 1.03		344.89	^f \pm 0.23		333.94	^b \pm 0.30	
5.0	358.27	^f \pm 1.17		341.95	^g \pm 0.17		332.26	^a \pm 0.34	

Mean values (n=3) followed by different lowercase letters in the same column are significantly different (Tukey's HSD, $p < 0.05$).



pH 7



pH 8



pH 9

Figure 5-6. Sappanwood water extract after 35 days in different environments, from left to right of each picture, at 25 °C with light exposure, at 25 °C in the dark, and at 4 °C in the dark.

Table 5-2. Color differences during storage of sappanwood extracts under various storage conditions and pH

Condition	Color Difference (ΔE)					
	0 day	3 day	7 day	14 day	21 day	35 day
pH 7						
25°C, Light	A 0.00 ± 0.00 f	B 3.11 ± 0.72 e	A 17.99 ± 0.54 d	A 23.79 ± 0.08 c	A 25.13 ± 0.32 b	A 50.60 ± 0.04 a
25°C, Dark	A 0.00 ± 0.00 e	B 2.17 ± 0.07 e	B 9.78 ± 0.50 d	B 19.42 ± 2.78 c	A 23.41 ± 1.03 b	A 49.37 ± 0.57 a
4°C, Dark	A 0.00 ± 0.00 e	A 11.27 ± 0.13 d	C 4.76 ± 0.23 d	C 5.24 ± 0.57 c	B 9.23 ± 0.83 b	B 36.69 ± 0.76 a
pH 8						
25°C, Light	A 0.00 ± 0.00 e	A 5.56 ± 1.80 d	A 21.10 ± 2.02 c	A 36.54 ± 0.57 b	A 38.14 ± 0.16 b	A 42.95 ± 0.16 a
25°C, Dark	A 0.00 ± 0.00 c	B 2.45 ± 0.21 c	B 5.49 ± 0.52 c	A 17.52 ± 1.41 b	B 33.01 ± 1.56 ab	A 41.22 ± 1.23 a
4°C, Dark	A 0.00 ± 0.00 d	B 2.62 ± 0.16 cd	C 1.00 ± 0.41 bc	B 2.64 ± 0.20 bc	C 3.55 ± 1.04 b	B 10.48 ± 1.36 a
pH 9						
25°C, Light	A 0.00 ± 0.00 c	A 2.06 ± 0.02 c	B 2.36 ± 0.46 c	A 3.02 ± 0.94 c	A 29.16 ± 3.89 b	A 43.72 ± 0.13 a
25°C, Dark	A 0.00 ± 0.00 b	AB 3.18 ± 0.57 b	A 4.02 ± 0.41 b	A 1.96 ± 0.92 b	AB 4.22 ± 2.17 b	AB 10.19 ± 3.66 a
4°C, Dark	A 0.00 ± 0.00 c	B 1.20 ± 0.60 bc	B 2.65 ± 0.35 bc	A 1.62 ± 0.91 b	B 2.13 ± 0.16 b	B 4.81 ± 0.94 a

Values expressed are means ± SD (n = 3). Different upper case letters in front of ΔE values in the same column and different lower case letters in row of each pH represent value that are significantly different (p < 0.5).

CHAPTER 6 SUMMARY AND CONCLUSIONS

The current research explored the possibility of developing a natural derived red colorant as a food additive from sappanwood water extract. Based on the current literature, the research focused on the water extracts and it was found that water extraction at room temperature (RT) and reflux at 5 hours (RF5) provided the better color saturation and redness, compared to the longer reflux time (RF24). This study is the first to report the chromaticity of the sappanwood water extract at pH 2-12. The results suggested the potential advantage of using the sappanwood water extract as a naturally derived red colorant for pink to red colors at a higher pH, which has been a limitation of the anthocyanins in food application.

Based on the Ames mutagenicity test, water extracts (RT and RF5) were not mutagenic. Cold water extraction (RT), thus, was chosen for further evaluation because it required less energy during extraction. RT water extract was sensitive to heat, light, and temperature and the best storage condition was at pH 9 and 4 °C (refrigerator) with no light exposure. In addition, sappanwood extract may be beneficial to health as indicated by antioxidant activities reported. The relatively low antimicrobial activity reported here suggested little potential for using sappanwood extract as a food preservative when used at concentrations for coloring purposes.

Overall, this research provided evidence for further exploration of sappanwood water extract as a potential color food additive. The main advantages include long history of consumption as well as, an ability to give red color to low acid foods.

APPENDIX A
EFFECTS OF EXTRACTION TIME ON MAXIMUM ABSORBANCE OF SAPPANWOOD
COLD WATER EXTRACTION

Background

Cold water extraction may be desirable compared to hot water extraction due to fewer unwanted compounds being extracted. Thus, cold water extract is an interesting subject to study red colorant from sappanwood. The total time of extraction can affect the yield, and should be determined as a preliminary study.

Materials and Methods

In an Erlenmyer flask, 3.0 g of sappanwood powder was added to 100 mL of deionized water and shaken for 2, 4, 5, 6, 7, and 8 hours on a platform shaker (New Brunswick Scientific Excella E1 Platform Shaker; Edison, NJ, U.S.A.). The solution obtained was first filtered through Whatman #1 filter paper and then through 0.25 μM syringe filter. The UV-vis spectra was collected in the extract from 400 – 600 nm. The experiment was done in triplicate.

Results and Discussion

The UV-vis spectra of the 6 treatments (Figure A-1) have similar characteristics, i.e. having maximum absorbance at between 444 to 448 nm. The results were agreed with previous studies which indicated the absorbance max of brazilein at 445 nm (Yan and others 2007). Since the 6 hour extraction yield the highest absorbance at 445 nm, this method of extraction was selected for the subsequent steps of this study.

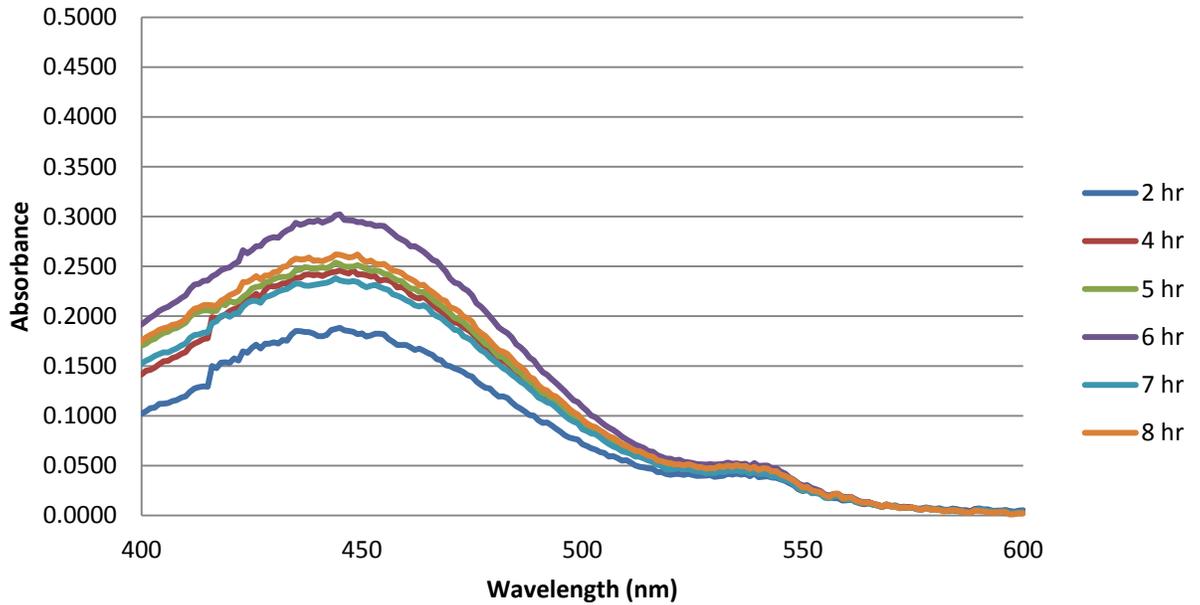


Figure A-1. Effects of extraction time on UV-vis spectra (from 400 to 600 nm) of sappanwood cold water extracts.

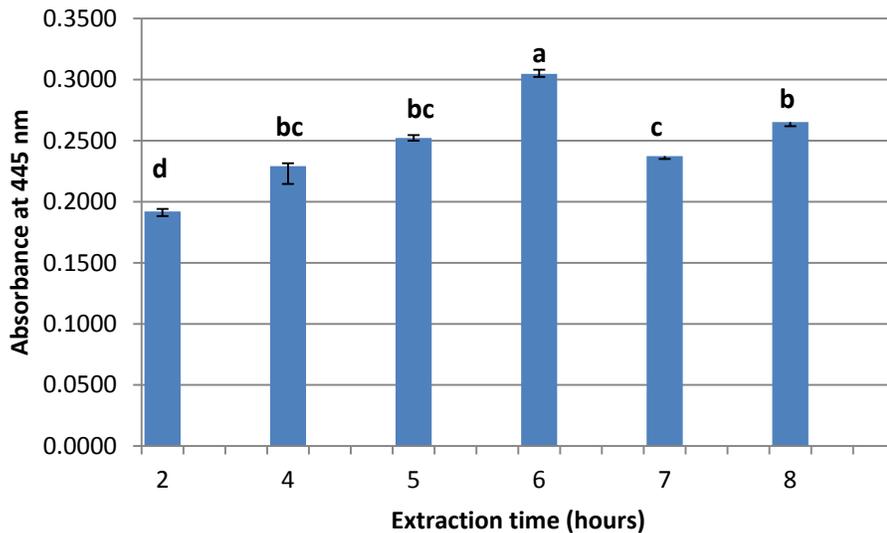


Figure A-2. Effects of extraction time on absorbance at 445 nm of cold water extracts of sappanwood powder. Data represents the mean of n=3.

* Columns with similar letters are not significantly different (Tukey's HSD, $p > 0.05$)

APPENDIX B EXAMPLE OF STATISTICAL ANALYSIS

The ANOVA Procedure

Class Level Information

Class	Levels	Values
extraction	3	RF24 RF5 RT

Number of Observations Read	9
Number of Observations Used	9

Dependent Variable: GAE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	418.8534000	209.4267000	13.82	0.0057
Error	6	90.8996000	15.1499333		
Corrected Total	8	509.7530000			

R-Square	Coeff Var	Root MSE	GAE Mean
0.821679	4.278027	3.892292	90.98333

Source	DF	Anova SS	Mean Square	F Value	Pr > F
extraction	2	418.8534000	209.4267000	13.82	0.0057

Tukey's Studentized Range (HSD) Test for GAE

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	15.14993
Critical Value of Studentized Range	4.33920
Minimum Significant Difference	9.7511

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	extraction
A	98.983	3	RF5
A			
B A	91.653	3	RT
B			
B	82.313	3	RF24

APPENDIX C
 PREPARATION OF SAPPANWOOD EXTRACT SOLUTIONS AT VARIOUS
 CONCENTRATIONS FOR COLOR STABILITY TESTING

Table C-1. Preparation of sappanwood extract solutions at various concentrations

Working solution preparation				In each test tube			
Solution #	Wt. of RT* (g)	Solution # and vol. (mL)	Water (mL)	Conc of working solution (mg/mL)	vol (mL) of working solution	vol (mL) of buffer	Conc of final solution (mg/mL)
A	0.1000	n/a	50	2.000	3	12	0.400
B	n/a	A = 25	25	1.000	3	12	0.200
C	n/a	B = 20	20	0.500	3	12	0.100
E	n/a	B = 10	30	0.250	3	12	0.050
F	n/a	A = 6	54	0.200	3	12	0.040
G	n/a	F = 30	30	0.100	3	12	0.020
H	n/a	G = 20	20	0.050	3	12	0.010
I	n/a	G = 10	30	0.025	3	12	0.005

*Freeze-dried sappanwood extract at room temperature as described for RT in Chapter 3.

APPENDIX D CHEMICAL OXIDATION OF BRAZILIN TO BRAZILEIN

Background

The main colorant in sappanwood is brazilin, which in a reduced form is colorless. The color of sappanwood extract becomes red when it is changed to the oxidized form, brazilein. Molecular structures for these compounds are described earlier in Chapter 3. In this preliminary experiment, the oxidation of brazilin as described by Wongsooksin and others (2008) was used to explain the possible oxidation mechanisms of phenols to quinones by reagents such as iodine (Becker 1965; Barret and Daudon 1990). Brazilin and its oxidized form were separated by liquid chromatography, and a comparison of HPLC profiles help to investigate changes resulting from these oxidation mechanisms. Mass spectrophotometry has been used as to identify brazilin and brazilein against previously published mass spectra of these compounds (Hulme and others 2005).

Although the oxidation of brazilin to brazilein was mentioned in several references, none was a direct comparison (before and after oxidation) using relative new technology such as mass spectrophotometry. The objective of this experiment is thus to identify brazilin in sappanwood extract as well as to influence the oxidation of the molecular structure of brazilin.

Materials and Methods

Brazilin was purchased from MP Biomedicals (Solon, OH, U.S.A.), HPLC grade methanol and iodine was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). The iodine solution was prepared by dissolving 33.8 g of iodine in 42.5 mL of methanol. The brazilin solution was prepared by dissolving 100 mg of brazilin in 0.5 mL warm methanol and mixing with 8.0 mL of hot deionized water (approximately 95 °C). Once the brazilin

solution was cooled to 60 - 70 °C, 1.6 mL of the iodine solution was added, and mixed well. The mixture was left overnight at room temperature. Brazilein, as a precipitate, was collected on filter paper (Whatman #1). The filtrate was washed several times with cold deionized water, followed by warm methanol. The brazilein obtained appeared to be a deep reddish-black color, it was dried in desiccator away from light, and then kept frozen in a tightly sealed glass vial.

The LC/MS analysis was performed by the Chemistry Department Mass Spectrometry Services facility, University of Florida Chemistry Laboratory, by Dr. Jodie Johnson. The HPLC analysis was performed the Agilent HPLC system 1100 series binary pump (Agilent; Palo Alto, CA, U.S.A.), equipped with Agilent 1100 G1314A UV/Vis detector at wavelength 254 nm. Compounds were separated on Phenomenex Synergi 4u Hydro-RP 80A (2 x 150 mm; 4 um; S/N=106273-5) column with C18 guard column (2mm x 4 mm) (Torrance, CA, U.S.A.). Mobile phases consisted of 0.2% acetic acid in H₂O (phase A) and 0.2% acetic acid in methanol (phase B) and all solvents and reagents were purchased from Burdick & Jackson (Morristown, NJ, U.S.A.). Flow rate was 0.17 mL/min. The gradient elution started with 100% of phase A for 45 minutes, then change to 5% phase A:95% phase B from 45 to 60 minutes. Electrospray ionization mass spectrometry (ESI-MS) was performed with ThermoFinnigan (San Jose, CA, U.S.A.), LCQ with electrospray ionization (ESI) in negative mode; and heated capillary temperature was 250 C. Injection volume was 20 µL.

Results and Discussion

HPLC chromatograms of brazilin and oxidized brazilin samples analyzed via reverse phase gradient C18 HPLC/254 nm UV/(+) & (-) ESI-MSn are shown in Figure D-

1 and Figure D-2 respectively. Brazilin samples contain both brazilin (MW 286) and brazilein (MW 284) while the oxidized sample contains only brazilein with no brazilin.

In the mass spectrometry with electrospray ionization in negative ion modes, MW 286 brazilin produced mainly the ion $[M-H]^-$ with m/z 285, while the MW 284 brazilein produced predominantly the ion $[M-H]^-$ with m/z (Figure D-3 and Figure D-4 respectively). Further dissociation of the $[M-H]^-$ ions of brazilin and brazilein produce product ions that agree with the previously published spectra by Hulme and others (2005) as detailed in Chapter 3.

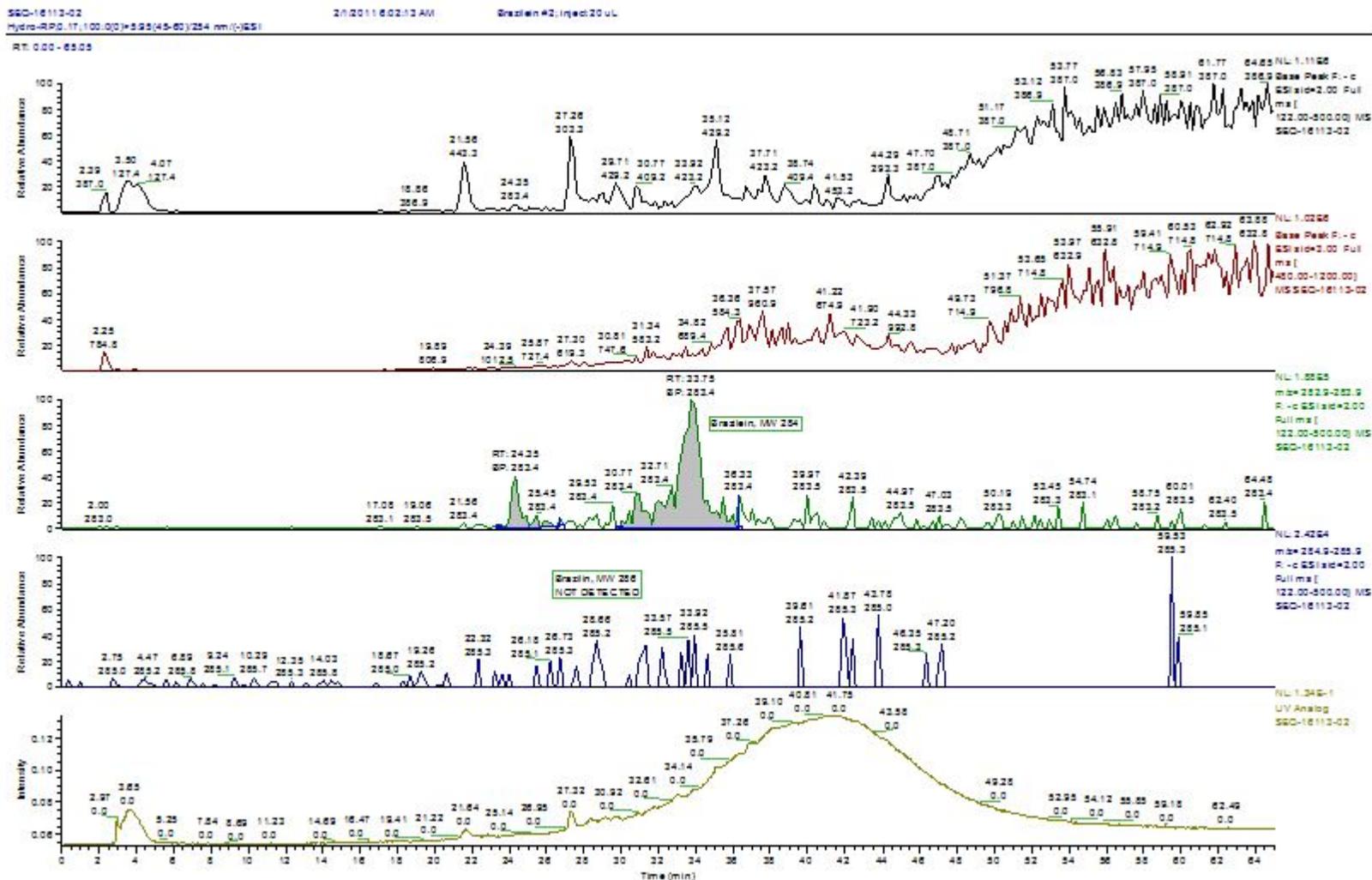
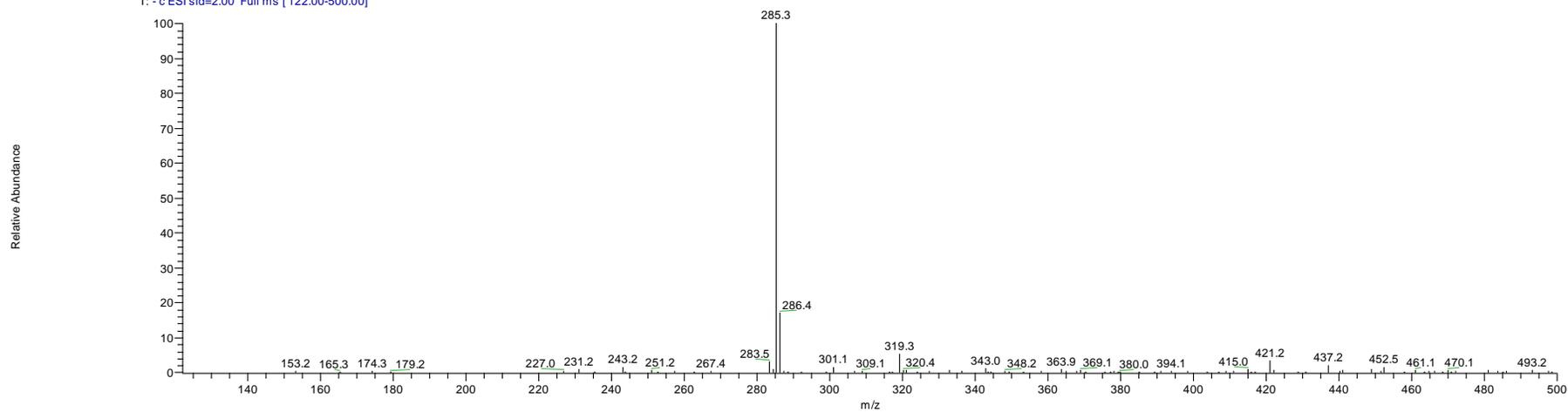


Figure D-2. Chromatogram of oxidized brazilin sample via C18 HPLC/UV/(-)ESI-MSⁿ. There are 2 brazilin ion peaks which are shown in shaded area.

SEQ-16112-01 2/1/2011 2:26:34 PM Brazilin #1; 20 uL
Hydro-RP:0.17:100.0(0)>5:95(45-60)/254 nm/(-)ESI

SEQ-16112-01 #1194-1216 RT: 25.66-25.86 AV: 3 SB: 4 24.79-25.26 NL: 1.05E7
T: -c ESI sid=2.00 Full ms [122.00-500.00]



SEQ-16112-01 #1689-1710 RT: 33.77-33.99 AV: 3 SB: 4 34.25-34.47, 33.27-33.47 NL: 2.30E6
T: -c ESI sid=2.00 Full ms [122.00-500.00]

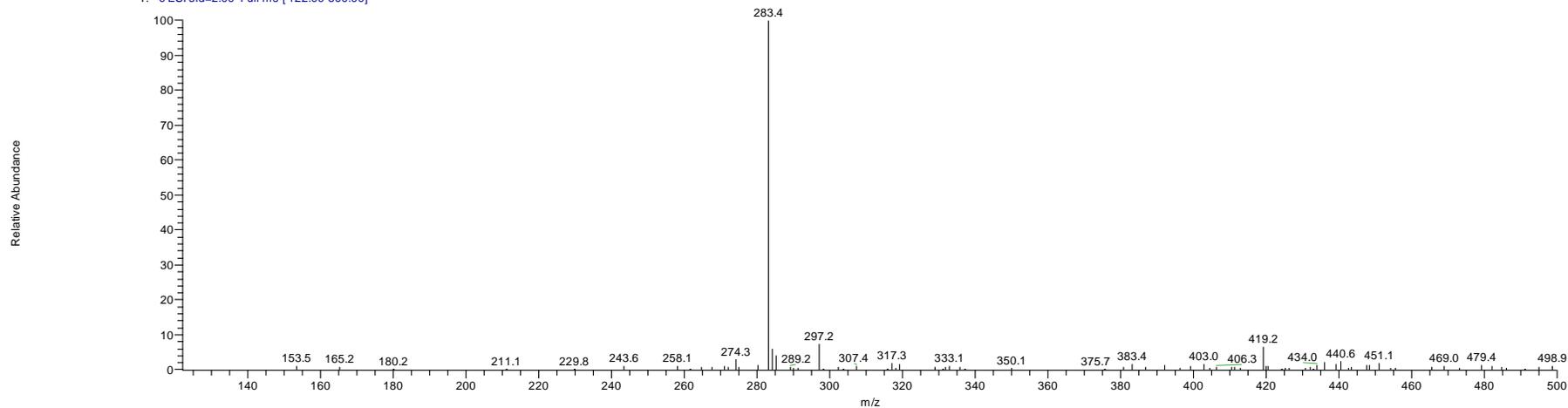
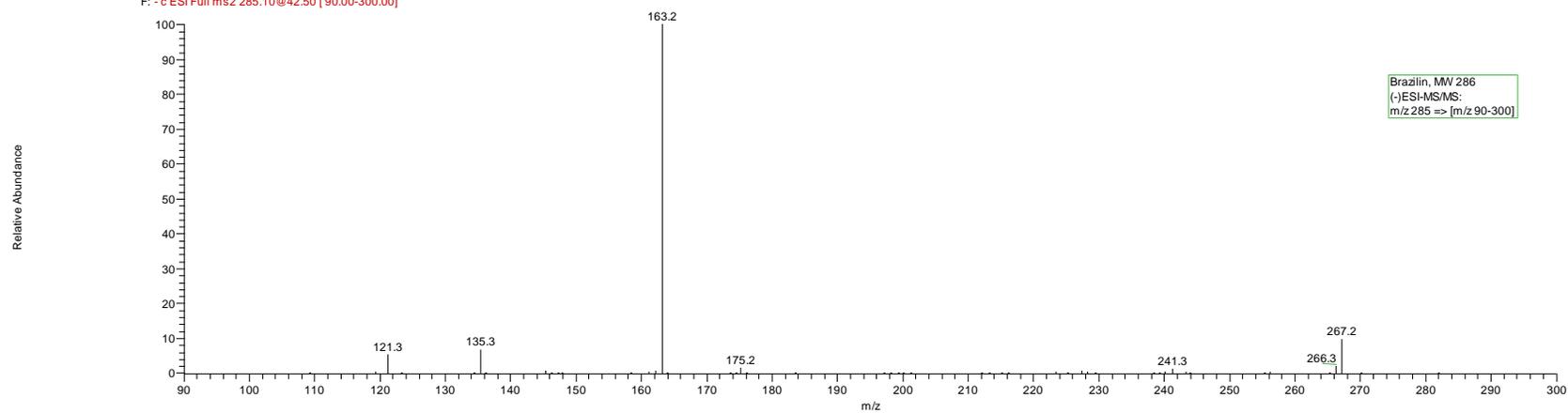


Figure D-3. Mass spectrum of brazilin (top) sample and oxidized brazilin sample (bottom) with (-)ESI-MS

SEQ-16112-01 2/1/2011 2:26:34 PM Brazilin #1: 20 uL
Hydro-RP.0.17;100.0(0)>5.95(45-60)/254 nm/(-)ESI

SEQ-16112-01 #1198-1233 RT: 25.72-26.12 AV: 5 SB: 4 24.79-25.26 NL: 2.77E6
F: - c ESI Full ms2 285.10@42.50 [90.00-300.00]



SEQ-16112-01 #1663-1719 RT: 33.36-34.06 AV: 7 SB: 3 34.25-34.47, 33.27-33.47 NL: 2.81E5
F: - c ESI Full ms2 283.20@42.50 [90.00-300.00]

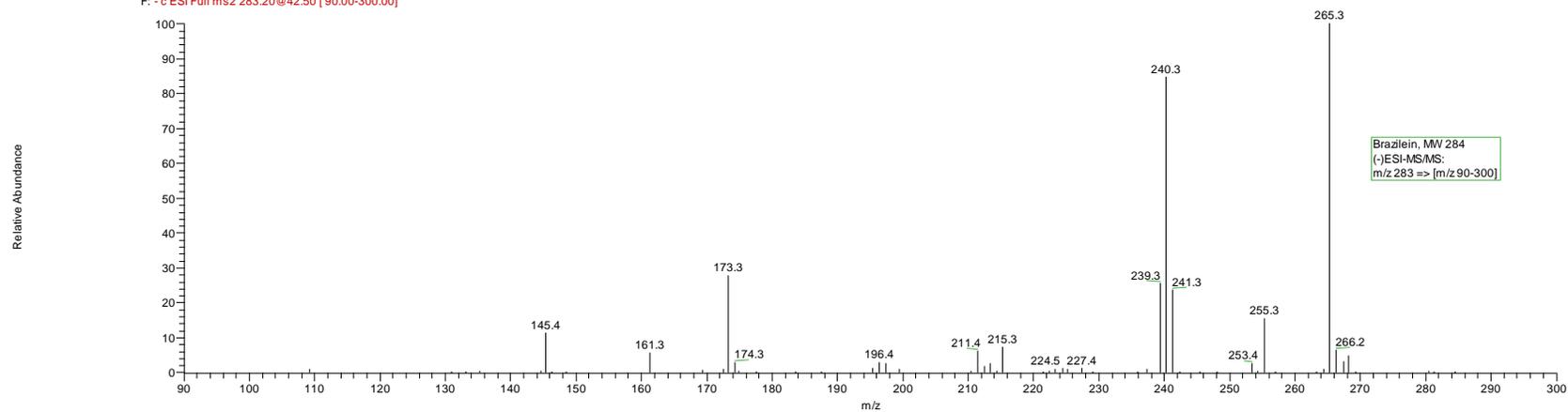


Figure D-4. The [M-H]⁻ ions of brazilin (top) and brazilein (bottom) were disassociated to produce a number of product ions.

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BIOGRAPHICAL SKETCH

Valeeratana Kalani Sinsawasdi was born in Hawaii and raised in Bangkok, Thailand. She earned her bachelor's degree in Food Science from Chiangmai University in Thailand, then worked in the international marketing department of Griffith Laboratories. She attended the University of Hawaii, and earned a master's degree in Food Science through an Asia-Pacific Scholarship.

After finishing graduate school, she worked in California as an HACCP coordinator for Stockton Further Processing, also at the same time, as a HACCP consultant for Angelina Foods. Then she worked as a Quality Assurance Manager for Dean Foods in the largest rBST-free milk processing plant on the West Coast, until moving back to Thailand.

In Bangkok, she worked for Unilever Thai Trading Ltd. Her first position was as a Product Development Manager for the local food division. Her last position before leaving the food industry for academia was as a Food Science Manager for Unilever Health Institute-Asia which served all Unilever companies in Asia and Australia.

During her current position at Mahidol University International College (MUIC), she taught many undergraduate level courses in the Food Science Department. She will continue to work with MUIC after completing her Ph.D. in Food Science at the University of Florida.