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Preparation of the Chinese sweet leaf tea extract and its anti-obesity effect in rodents

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**PREPARATION OF THE CHINESE SWEET LEAF TEA EXTRACT AND ITS ANTI-
OBESITY EFFECT IN RODENTS**

A Thesis

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
Requirements for the degree of
Master of Science**

in

The School of Renewable Natural Resources

**by
Gar Yee Koh
B.S., Louisiana State University, 2007
December 2009**

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ABSTRACT

The aqueous leaf extract of *Rubus suavissimus* (Chinese sweet leaf tea) was active in inhibiting angiogenesis, which could be used to suppress adipogenesis and cause weight loss. Following this revelation and approach, the anti-obesity effect was thoroughly investigated in normal and obese rat models.

The investigations began with the preparation of quality-controlled extract. First, the crude extraction methods were examined leading to the characterization of the chemical composition. Second, the impure components were removed by alcohol precipitation and column chromatography, resulting in a standardized sweet leaf tea extract (RUS). Third, three bioavailable compounds identified from the urine analyses were re-constituted to form the purified sweet leaf tea extract (GER), which accounted for 27% by weight of the RUS. These standardized and purified sweet leaf tea extracts were then evaluated for their anti-obesity effect in normal and obese-prone rats.

Both RUS and GER reduced body weight gain by 7% ($p = 0.999$) in normal rodents but statistical analyses failed to show any significance due to small sample size. Amazingly, it was discovered that the use of the tri-compounds combination (GER) produced the same anti-obesity effect as the standardized extract (RUS). Consequently, new experiments were focused on the GER using obese-prone rats as the study model. GER showed a significant anti-obesity effect by reducing 22% ($p \leq 0.001$) weight gain compared to the high-fat diet control group. Total abdominal fat was reduced significantly by 48%, indicating that the lost body mass was mainly due to the loss of body fat. Other benefits of the Chinese sweet leaf tea extract included lowered blood glucose, cholesterol, and triglycerides. Food intake was not affected. No adverse effects were observed.

This research provides exciting insights about the novel use of the Chinese sweet leaf tea as a safe and effective anti-obesity agent beyond its historic use as a natural sweetener. Human clinical investigations are strongly warranted to determine the effective doses and long-term use safety in combating obesity or managing body weight.

CHAPTER 1. INTRODUCTION

Epidemiological and experimental studies have provided convincing evidence that the consumption of plant-derived foods, such as vegetables, fruits, nuts, spices, and grains, exerts beneficial effects on human health due to the presence of phytochemicals or non-nutrient secondary metabolites in them. Crude herbs, for example, have been used as traditional medicines in different cultural practices to treat or prevent diseases, including cancer (Stoner and Mukhtar 1995; Pezzuto 1997), metabolic syndrome (Xie et al., 2002; Ekanem et al., 2007; Lemaure et al., 2007; Modak et al., 2007; Lee et al., 2008; Yin et al., 2008), inflammation (Fang et al., 2008; Rogerio et al., 2008; Papoutsi et al., 2008), and infectious diseases (Patel et al., 2004). In the past few years, bioactive components found in plant-derived food, such as polyphenol and terpenoids, have been commercialized as food or non-food products due to the increased demand from the health conscious consumers (Espin et al., 2007; Itokawa et al., 2008). *Rubus* species (Rosaceae), among the common herbal medicines, have long been used for therapeutic purposes (Patel et al., 2004). In the screening of plant-sourced angiogenesis inhibitors, a perennial plant widely distributed in southwestern China, *Rubus suavissimus*, was tested positive for antiangiogenic activity and gallic acid (**Fig. 1.1**) may be partially responsible for the activity (Liu et al., 2006).

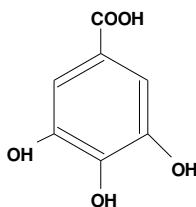


Figure 1.1. Gallic Acid Structure

Currently, there are 250 species of *Rubus* established worldwide in the Rosaceae family. *Rubus* species have been used traditionally as therapeutics and shown anti-cancer, anti-bacterial, anti-diabetes, and anti-anemia properties (Patel *et al.*, 2004). *R. suavissimus* S. Lee is a perennial

shrub widely abundant in Guangxi and Guizhou provinces of China. The leaf of *R. suavisissimus* is the material to make beverage leaf tea by the local residents. Due to its intense natural sweet taste, it is often called tiancha in Chinese, or “Chinese sweet tea” (Liu *et al.*, 2006). The sweet taste from the sweet leaf is attributed to the presence of diterpene glucosides where the major sweet principle comes from rubusoside (Patel *et al.*, 2004; Liu *et al.*, 2006). These sweet diterpene glucosides were limited to the leaves and fruits of *R. suavisissimus* (Chou *et al.*, 1987) and undetectable in the roots. Instead, triterpene glucosyl esters, such as the suavisissimoside F1, were specifically isolated from the roots of *R. suavisissimus* (Gao *et al.*, 1985). Other diterpene glucosides contributing to the sweetness and bitterness of the tea leaf include the sweet glycosides, suavioside A (Hirono *et al.*, 1990), suaviosides B, G, H, I, and J as well as the bitter glycosides, suaviosides C1, D2, and F (Ohtani *et al.*, 1992). Rubusoside (**Fig. 1.2**), a sweet steviol glycoside as well as a dominant diterpene glucoside, is widely used as a natural sweetener and food additive in the food industry (Tanaka, 1997; Sugimoto *et al.*, 2002) due to its intense sweetness. The sweetness of rubusoside is said to be about 115 times sweeter than sucrose at a concentration of 0.025%. However, it has a slightly bitter aftertaste which affects the quality of rubusoside as a candidate for natural sweetener (Hirono *et al.*, 1990; Patel *et al.*, 2004; Liu *et al.*, 2006). Therefore, improvements in the sweetness and quality of rubusoside through enzymatic 1, 4- α -transglucosylation were investigated previously and a significant improvement of sweetness was observed in most di- and tri-glycosylated products (Darise *et al.*, 1984; Kitahata *et al.*, 1989; Ohtani *et al.*, 1991; Tanaka, 1997).

In addition to the use of Chinese sweet leaf as a natural sweetener, it has been applied as a folk medicine to treat various diseases. In southern China, the sweet leaf is used as a traditional remedy to treat hypertension, diabetes, and atherosclerosis, maintaining healthy kidneys, as well as to relieve cough (Huang and Jiang, 2002). Recent studies have also demonstrated that the

Chinese sweet leaf tea extract exhibits anti-inflammatory, anti-allergy (Ishikura et al., 1995; Kotaro, 1997; Ono, 2004), and anti-angiogenesis activities (Liu *et al.*, 2006). As a potential natural inhibitor of angiogenesis, sweet leaf tea extract has been reported capable of reducing corneal neovascularization in experimental rodents (Oner et al., 2007). Moreover, the ability of sweet leaf extract in inhibiting the transcription factor nuclear factor kappa B or, NF- κ B (Liu *et al.*, 2005) and α -amylase activity (Li et al., 2007), may prevent certain metabolic diseases such as diabetes and obesity. This may further support the use of the tea leaf as a traditional folk medicine in treating or regulating glucose metabolism. More recently, a validated chemical fingerprint analysis was developed, which further provides a reliable quality assessment of the Chinese sweet leaf tea extract (Chou et al., 2009). In the chemical fingerprint analysis, Chou et al. (2009) has identified five marker compounds in the leaves of Chinese sweet tea plant, which include the gallic acid, rutin, ellagic acid, rubusoside, and steviol monoside. With the development of chemical fingerprint analysis for the Chinese sweet leaf tea extract, qualitative and quantitative measurements of the extract can be determined more accurately. This could translate to reduced batch-to-batch variations.

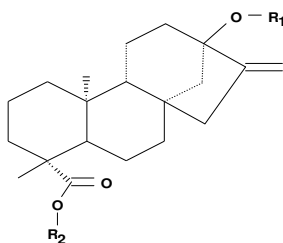


Figure 1.2. Rubusoside Structure: R₁ = R₂ = Glucose

Since most studies on Chinese sweet leaf tea were currently focused on the chemical analyses, a validated and thorough study relating to the *in vivo* studies is scarce. Thus, it brought up another question whether these bioactive components found in the leaves of *R. suavissimus* are orally active and can produce a positive response in live animals. Indeed, to ensure consistent

and reliable results from either *in vitro* or *in vivo* studies, quality controlled extracts with promising bioactivity are essential. In the present study, preparation of the extract using different extraction methods and alcohol precipitation was examined. The prepared and purified extract was evaluated for its anti-obesity effect. It is hoped that present study would provide a defined pharmacological effect of the Chinese sweet leaf extract and the effect can be repeated in future human studies.

The objectives of this study are (1) to compare the novel extraction techniques with the traditional extraction method in the production of high quality leaf extract, (2) to develop an efficient and cost effective method in purifying the crude extract of the leaves of *R.suavissimus*, (3) to examine the effect of the purification procedure on the changes of chemical composition between the purified and precipitated leaf extract, (4) to determine the bioactive and bioavailable chemical compounds *in vivo* via chemical analyses, and (5) to examine anti-obesity effect of the Chinese sweet leaf tea extract in normal and high fat diet-induced rats. The information shortage on the sample preparations and its pharmacological function on body weight changes prompted the investigation over the Chinese sweet leaf tea extract. Hopefully, with the detailed investigations, we will be able to reveal the functions of the Chinese sweet leaf tea extract beyond its historic use as a natural sweetener or beverage leaf tea.

CHAPTER 2. EXTRACTION OF BIOACTIVE COMPONENTS IN THE CHINESE SWEET TEA PLANT (*RUBUS SUAVISSIMUS* S. LEE) BY DIFFERENT METHODS

2.1 Background

Rubus suavissimus is a perennial shrub naturally abundant in Southern China. Due to their intense sweet taste, the leaves of the *R. suavissimus* (Chinese sweet leaf tea plant) have been used in making beverage leaf tea by the local residents. Rubusoside, one of the dominant sweet principles in the Chinese sweet leaf tea plant, is 115 times sweeter than sucrose at a concentration of 0.025%, making it a good candidate for natural sweetener (Ohtani et al., 1992; Sugimoto et al., 2002). In addition, the Chinese sweet leaf has also been applied as a folk medicine to treat various diseases such as hypertension, diabetes, atherosclerosis, to maintain healthy kidneys and relieve coughs (Huang and Jiang, 2002), as well as to alleviate inflammation and allergy (Kotaro, 1997; Ono, 2004; Fang et al., 2008). Recently, the aqueous leaf extract of *R. suavissimus* was also found to inhibit angiogenesis, partially via gallic acid that was present (Liu et al., 2006).

While our research focuses on analyzing the water extract of Chinese sweet leaf tea (Chou et al., 2009) and testing it for bioactivities (Liu et al., 2006; Oner et al., 2007) because of the historic beverage use, the question centers around whether a sweet-taste-based tea preparation stands to the bioactivity test and validation. This is because a thorough and validated study on the preparation of the tea leaf extract using non-traditional methods has not yet been conducted nor evaluated. We are concerned if the traditional hot water extraction of the Chinese sweet leaf tea has actually maximized the recovery of desired bioactive components rather than the sweetening components only. This concern prompted the investigation to determine if the traditional extraction method oriented for preparing beverage tea is suitable in the preparation of the functional tea extract characterized by the bioactive compounds. Since the initial crude

extraction method defines the extractable composition for further purifications using methods such as alcohol precipitation (Koh et al. 2009), the initial crude extraction method is a crucial but often ignored step that warrants special examination.

Maceration using food-grade and food-compatible organic solvent and hot water extraction are the most common extraction methods in the preparation of the traditional herbal medicines. Chinese sweet leaf tea, for example, is conventionally prepared by the hot water extraction or the boiling method. Li et al. (2007) reported that these conventional extraction methods were effective in extracting antioxidant from a wide variety of plants and are applicable to both food industry and individual consumers at home. During the maceration process, plant materials are soaked in an organic solvent for certain lengths of time often assisted with agitation to facilitate the diffusion of metabolites into the solvent. However, maceration requires large amounts of solvent and lengthy time. Therefore, the simple maceration method, comparing to other novel extraction techniques such as pressurized liquid extraction (PLE), is labor-intensive and time-consuming for a complete recovery of desired components. Hot water extraction is another common method used in tea preparations. Although the hot water extraction takes much less time, the extracted components through the method often are water soluble (e.g., polysaccharides).

In addition to the conventional extraction methods described above, recently developed new extraction techniques such as PLE can be employed for the initial crude extraction of the Chinese sweet leaf tea. These new techniques generally have the advantages of reducing the consumptions of organic solvents, decreasing the risk of sample degradation, and saving time and labor (Deng et al., 2007). These advantages result in the improvement of overall extraction efficiency and effectiveness. PLE, initially designed for extracting environmental pollutants (Benthin et al., 1999; Ong et al., 2004), is becoming a widely used technique in the

pharmaceutical and food industries. (Shen and Shao, 2005; Herrero et al., 2006; Jiang et al., 2006; Li et al., 2006; Peres et al., 2006; Smelcerovic et al., 2006; Chukwumah et al., 2007; Jiang et al., 2007).

PLE uses organic solvent at elevated temperatures up to 200°C and relatively high pressure between 4 and 20 MPa (Ong et al., 2004; Deng et al., 2007). With the elevated temperature and pressure, the viscosity and surface tension of the solvent are decreased, and thereby accelerate the solubility and diffusion rate of compounds (Huie, 2002; Deng et al., 2007). Studies on medicinal plants (Waksmundzka-Hajnos et al., 2004; Li et al., 2006; Jiang et al., 2006; Peres et al., 2006; Jiang et al., 2007) demonstrated the advantages of PLE in reducing the solvent volume and extraction time. Under an optimized condition, only one extraction cycle was needed to recover most active constituents in plants (Jiang et al., 2006; Li et al., 2006) whereas soxhlet and sonication often require multiple extraction cycles.

The current study was to investigate the effects of alternative solvent and new extraction apparatus on the extraction of Chinese sweet leaf tea using the traditional hot water extraction as the control. These effects were evaluated through the qualitative and quantitative analyses of various resulting crude extracts on high performance liquid chromatography (HPLC). Five marker components, gallic acid, rutin, ellagic acid, rubusoside, and steviol monoside were quantified. Experiments were designed to test the hypothesis that the traditional hot water extraction method is not sufficient to extract these five functional compounds.

2.2 Materials and Methods

2.2.1 Plant Materials

Air-dried leaves of the Chinese sweet leaf tea plant (*Rubus suavissimus* S. Lee) (Batch#SLT20070206) were provided by Guangxi Normal University in Guilin, China.

2.2.2 Sample Preparations

2.2.2.1 Hot Water Extraction (HW)

Dried and ground leaves were weighed into an Erlenmeyer flask and soaked for an hour in deionized and distilled water at a ratio of 1:15 w/v. The water solution was brought to boil for 60 min. Then, the solution was filtered with a filter paper (Whatman #4) to separate the solid from the liquid. The solid residuals were rinsed with half of the initial solvent volume, filtered, and combined with the first extraction. The combined solutions were later concentrated and freeze-dried to a powdered extract and the extraction yield was obtained (% w/w). This procedure was done in three replications.

2.2.2.2 Maceration (MA)

Dried and ground leaves were weighed into an Erlenmeyer flask and extracted at a leaf to solvent ratio of 1:15 w/v with aqueous ethanol of 0%, 20%, 50%, 70%, or 95%, respectively. To perform the extractions, the samples were mixed and sealed well with parafilm to avoid contamination and to minimize solvent evaporation. Then, the samples were placed on an orbital shaker at 80 rpm for 72 hours. After 72 hours, the extract solutions were separated from the solids through filtration with filter papers (Whatman #4) and the solid residuals were rinsed with half of the initial solvent volume. Supernatant solutions were later combined, condensed, and freeze-dried to obtain the powdered extracts and extraction yield (% w/w). Dried extracts were stored in room temperature until HPLC analyses. Each treatment was done in three replications.

2.2.2.3 Low-pressurized Liquid Extraction (LP)

The low-pressurized liquid extraction was performed with an extractor (TECNOLAB, Via Vitale Rosi, Spello, Italy) equipped with a 2-L extraction cell. Dried and ground leaves were weighed into the extraction cell and extracted with aqueous ethanol of 0%, 20%, 50%, 70%, or 95% at a leaf to solvent ratio of 1:15 w/v, respectively. Extraction was conducted at 85 psi (5.86 bars) for

60 min (6 extraction cycles with 8 min of compression and 2 min of decompression) at 20°C. Extract solutions were discharged and residuals were rinsed with half of the initial solvent volume. The initial and rinsed liquids were combined, filtered with filter paper (Whatman # 4), and then freeze-dried to obtain powdered extracts and extraction yield (% w/w). Dry extracts were stored in room temperature until HPLC analyses. The procedure was done in three replications.

2.2.2.4 High-pressurized Liquid Extraction (HP)

The high-pressurized liquid extraction (ASE 150, Dionex, Bannockburn, IL) was conducted under the pressure of 1500 psi (103 bars) and temperature at 80°C. Under these conditions, the leaf samples were extracted for 30 min (15 min per cycle) using 0%, 20%, 50%, 70%, or 95% aqueous ethanol, respectively. After the extraction, the samples were rinsed with an equal volume of initial solvent and purged for 100 seconds. All extractions were done with a 34-ml extraction cell containing 5 g of leaf material each at a leaf to solvent ratio of approximately 1:15 w/v. Extraction and rinse solutions were collected and combined, then evaporated and freeze-dried to obtain powdered extracts and extraction yield (% w/w). Dry extracts were stored in room temperature until HPLC analyses. The procedure was done in three replications.

2.2.3 HPLC Analysis

2.2.3.1 Reference Standards

Gallic acid (GA; Purity > 98%), rutin (RUT; Purity > 95%), and ellagic acid (EGA; Purity > 95%) were purchased from Sigma Chemical Company (St. Louis, MO). The reference standards of rubusoside (RUB) and steviol monoside (STM) were isolated by our own lab and identified by spectral data (UV, MS, ^1H NMR, ^{13}C NMR and 2D-NMR). Both RUB and SM have purities greater than 98% by HPLC–PDA analyses based on a peak-area normalization method.

2.2.3.2 HPLC Conditions

An HPLC system consisting of a Waters (Milford, MA) 600 pump, a 717 auto-sampler, and a UV/Vis Photodiode Array (PDA) 2996 Detector were used for all analyses. Chromatographic separations were run on an Alltech Prevail C18 column (250 mm × 4.6 mm, 5 μm) with a C18 Guard column (7.5 mm×4.6 mm, 5 μm). The mobile phase was consisted of solvent A (0.17% phosphoric acid in acetonitrile) and solvent B (0.17% phosphoric acid in water). The elution profile for A is: 0–65 min, linear gradient of 5–30%; 65–85 min, linear gradient of 30–60%; 85–90 min, linear gradient of 60–70%; and 90–100 min, isocratic 70%. A pre-equilibration period of 20 min was used between individual runs. Column temperature was maintained at 25°C. The flow rate was 1.0 mL/min and the injection volume was 10 μL. All compounds were detected at dual wavelengths of 254 and 205 nm to derive at combined chromatograms.

2.2.4 Similarity Analysis

A similarity test among the 16 samples resulted from HW (control) and 15 treatment combinations consisting of three extraction methods and five solvent systems was performed using the software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (2004A). The matching of the fingerprints of each sample was done by a multipoint calibration mode based on retention time and UV spectra. With the test, a reference chromatogram was generated from the samples as a standard fingerprint. Then, the similarity of each chromatogram against this reference chromatogram was calculated.

2.2.5 Statistical Analysis

Data were analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System (SAS, Cary, NC). The effects of solvent systems (SOLVENT) and extraction methods (METHOD) on the yield of five marker compounds were analyzed. Two-way factorial arrangements were examined at 3 (extraction methods with MA, LP, and HP) and 5 levels

(aqueous ethanol concentrations at 0, 20%, 50%, 70%, and 95%), respectively. Tukey's post hoc test was performed to compare the means of the main effects as well as their interactions. Significance of all tests was set at $p \leq 0.05$ and all results reported were expressed as mean \pm SEM unless otherwise specified.

2.3 Results

2.3.1 Effects of the Extraction Methods and Solvent Systems on the Yields of Marker Compounds in the Chinese Sweet Leaf Tea Extract

Compared to the commonly used hot water extraction (HW), the LP method extracted significantly less components regardless of the solvent systems used (**Fig. 2.1**). Similarly, the extraction yield (the extractable solids) was the lowest at the 95% solvent system regardless of the extraction method used. Under the above conditions, the hot water extraction was superior in term of extraction yield. Except at the 95% solvent system, the HP and MA methods in combination of all solvent systems resulted in either equal or better extraction yield than the hot water extraction method. In fact, the highest extraction yield (about 35% w/w) was attained by the HP method when combined with the 20% to 70% aqueous ethanol, superior to the hot water extraction method with a yield of 28.6% w/w.

To further examine the effect of the extraction methods and solvent systems as well as their interaction on each of the marker compound, a two-way factorial arrangement was conducted. Results showed that the main effects, extraction methods and ethanol concentrations, as well as their interactions had significant influences on the concentrations of all markers compounds (**Table 2.1**).

In the extracts obtained using various combinations of extraction methods and solvent systems, the effects on the concentrations of each marker compound varied. The MA method extracted significantly more gallic acid than the HW method, especially when extracted with

20% ethanol, to a concentration of 0.26% w/w, which is almost 5-fold more than that extracted with the HW method (**Fig. 2.2**). Most of the other extraction methods or solvent systems extracted about the same amount of gallic acid as the commonly used hot water extraction, although some combinations did either slightly better or poorer.

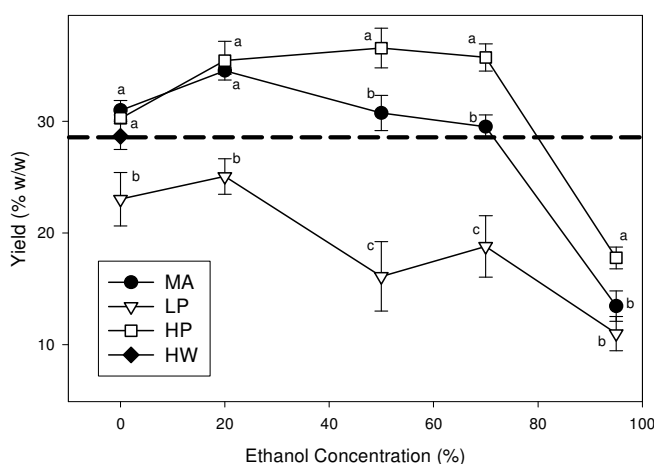


Figure 2.1. Effect of ethanol concentrations on the total yield (% w/w) of the Chinese sweet leaf tea (*Rubus suavissimus*) extract prepared by the maceration (MA), low-pressurized (LP), and high-pressurized (HP) liquid extraction methods. The commonly used hot water extraction (HW) is used as reference shown in a dash line. Different letters indicate a significant difference in the extraction yields among the extraction methods at the particular ethanol concentration. Values are expressed as mean \pm standard deviation (vertical line; $n=3$).

Unlike gallic acid, the concentrations of rutin, ellagic acid, rubusoside, and steviol monoside extracted by the MA, LP and HP methods increased with the ethanol concentrations in a non-linear fashion and reached their maximum yields at 70% or higher (**Fig. 2.2**). Interestingly, the concentrations of these compounds extracted by the MA, HP, or LP were not affected by the solvent systems ranging from 20% EtOH to 70% EtOH. The LP method, however, behaved differently from the MA or HP method. Instead of increases, the 95% aqueous ethanol system caused rapid decreases of rutin, ellagic acid, rubusoside, and steviol monoside by 22%, 12.5%, 11% and 8% with LP method, respectively, from their peak values seen at the 70% EtOH.

Table 2.1. Statistical results of the main effects, Method and Solvent, as well as their interactions (Method*Solvent) on the concentrations of the five marker compounds, gallic acid (GA), rutin (RUT), ellagic acid (EGA), rubusoside (RUB), and steviol monoside (STM).

Compounds	Num DF	Den DF	F-Value	Pr > F
<u>GA</u>				
Method	2	30	373.18	<.0001
Solvent	4	30	364.34	<.0001
Method*Solvent	8	30	121.27	<.0001
<u>RUT</u>				
Method	2	30	12.25	0.0001
Solvent	4	30	101.29	<.0001
Method*Solvent	8	30	3.59	0.0049
<u>EGA</u>				
Method	2	30	106.65	<.0001
Solvent	4	30	473.09	<.0001
Method*Solvent	8	30	18.77	<.0001
<u>RUB</u>				
Method	2	30	79.60	<.0001
Solvent	4	30	889.00	<.0001
Method*Solvent	8	30	41.39	<.0001
<u>STM</u>				
Method	2	30	132.44	<.0001
Solvent	4	30	1354.23	<.0001
Method*Solvent	8	30	27.95	<.0001

Regardless of any method used, the organic solvent systems were superior to the HW method in extracting rutin, ellagic acid, and steviol monoside. Steviol monoside, indeed, appeared to be non-extractable with the HW or other methods using water. However, a different phenomenon was observed in the extraction of rubusoside. **Fig. 2.2** showed that the concentration of rubusoside prepared by the HW method was almost 3-fold higher (17.9% w/w) than other water extraction methods (i.e. MA, LP, or HP) and it was not significantly different from those extracted with 20% to 70% aqueous ethanol using the MA, LP, or HP method. Still, the highest concentration of rubusoside (24% w/w) was achieved with the MA or HP method in

combination with the 95% solvent system (**Fig. 2.2**), which was approximately 25% higher than that extracted by the HW method.

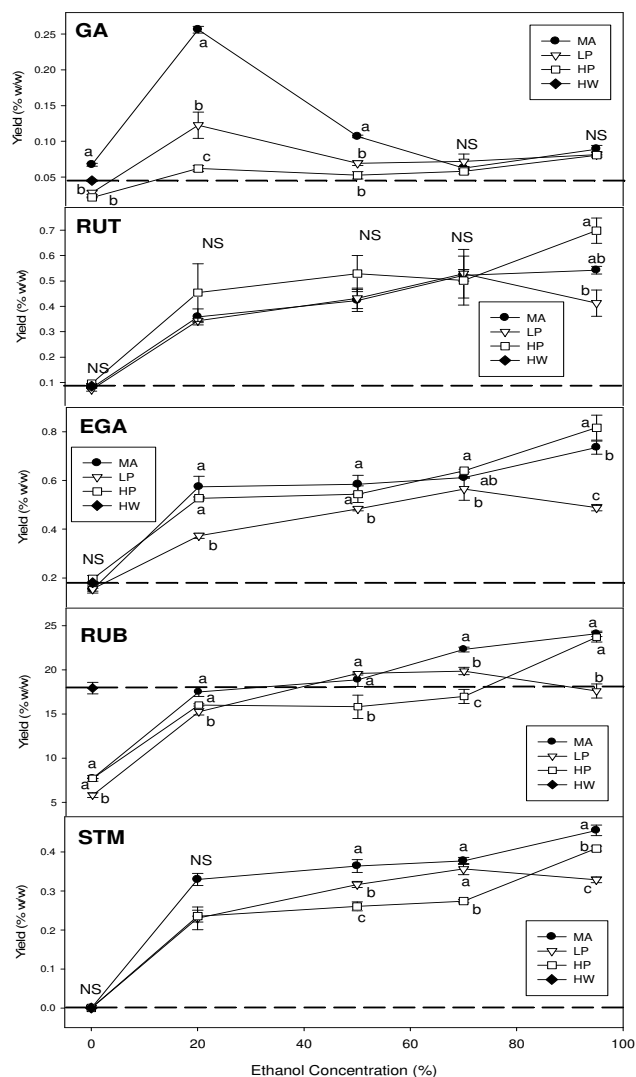


Figure 2.2. Effect of ethanol regimens on the concentration (% w/w) of the five marker compounds in the Chinese sweet leaf tea extract (*Rubus suavissimus*) prepared by the maceration (MA), low-pressurized (LP) and high-pressurized (HP) liquid extractions. Hot water extraction (HW) is used as a reference shown in a dash line. Different letters indicate a significant difference in the yield of compounds among the extraction methods at the particular ethanol concentration. Values are expressed as mean \pm standard deviation (vertical line; n=3).

2.3.2 Chromatographic Fingerprint Analysis of the Extracts

Overall, the MA and HP methods extracted higher amount and diverse species of chemical components. To best illustrate this statement, the chemical compositions of their extracts were

visualized in chromatographic fingerprints focusing on not only the five marker compounds but also other unknown components that possess characteristic UV absorption peaks (e.g., Peaks 1-8 in **Fig. 2.3**).

The UV absorption profiles showed more abundant peaks in the extract prepared by the HP than those by the MA method. Gallic acid showed the highest peak area at 20% EtOH, while other marker compounds maximized at 95% EtOH. Among the unknown components, Peak 1 appeared to be a single component, with a retention time of approximately 12 minute, displayed its highest absorption peak area at lower ethanol solvent systems (i.e., 20% and 50% EtOH). In the 95% EtOH system, peak 1 decreased significantly. This phenomenon was similar in either MA or HP method. Peak 2 to peak 7, however, spiked at either 70% or 95% EtOH. Indeed, peak 8 appeared to have lower absorption area in the extract prepared by the HP than that by the MA method. In the comparison of all known and unknown compounds, obviously, water extracts have less number of peaks and lower degrees of chromatographic absorption profiles than the extracts prepared by aqueous ethanol.

2.3.3 Similarity Analysis among Extracts

In order to access the similarities of extracts resulted from the HW method and other 3 × 5 treatment combinations, chromatograms resulted from each treatment were compared against a standard chromatogram using a software (Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (2004A)). Generally, the closer the similarity value, the more similar the samples are. In the study, the similarities of the samples against the reference chromatogram ranged from 0.276 (least similar) to 0.729 (most similar) (**Table 2.2**). In the comparison of HP and MA methods across the five solvent levels, the extracts' similarities reached 98% with the 95% EtOH treatment and only 53% with 20% EtOH, in agreements with the HPLC analyses based on the known five compounds. For the extracts prepared by the LP

method, however, the similarities ranged from 88.7% (70% EtOH) to 98.1% (20% EtOH) when compared to the MA method. More interestingly, the similarities of extracts prepared by MA, LP, and HP method with 95% EtOH were 98.4%, 94.56%, and 99.9%, respectively, when compared to the extract prepared by the HW method. It is therefore to conclude that the solvent system, not the extraction methods, was a dominant factor influencing the similarities of the extract, based on all UV-detected components (known and unknown).

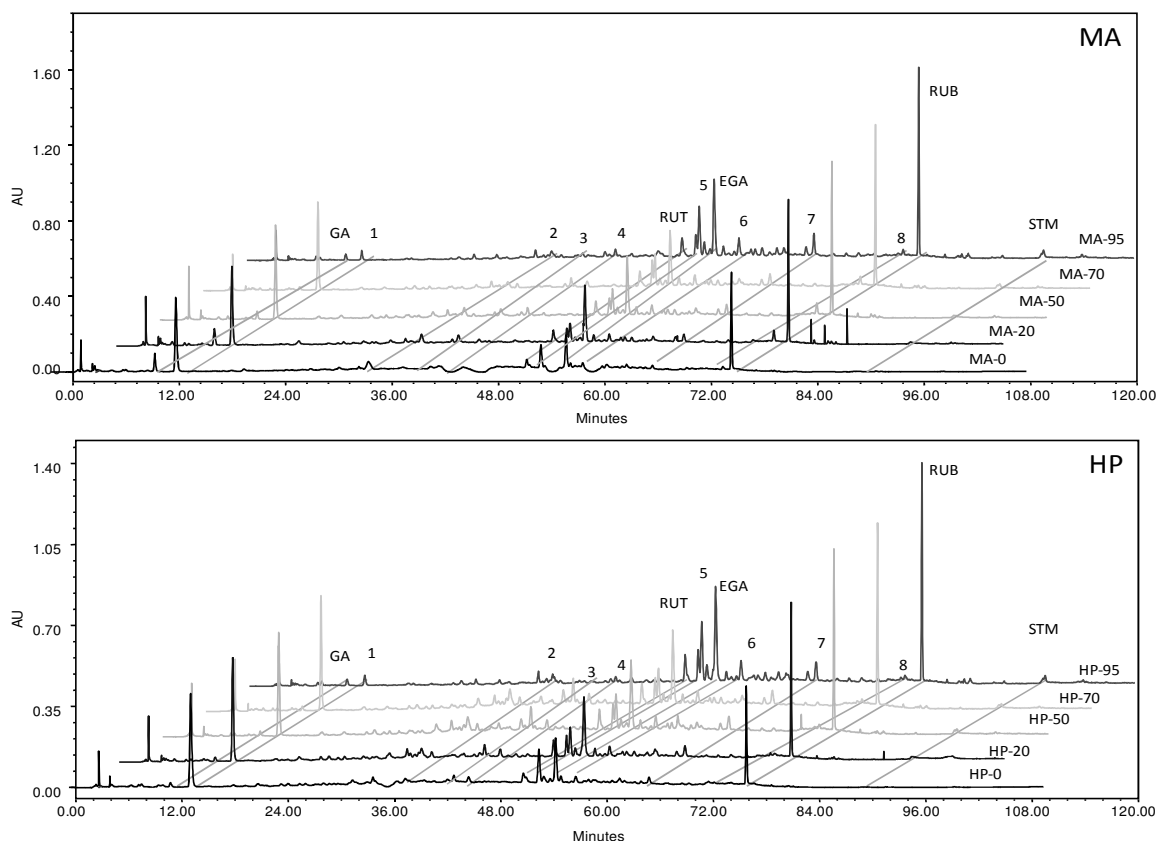


Figure 2.3. Chromatographic fingerprint of the Chinese sweet leaf tea (*Rubus suavissimus*) extracts prepared by maceration (MA) and high-pressurized liquid extraction (HP). Selected chromatograms demonstrated the extractions using the aqueous ethanol at 0% (0), 20% (20), 50% (50), 70% (70), and 95% (95). Presence of the five marker compounds is indicated as gallic acid (GA), rutin (RUT), ellagic acid (EGA), rubusoside (RUB), and steviol monoside (STM). Peak 1-8 are unknown compounds. NOTE: Alignment may be slightly off for some fingerprints due to graphic demonstration purpose.

Table 2.2. Similarity analyses among the 16 Chinese sweet leaf tea extracts prepared by hot water (HW), maceration (MA), low-pressurized liquid extractor (LP), and high-pressurized liquid extractor (HP), using either water (0), 20% (20), 50% (50), 70% (70), or 95% (95) aqueous ethanol over a reference chromatogram.

Sample	Similarity	Sample	Similarity	Sample	Similarity
HW-0	0.717	LP-0	0.677	HP-0	0.600
MA-0	0.654	LP-20	0.535	HP-20	0.276
MA-20	0.525	LP-50	0.577	HP-50	0.643
MA-50	0.664	LP-70	0.606	HP-70	0.725
MA-70	0.683	LP-95	0.678	HP-95	0.716
MA-95	0.729				

2.4 Discussion

Recent pharmacological studies on the extract of the Chinese sweet leaf tea have revealed significant health benefits and bioactivities due to the presence of phytochemicals in the plant extract (Ishikura et al., 1995; Kotaro, 1997; Ono, 2004) especially gallic acid (Liu et al., 2006) and rubusoside. It is predicted that the overall observed bioactivity of a prepared extract may be a concerted effort of more than one single component (Liu et al., 2006; 2009 unpublished data). Although traditionally the quality of the extract was determined on the basis of sweetness, the extract is now measured for the presence of other bioactive components due to the multiple bioactivities reported recently. For example, gallic acid is one of the active compounds with potent anti-angiogenic (Liu et al., 2006) and alpha-glucosidase inhibitory (Li et al., 2007) activities. Ellagic acid, as a strong antioxidant (Devipriya et al., 2007), possesses the properties of anti-cancer (Bell and Hawthorne, 2008; Zhang et al., 2008), anti-inflammatory (Papoutsis et al., 2008), and α -amylase inhibition (Li et al., 2007). Rutin has strong antioxidant (Kartika et al., 2007; Vukics et al., 2008) and anti-inflammatory activities (Rogerio et al., 2008). Although scientific research on rubusoside was mostly focused on the development of natural sweetener

(Tanaka, 1997; Sugimoto et al., 2002), preliminary data has supported that rubusoside may be potent in the inhibition of bacterial activities (unpublished data).

Liu et al. (2006) showed that the crude water extract of the Chinese sweet leaf tea had potent anti-angiogenesis activity, which was partially exerted by the presence of gallic acid. Clearly, a higher level of gallic acid is desirable in the Chinese sweet leaf tea extract. However, the overall anti-angiogenic activity was not explained by gallic acid alone, which strongly suggesting the needs of inclusion of other yet-to-be determined compounds. To closely monitor the changes of each chemical component at different levels of extraction conditions, detailed fingerprint analyses on the Chinese sweet leaf tea extract were developed (Chou et al., 2009). In addition to the five marker compounds, it is also imperative to retain other unknown components until proven not useful to avoid the loss of bioactivity. For example, peak 1 demonstrated a significant behavior when extracted with 95% EtOH (**Fig. 2.3**) suggesting its affinity to water than the ethanol. Peak 8, on the other hand, was extracted more sufficiently with the MA than the HP method (**Fig. 2.3**).

In addition to a valid HPLC method, sample preparation is the most critical procedure in recovering majority of the bioactive compounds, if not all, prior to further purification or bioactivity screening. Hot water extraction is a commonly applied method in making the beverage leaf tea and thus it was chosen as a reference method in the present study. Although our comparisons basically confirmed the validity of it in extracting the sweetening agent rubusoside and is economical from the solvent point of view, the hot water extraction method has some typical disadvantages. For example, the method requires a large volume of solvent (water) and extracts more unwanted water-soluble components such as carbohydrates thus contributes to a higher impurity of the extract. To optimize the extraction efficiency with purer extracts, extraction with the use of organic solvents through maceration and pressurized-liquid extractions

were proven viable in this study. In our study, aqueous ethanol was selected as the extraction solvent to recover some poorly water-soluble components in addition to the water-soluble components (Liu 2008). For the known compounds, the water solubility of gallic acid is 11 mg/mL, rutin and ellagic acid are nearly water-insoluble, steviol monoside is about 20 mg/mL, but rubusoside dissolves equally well in either water or ethanol. Therefore, the use of organic solvents is likely to increase the extraction efficiency of poor to water-insoluble components. Our results confirmed that this was the case with the sweet leaf tea. Gallic acid in a 20% ethanol dissolved more than that in hot water. In every other occasions, 95% ethanol dissolved rutin, ellagic acid, and steviol monoside significantly more than hot water. These results further confirmed that the bioactive components, such as gallic acid and ellagic acid, found in the sweet leaf tea extract, have better solubility in aqueous ethanol than in water. In the study of eight major classes of legumes, Xu and Chang (2007) reported that antioxidant activity of these legumes reached an optimum level when extracted with 70% aqueous ethanol. Our findings are in agreements with others where the aqueous ethanol, as compared to water, had stronger capability in extracting desired marker compounds.

In addition to the solvent system, the chemical compositions in an extract were highly dependent on the extraction conditions. Our results showed that gallic acid was highly sensitive to the temperature and pressure resulted from HP. The yield of rubusoside, on the other hand, was elevated in the hot water extraction method. Ellagic acid, rutin, and steviol monoside, were extracted maximally with 95% EtOH using either MA or HP method. HP has been reported to increase the extraction efficiency of botanical plants (Huei, 2002; Deng et al., 2007) by reducing the use of solvent volume and labor intensiveness. Our study further confirmed the increased efficiency of HP in extracting the major components in the Chinese sweet leaf tea. However, the decreased gallic acid level in the HP extract compared to the MA extract was a major drawback

for this method. Despite the yield of gallic acid, HP in combination with 95% ethanol was superior to the HW method in extracting all other marker compounds and the yields were comparable to the MA method.

The present study found that not a single extraction procedure could extract all marker components to respective maximum levels. Liu (2008) reported that the combination of extracts from several sequential extractions may be necessary in recovering majority of the components in botanical raw material for further bioactivity screening due to the diverse chemical properties of the bioactive components present in the plant. Therefore, to maximize the levels of all five major marker compounds in the extraction of Chinese sweet leaf tea, a sequential extraction may be useful. For instance, to obtain an extract that contains maximal levels of all five compounds, a sequential extraction method must consist of the extraction of raw material first with the 20% ethanol via MA to recover gallic acid, then with 95% ethanol to recover other compounds, using either the MA or HP method, from the same raw materials. However, to maximally extract the rubusoside from the raw material, HW method would be sufficient considering the unnecessary use of ethanol. As the sample preparation prompted the result consistency over further biological studies, an effective and efficient extraction method with standard protocol is warranted to reproduce the extract with elevated bioactivity level.

2.5 Summary

This is the first study evaluating the crude extraction of the Chinese sweet leaf tea using different extraction methods and extraction solvent systems. Based on the sweetness of the extract, the HW method was satisfactory in producing significantly higher amount of rubusoside. However, if the extraction aims to maximally extract bioactive components, organic solvent systems will have to be used. To maximally extract all five marker compounds, a sequential extraction procedure can be used to accomplish this goal.

CHAPTER 3. PURIFICATION OF A WATER EXTRACT OF CHINESE SWEET TEA PLANT (*RUBUS SUAVISSIMUS* S. LEE) BY ALCOHOL PRECIPITATION*

3.1 Background

Rubus suavissimus S. Lee (Rosaceae) is a perennial shrub widely abundant in Guangxi and Guizhou province of China. The leaf of *R. suavissimus* is the material to make beverage leaf tea by the local residents. Due to its intensely sweet flavor, it is better known as “tiancha” in Chinese, or “Chinese sweet tea”. The sweet taste from the leaf is attributed to the presence of diterpene glucosides, dominated by the major sweet principle rubusoside (Tanaka et al., 1981). Rubusoside has a slightly bitter aftertaste, but it is about 115 times sweeter than sucrose at a concentration of 0.025%, making it a good candidate for a natural sweetener (Ohtani et al., 1992; Sugimoto et al., 2002). Other diterpene glucosides contributing to the sweetness and bitterness of the leaf include the sweet glycosides, suavioside A (Hirono et al., 1990), suaviosides B, G, H, I, and J, as well as the bitter glycoside, suaviosides C1, D2, and F (Sugimoto et al., 2002).

In addition to the use of rubusoside as a natural sweetener, Chinese sweet leaf has also been used as a folk medicine to treat various diseases. For example, in southern China, it is used as a traditional remedy for alleviating hypertension, diabetes, atherosclerosis, and maintaining healthy kidneys as well as to relieve coughs (Huang and Jiang 2002). Recent studies have also demonstrated that sweet leaf exhibits anti-inflammatory, anti-allergic (Ono, 2004; Kotaro et al., 1997), and anti-angiogenic activities (Liu et al., 2006). As a potential natural inhibitor of angiogenesis, sweet leaf tea extract has been reported to be capable of reducing corneal neovascularization in experimental rodents (Oner et al., 2007). Furthermore, the ability of sweet leaf to inhibit the transcription factor NF- κ B (Liu et al., 2005) and α -amylase activity (Li et al., 2007) may also prevent certain metabolic diseases such as diabetes and obesity.

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Traditionally, the sweet leaf as beverage or folk medicine was prepared using boiling water or decoction. This preparation recovered bioactive compounds such as gallic acid, rutin, ellagic acid, rubusoside, and steviol monoside as well as other yet-to-be identified compounds (unpublished data) in a water extract of the sweet leaf. These bioactive compounds may play an important role in the development of pharmaceutical and food products. For instance, gallic acid is one of the active compounds that has potent anti-angiogenic (Liu et al., 2006) and α -glucosidase inhibitory (Li et al., 2007) activities. Ellagic acid and rutin, on the other hand, are strong antioxidants (Kartika et al., 2007; Vukics et al., 2008), and both compounds may also be responsible for anti-inflammatory activities (Papoutsis et al., 2008; Rogerio et al., 2008). In addition, ellagic acid also possesses potent α -amylase inhibitory (Li et al., 2007) and anti-cancer (Bell and Hawthorne, 2008; Zhang et al., 2008) properties. Although this preparation method may have extracted the majority of the bioactive components from the leaf material, it also pulls out a large amount of water-soluble polysaccharides and possibly other macromolecules such as proteins that are not bioactive, resulting in a crude leaf extract that has room for additional purification.

A purified extract with potentially improved bioactivity is highly desirable in many ways, including but not limited to reaching an effective dose range in a practical dosage. The first line of purification is often associated with the removal of polysaccharides that are not bioactive yet are highly extractable by boiling water. Alcohol precipitation is often used to achieve this initial purification of an aqueous extract. This alcohol precipitation method is simple, rapid, easily scalable, and cost effective in the removal of polysaccharides. Conventional applications of alcohol precipitation methods are mainly seen during the purification of plant DNA and RNA (Wang et al., 2004; Gemini et al., 2007) as well as isolation of biologically active polysaccharides (Schmourlo et al., 2005; Wu et al., 2007; Yang et al., 2008). However,

employing alcohol precipitation to purify active components from plant extracts is less common. Alcohol precipitation of crude plant extracts could separate macromolecules and polymers from small molecules including those of 1000 Daltons or less such as gallic acid, ellagic acid, rutin, and rubusoside. Therefore, it is hypothesized that during the precipitation process, most of the polymers, such as polysaccharides and proteins, will precipitate while the small molecules will stay in the supernatant solution. In the present study, this hypothesis was tested through a series of experiments and quantitative analyses. Qualitative and quantitative analyses of the purified extracts were performed using high performance liquid chromatography (HPLC) with the focus on the five bioactive components as adopted from Chou et al. (2009). Total polysaccharide in the precipitated extracts was measured by the phenol-sulfuric acid colorimetric method. It is hoped that this simple but effective method could be demonstrated for the sweet leaf tea extract and used to achieve satisfactory degrees of purification of many other bioactive botanical extracts.

3.2 Materials and Methods

3.2.1 The Sweet Leaf Material

The sweet leaf tea plants (*Rubus suavissimus* S. Lee; Rosaceae) used in this study grew on a farm in Guizhou province, China. A voucher specimen was obtained and deposited in the Herbarium of the Louisiana State University. Fresh sweet leaves from the sweet leaf tea plants were harvested in June and air-dried.

3.2.2 Preparation of the Crude Extract

The crude extract was prepared by the industry. The air-dried leaves were extracted with distilled water at approximately 1:15 w/v ratio. After soaking for 1 h, the decoction was brought to boil for 60 min. The liquid extract was separated from the solids by filtration with an approximately 100- μ m filter screen and cloth, and by still precipitation. In the industry operation, filtration and still gravity centrifugation were more cost effective than other separation methods such as

centrifugation. Thus, the particular procedure was chosen in the preparation of crude extract. Later, the filtered supernatant liquid extract was concentrated and subsequently spray-dried to powder and designated as the crude extract RUS (batch RUS20040306).

3.2.3 Alcohol Precipitation (AP)

The crude extract (RUS) prepared above was converted to aqueous solutions first by re-constituting the extract in deionized water at 1:4 w/v ratio, with the assistance of heat and stirring as needed. This aqueous solution was also used as a control regimen expressed as 0% AP (AP-0) subjected to no alcohol precipitation. For other regimens, appropriate volumes of ethanol (EtOH) were then added to the water extracts to achieve final ethanol concentrations of 10% (RUS-10), 20% (RUS-20), 30% (RUS-30), 40% (RUS-40), 50% (RUS-50), 60% (RUS-60), 70% (RUS-70), 80% (RUS-80), 90% (RUS-90), and 95% (RUS-95), respectively. These AP solutions were sealed with parafilm to avoid contaminations and minimize evaporation. Then, the solution was let stand for an hour at 4°C. Supernatant and precipitant were separated through centrifugation. The supernatant was removed and the precipitant was rinsed five times, each time with approximately 20 mL of appropriate ethanol regimens. Supernatant solutions were combined and filtered with filter papers (Whatman#4) (Whatman, Maidstone, Kent ME14 2LE, UK), then concentrated to remove ethanol, and freeze-drying to powder. The precipitants, on the other hand, were subjected to freeze-dry to yield powdered samples. Dry weights of supernatant and precipitant samples from each regimen were obtained and the yield (% w/w) was calculated respectively. Each regimen was done in five replicates.

3.2.4 Measurement of Polysaccharide in the Precipitants

3.2.4.1 Development of a Standard Curve

The phenol-sulfuric colorimetric method was a modified method adapted from Gao et al. (2004), and glucose was used as a standard in the determination of total polysaccharides (PSAC) in the

precipitant samples. A glucose stock solution was prepared at a concentration of 0.04 mg/mL. Subsequently, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, 1.0 mL, 1.2 mL, 1.4 mL, or 1.6 mL of the glucose stock solution was transferred to a test tube and brought up to 2 mL with deionized water, respectively. A blank solution was prepared with 2 mL of deionized water without glucose. These preparations resulted in a range of glucose concentrations from 0.008 mg/mL to 0.064 mg/mL. Then, 0.5 mL of 6% phenol solution (Sigma-Aldrich, St. Louis, MO) was added into each test tube, followed by addition of 5.5 mL of 98% sulfuric acid. Each tube was mixed well and placed at room temperature for 30 minutes. The optimum absorbance of the reacted solution was measured at 490 nm using the Ultraviolet (UV)-Visible Spectrophotometer (Beckman DU 720, Fullerton, CA). A standard curve was obtained by three replications.

3.2.4.2 Precipitant Sample Purification

For the purification process, 20 mg of precipitant sample was dissolved in absolute EtOH at a ratio of 1:20 w/v. The solution was sonicated for 30 min and centrifuged at $2060 \times g$ for 10 min. The supernatant was discarded and the precipitant (containing polysaccharides) was dried. The dried precipitant was then dissolved in a 25-mL volumetric flask with deionized water. The solution was centrifuged to remove excessive insoluble residue, if any. This was the sample stock solution.

3.2.4.3 Determination of the Glucose-Equivalent Polysaccharide Content

Pipetted 0.1 mL of the sample stock solution prepared above into a test tube and brought up to 2 mL using deionized water. A blank solution was prepared with 2 mL of deionized water without sample stock solution. Then, 0.5 mL of 6% phenol solution was added into each test tube followed by the addition of 5.5 mL of 98% sulfuric acid. Each tube was mixed well and placed at room temperature for 30 min. The absorbance of reacted solution was measured at 490 nm using the UV-Visible Spectrophotometer. Glucose concentration and amount were obtained based on

the standard curve. Polysaccharides content was expressed as glucose-equivalent polysaccharide in percentage.

3.2.4.4 Recovery Rate

Dissolve 10 mg of a precipitant sample with a known amount of glucose-equivalent polysaccharide in deionized water at a ratio of 1: 20 w/v. Then, 0.1 mL of the above solution was pipetted into a test tube followed by an addition of glucose in the amount equivalent to that found in the respective precipitant sample. The solution was then brought to 2 mL with deionized water and 0.5 mL of 6% phenol was added into each test tube followed by an addition of 5.5 mL of 98% sulfuric acid. The contents of each tube were mixed well and placed at room temperature for 30 min. The absorbance of reacted solution was measured at 490 nm using the UV-Visible Spectrophotometer. Recovery rate was calculated based on the following formula with five replications:

Recovery rate (%): [(Total Polysaccharides (mg) – Known Polysaccharides (mg) from precipitant sample) / Added Glucose (mg)] x 100

3.2.5 HPLC Analysis of the Purified Supernatant Sample

3.2.5.1 Reference Standards

Gallic acid (GA; Purity > 98%), rutin (RUT; Purity > 95%), and ellagic acid (EGA; Purity > 95%) were purchased from Sigma Chemical Company (St. Louis, MO). The reference standards of rubusoside (RUB) and steviol monoside (STM) were isolated by our own lab and identified by spectral data (UV, MS, ¹H NMR, ¹³C NMR and 2D-NMR). Both RUB and SM have purities greater than 98% by HPLC–PDA analyses based on a peak-area normalization method.

3.2.5.2 HPLC Condition

An HPLC system consisting of a Waters (Milford, MA) 600 pump, a 717 auto-sampler, and a UV/Vis Photodiode Array (PDA) 2996 Detector was used for all analyses. Chromatographic

separations were carried out on an Alltech Prevail C18 column (250 mm × 4.6 mm, 5 μm) with a C18 Guard column (7.5 mm×4.6 mm, 5 μm). The mobile phase consisted of solvent A (0.17% phosphoric acid in acetonitrile) and solvent B (0.17% phosphoric acid in water). The elution profile for A was: 0–65 min, linear gradient of 5–30%; 65–85 min, linear gradient of 30–60%; 85–90 min, linear gradient of 60–70%; and 90–100 min, isocratic 70%. A pre-equilibration period of 20 min was used between individual runs. Column temperature was set at 25°C. The flow rate was 1.0 mL/min and the injection volume was 10 μL. All compounds were detected at dual wavelengths of 254 (for GA, RUT, and EGA) and 205 (for RUB and STM) to derive combined chromatograms.

3.2.6 Statistical Analysis

Data were analyzed with Statistical Analysis System (SAS, Cary, NC). Regression analysis was performed to examine the correlation between the response yield and AP regimen. Statistical significance of all tests was set at $P \leq 0.05$.

3.3 Results

3.3.1 Aqueous Sample Preparations Prior to Alcohol Precipitation

Prior to performing alcohol precipitation of the aqueous extract samples, various extract-to-water ratios ranging from 1:4 w/v to 1:8 w/v were tested to determine the amount of precipitant (thus the reciprocal amount of the purified extract) caused by extract solubility itself. It was found that at the ratio of 1:4 w/v (i.e., 250 mg/ml), approximately 11% of the extract had already precipitated in water prior to the addition of alcohol. The yield of precipitant showed a constant rate of insignificant decrease of 0.2% w/w between the ratios of 1:4 w/v and 1:6 w/v, then leveled off at 10.6% w/w. Based on these results, the partitioning of each sample due to alcohol precipitation was adjusted and normalized by eliminating the insoluble effect averaging at 11%.

3.3.2 Yields of the Purified and Precipitant Extracts in response to Alcohol Precipitation

AP was successful in partitioning the crude extract into a soluble supernatant (the purified extract) and insoluble precipitant. The yields of the purified extracts in the form of supernatants decreased exponentially from 94% to 55% as the aqueous ethanol concentrations increased from 0% (control) to 95% (**Fig. 3.1**). The highest effect of alcohol precipitation occurred at the 95% AP regimen, which precipitated 36% of the crude extract mass as impure components, leaving 55% of the weight in the supernatant solution, which was later dried to become the purified extract. The unaccounted 9% was a loss during the process of filtration, concentration, or freeze-drying.

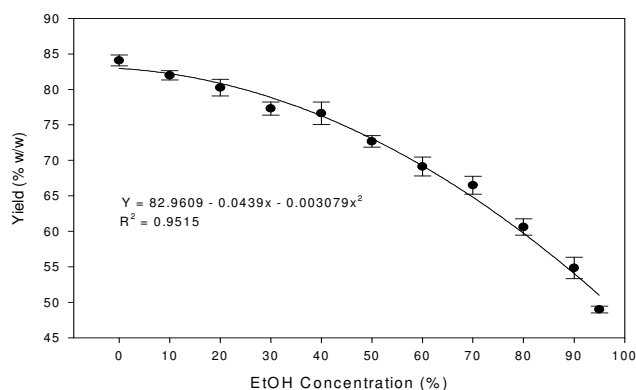


Figure 3.1. Yield of the purified extract (supernatant) as a result of alcohol precipitation (AP) regimens (n=5). Quadratic regression model was fitted. Values are expressed as mean \pm standard error (vertical line; n=5).

3.3.3 Changes of Marker Compounds in the Purified Extract to Alcohol Precipitation

As the ethanol concentrations increased in the aqueous solutions, the contents of the combined five marker compounds increased in the solutions (**Fig. 3.2**). In the 95% aqueous ethanol solution where the content of the five markers was highest, the marker compounds accounted for over 20% by weight of the purified extract, a significant 8% increase from the 10% AP regimen.

To further illustrate the changing trend of each individual compound, regression models were used to determine the relationships. Generally, all markers were continuously concentrated by increasing AP levels, but the pace of and maximum change differed among the markers (**Fig. 3.3**). Among them, GA, EGA, RUB, and STM had quadratic relationships ($P < 0.001$) characteristic of a slow or nearly zero rate of increase at lower alcohol concentrations followed by a rapid rate of increase. For example, the contents of GA, EGA, RUB, and STM in the purified extract seldom changed up to 40% AP regimens, but increased linearly and significantly after this point to a maximum at 95% AP regimen. In contrast, the relationship of RUT and AP regimens was linear ($P < 0.001$). RUT content increased at a constant rate as AP regimens moved upward. Overall, at 70% AP, the yield of GA and STM in the purified extract increased almost 10-fold compared to the raw material while the RUB concentration was 3-fold higher than the raw leaf material (Table 3.1). The yield of RUT and EGA stayed about the same even after the purification process possibly due to chemical properties of the compounds itself (Table 3.1).

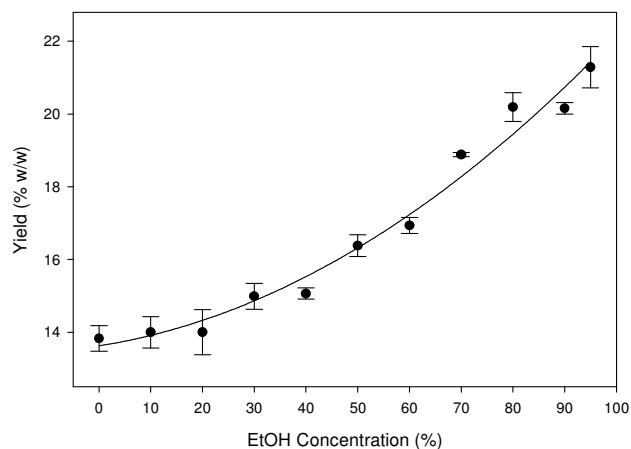


Figure 3.2. Changes in the contents of the total five marker compounds in the purified extract in response to alcohol precipitation (AP). Quadratic regression model was fitted. Values are expressed as mean \pm standard error (vertical line; $n=3$).

Table 3.1. The yield (% w/w) of extract and all five marker compounds in the leaf, crude, and purified extract of the Chinese sweet tea plant (*Rubus suavissimus*).

	Yield (% w/w)					
	Extract	Gallic Acid	Rutin	Ellagic Acid	Rubusoside	Steviol Monoside
Raw Leaf	-	0.13 ± 0.02	0.11 ± 0.03	0.71 ± 0.14	4.87 ± 0.80	0.08 ± 0.02
Crude Extract	28.64 ± 1.18	0.72 ± 0.03	0.11 ± 0.00	0.87 ± 0.03	7.84 ± 0.19	0.64 ± 0.01
Purified Extract	66.50 ± 1.23	1.03 ± 6.54	0.19 ± 0.00	0.67 ± 0.01	12.53 ± 0.06	0.96 ± 0.03

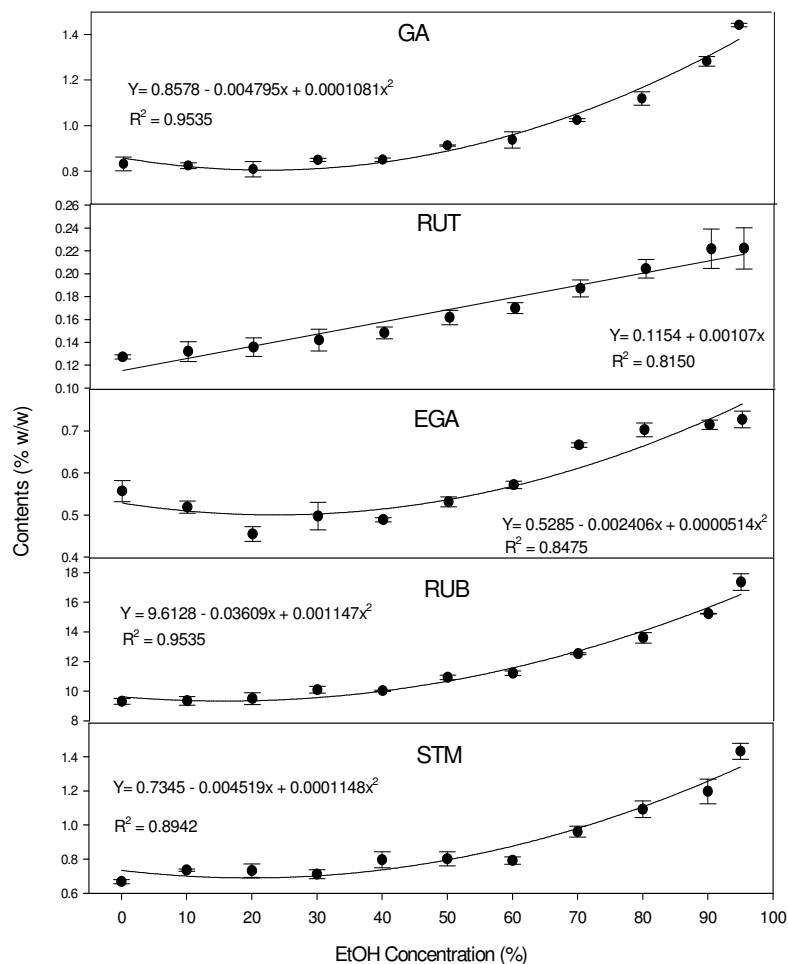


Figure 3.3. Changes of the individual marker compound in the purified extract (supernatant) corresponding to ethanol concentrations in the AP experiments. A simple linear regression model was fitted for RUT. Quadratic linear models were fitted for the contents of GA, EGA, RUB, or SM. All values are expressed as mean ± standard error (n=3).

3.3.4 Changes of Chemical Components in the Precipitant to Alcohol Precipitation

Marker compounds were minor components in the precipitant to begin with. At a low alcohol concentration of 10%, marker compounds accounted for approximately 8% (w/w). As the alcohol concentrations increased from 10% to 50%, the contents of the marker compounds decreased at nearly a linear rate to 2.8%, reflecting an obviously higher affinity of marker compounds with ethanol than with water. Further increases in alcohol concentrations of 60% or higher caused an additional 1.2% decrease of the marker compounds, to rest at a final 1.6% in the precipitant (**Fig. 3.4**).

Polysaccharide (PSAC), on the other hand, was a major component in the precipitant, a target component to be removed by alcohol precipitation. The total polysaccharides content was measured using the phenol-sulfuric acid colorimetric method. A standard curve ($n=3$) was developed and validated using glucose as a standard compound. The R^2 was 0.993 and the recovery rate was 98.79% with a relative standard deviation (RSD) of 2.74. All polysaccharides measurements were expressed as glucose-equivalent polysaccharide. Polysaccharides content in the precipitant linearly increased fourfold but stopped at 70% alcohol concentration when maximal saturation was reached (**Fig. 3.4**). At that point, polysaccharides accounted for 22.97% of the precipitant and approximately 11% of the crude extract by weight. Leveling off of polysaccharide content at the 70% AP regimen was an indication of complete removal of polysaccharides. The observed continued slight increases in the precipitant yield were accompanied by the slight re-bounce of the unknown components. These components analyses, hence, demonstrated the positive effect of AP in purifying the bioactive compounds (markers) in the sweet tea extract through the removal of polysaccharides. The other unknown constituents in the precipitant followed the similar response patterns to the marker compounds except at 80% AP or higher, where there was a slight re-bounce (**Fig. 3.4**).

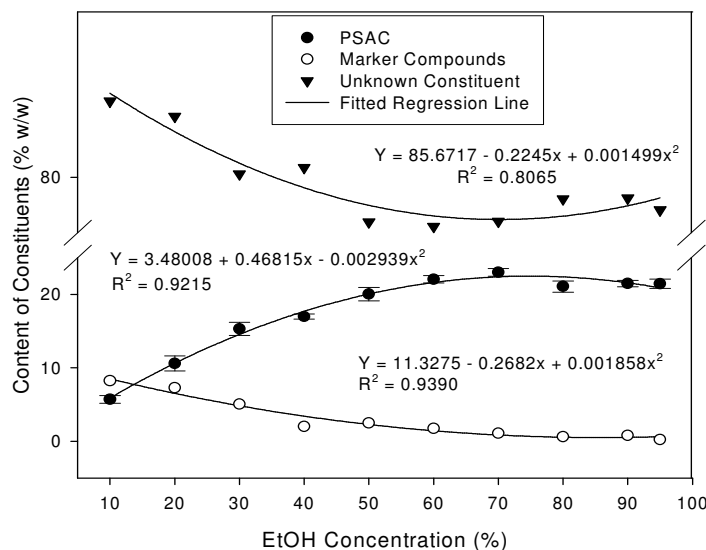


Figure 3.4. Changes in the contents of the five marker compounds, polysaccharides (PSAC), and unknown constituents in the precipitant in response to alcohol precipitation (AP). Quadratic regression model was fitted. Values are expressed as mean \pm standard error (vertical line; $n=3$).

3.3.5 Chromatographic Fingerprint of the Purified and Precipitated Extracts

Fingerprints were developed for the purified and precipitated samples to gain insight into the differences in composition benchmarked by the five marker compounds and unknown but characteristic peaks (1-5) that responded noticeably to alcohol precipitation.

At the 10% alcohol concentration, more GA, Peak 1, Peak 2, and RUB were in the supernatant solution than in the precipitant; less Peak 3, Peak 4, RUT, and Peak 5 were in the solution than in the precipitant; much less EGA was in the solution than in the precipitant; and SM was equal in the solution and precipitation (**Fig. 3.5A**). At the 50% alcohol concentration, the majority of GA, Peak 1, Peak 2, Peak 3, and RUB were in the supernatant solution; Peak 4, RUT, and Peak 5 were equally split between the solution and precipitant; more EGA was in the precipitant than in the solution; and SM was all in solution (**Fig. 3.5B**). When the alcohol concentration increased to 95%, some obvious shifts of partitioning patterns were observed. GA, Peak 3, RUT, Peak 4, Peak 5, and RUB were almost completely in solutions, similar to STM

already started at the 50% AP regimen (**Fig. 3.5C**). Peak 2 remained unchanged. Conversely, Peak 1 and EGA changed to opposite directions relative to their responses to 50% alcohol concentration. Most of Peak 1 (or the component it represented) went to the precipitant at the 95% AP regimen, whereas most of it stayed in the solution at the 50% AP regimen. While more EGA was in the precipitant at the 50% AP regimen, it was apparently much less at the 95% AP regimen.

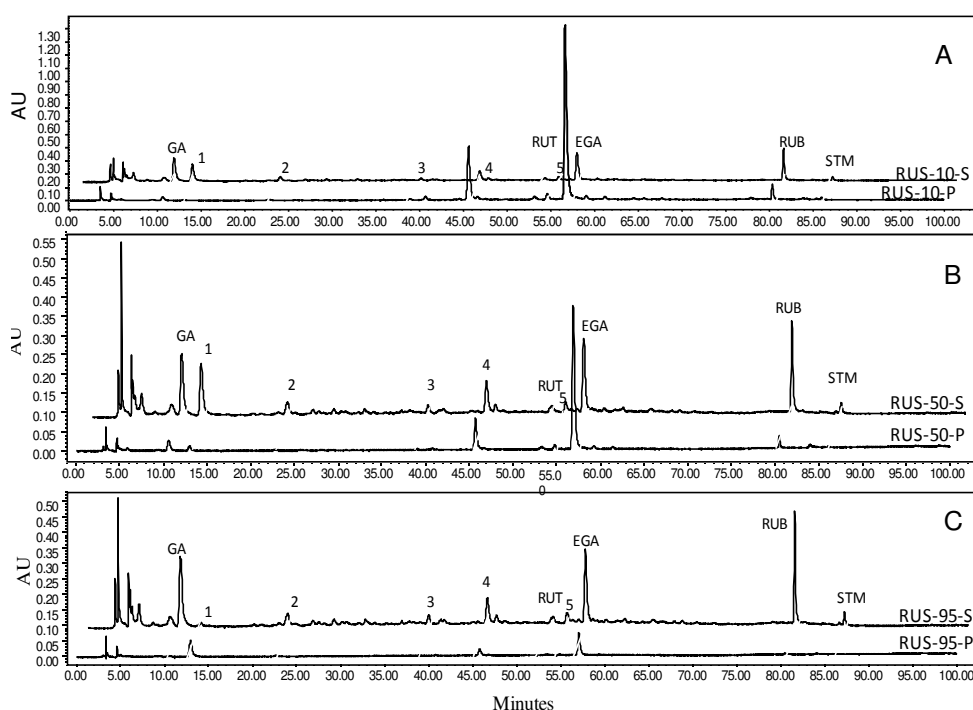


Figure 3.5. Chromatographic fingerprint of the Chinese sweet leaf tea (*Rubus suavissimus*) extracts subjected to alcohol precipitation (AP). Selected chromatograms demonstrate the partitioning of the five marker compounds between the supernatant (S) and precipitant (P) fractions as results of 0% EtOH (RUS-0), 50% EtOH (RUS-50), or 95% EtOH (RUS-95) treatments. Presence of the five marker compounds is indicated as gallic acid (GA), rutin (RUT), ellagic acid (EGA), rubusoside (RUB), and steviol monoside (SM). Peaks 1- 5 are unknown compounds. NOTE: Alignment may be slightly off for some fingerprints due to graphic demonstration purpose.

3.4 Discussion

Alcohol precipitation allows the separation of small molecules from polymers, and therefore is a useful initial purification procedure for desired chemical compounds. This procedure has been applied in many studies to separate biologically active polysaccharides (Schmourlo et al., 2005; Wu et al., 2007; Yang et al., 2008) from other components in botanical samples. In our current study, however, we found that the bioactive compounds in the crude extract of the Chinese sweet tea plant are secondary metabolites with low molecular weight, rather than those of macromolecules such as polysaccharides. Thus, in order to eliminate polysaccharides or other polymers in the crude extract prepared by boiling water, a simple yet effective method was sought to purify the bioactive small molecules in the crude extract. In this study, the alcohol precipitation method, commonly used to obtain pure polysaccharides, was examined for its effectiveness of removing rather than purifying polysaccharides from the crude water extract of the Chinese sweet leaf tea. Schmourla et al. (2005) demonstrated that there was a clear difference of antifungal activity in medicinal and food plants between the supernatant and precipitant phases using AP as a purification procedure. The separation which resulted from their respective study did enhance the antifungal activity of certain plants.

Another study on the honeysuckle flowers (Yang et al., 2006) also clearly illustrated the improvement of chlorogenic acid purity from 31.62% to 37.72% after the precipitation of crude extract with 60% ethanol. Our current finding that the contents of selected bioactive marker compounds were significantly increased in a concentration-dependant manner was similar. Using the alcohol precipitation method, the crude extract of sweet tea was purified by one-fold through the removal of 11% polysaccharides and other yet-to-be identified components. The separation of mass to this magnitude by a single step shows the efficiency and cost-effectiveness of alcohol precipitation in obtaining purer botanical samples.

Most interestingly, the degree of purity of the finished product can be readily controlled so that the purified extract contains the levels of desired components. For example, to safely remove all polysaccharides without removing unnecessary unknown components, 70% alcohol concentrations would be sufficient and appropriate, and alcohol strength higher than 70% would cause additional precipitation of unknown components, such as that represented by Peak 1. Although the exact mechanism is unknown, it may be due to the intrinsic affinity nature of the compound itself in Peak 1. While Peak 1 was found highest in the supernatant at 70% ethanol, either too high (e.g., 95%) or too low (e.g., 10%) returned this compound to the precipitant. Apparently, this compound has higher affinity to water than to the ethanol. Another example in our study was EGA (ellagic acid) where the absorption peak was found highest in the precipitant at 10% alcohol. In fact, as the concentration of ethanol increased from 10% to 95%, the EGA content decreased by almost 15-fold in the precipitant. On the other hand, in the supernatant solution, the yield of EGA increased with the alcohol regimens and then reached a plateau at 80% AP. These phenomena clearly suggested greater affinity of EGA to ethanol than water. Liu (2007) also reported that most starch, protein, polysaccharides, inorganic salts, or other polymers can be removed by AP when using 80% ethanol. Due to the concern of losing small molecules with high alcohol concentrations (e.g., higher than 80%), 60% to 75% ethanol is usually recommended to avoid additional loss of bioactive metabolites (Liu, 2007). Therefore, our detailed quantitative and qualitative fingerprint analyses over the fractionated extracts as a result of alcohol precipitation provide the best understanding of the partitioning behaviors of each known component as well as some unknown compounds so specific alcohol precipitation regimens can be adopted for the desired extract purity and composition.

The crude extract of the Chinese sweet leaf prepared by boiling water contained approximately 11% w/w of polysaccharides as determined by the phenol-sulfuric acid

colorimetric method. Phenol-sulfuric assay is a simple, convenient, and sensitive method to measure the concentration of polysaccharides in plant extracts. The reaction mechanisms may involve the condensation with phenol after the parallel dehydrations of carbohydrates with sulfuric acid (Scherz et al., 1998). Under a proper condition, the phenol-sulfuric colorimetric method has approximately $\pm 2\%$ of accuracy (Hodge and Hofferter, 1962) and has also been applied in microplate format due to its simplicity and sensitivity (Masuko et al., 2005). Our study indicated that the recovery rate of the phenol-sulfuric acid colorimetric method was 98.79% with an RSD of 2.74. These data confirm the validity of this method in measuring the content of polysaccharides in complex botanical samples.

Purification of crude plant extracts is widely used in botanical research to augment the initial observation of biological activities. Chou et al. (2009) have presently developed a validated HPLC method in assessing the quality of the Chinese sweet leaf tea extract possessing multiple bioactivities. This would further help us to quantify and qualify the end products resulted from AP. Because the stability, safety, and effectiveness of AP depend on several factors such as the extract concentration, alcohol volume and concentration, reaction time length, temperature, stirring procedure during precipitation, and chemical and physical properties of raw material used in precipitation (Liu, 2007), it is important to verify and control the overall quality and thus the bioactivity of the extracts under the conditions of AP. In the study of antifungal activity, Schmourla et al. (2005) demonstrated the lost activity in some medicinal plants after the separation of the plant extracts into precipitant and supernatant. The crude aqueous extract of the Chinese sweet leaf displayed potent antiangiogenesis activities due to the presence of gallic acid (Liu et al., 2006). Therefore, to purify the crude sweet leaf tea extract for increased antiangiogenesis activity, achieving an increased level of gallic acid is clearly desirable. On the other hand, since gallic acid is not the only compound that explained the overall antiangiogenic

activity of the sweet leaf tea extract, it is imperative to retain other possible bioactive compounds until proven not useful. This also further explained the reasons we adopted a longer analytical time (100 min) to our HPLC methods which would help us optimize the separation of compounds, known or unknown, that may exhibit bioactivity (Chou et al., 2009). In addition to the quantity of each known marker compound, the ratios of these compounds are of great importance to us because of the prospect of concerted action potential. This argument might be exemplified by the observed lost activity in some medicinal plants after AP regimens (Schmourlo et al., 2005), which could be a result of changes of the ratios of active compounds that could have modified the intrinsic synergistic or additive properties. Because the alcohol precipitation used in this study resulted in a whole spectrum of extracts differing in ratios, thus in composition, these samples warrant differential bioactivity examinations. For this purpose, detailed fingerprint analyses over the results of a simple purification procedure were proven worthy.

3.5 Summary

This is a first report on the purification of sweet leaf crude extract using alcohol precipitation. Our study confirmed a clear separation of selected marker components from polysaccharides in response to alcohol precipitation. The level of rubusoside, one of the marker compounds and the characteristic sweetening agent, was doubled in a 95% alcohol solution via a complete removal of 11% polysaccharides and other macromolecules or ethanol insoluble components. Overall, 70% to 80% EtOH was the best range for purifying the five markers without risking the loss of many unknown compounds. By employing this purification method, a significantly purer extract can be obtained for potential improved bioactivity. Alcohol precipitation, therefore, proves to be a useful tool in purifying polysaccharide-rich plant extracts such as the sweet leaf tea extract.

CHAPTER 4. ABSORPTION OF BIOACTIVE COMPONENTS IN THE CHINESE SWEET LEAF TEA EXTRACT (*RUBUS SUAVISSIMUS* S. LEE) AND ITS ANTI-OBESITY EFFECT IN NORMAL RATS

4.1 Background

The prevalence of overweight and obesity are growing worldwide, overtaking the incidences of underweight, malnutrition, and other infectious diseases. It is estimated that there were over one billion of overweight individuals and more than 300 millions who are obese (Aronne, et al., 2007). Generally, obesity is defined by the body mass index (BMI) greater than 30 kg/m². In addition to body weight gain, obesity also causes the accretion of adipose tissues where health may be adversely affected (Frühbeck, 2008). Nonetheless, obesity also increases the risk of other health-threatening diseases such as diabetes, hypertension, cardiovascular disease, and cerebrovascular disease (Haslam et al., 2005; Aronne et al., 2007; Shah et al., 2008).

Search for ways to manage a healthy body weight has been a thirst for individuals and biomedical researchers. Because of the lack and limitation of weight loss medicines, traditional herbal medicines and food ingredients that are physiologically functional become once again important sources of weight management agents. There were numerous reports on the weight loss and treatment of metabolic disorders via the application of functional foods and herbal medicines (Han et al., 2002; Park et al., 2006; Choi et al., 2007; Ekanem et al., 2007; Lei et al., 2007; Lemaure et al., 2007; Kamisoyama et al., 2008; Lee et al., 2008). Among the potential functional food ingredients, green tea (*Camellia sinensis*) is the one most extensively studied in the prevention of metabolic syndrome (Dulloo, 1999; Sayama et al., 2000; Zheng et al., 2004; Nagao et al., 2007; Boschmann et al., 2007; Bose et al., 2008; Ito et al., 2008; Maki et al., 2009; Nagao et al., 2009). Studies have shown that green tea increases fat oxidation and thermogenesis, contributing to the weight reduction in obese men (Dulloo, 1999; Boschmann et al., 2007). Other food and herbal ingredients, such as ginger roots (*Zingiber officinale*) (Al-Amin et al., 2006;

Goyal et al., 2006), capsaicin or capsinoids (Snitker et al., 2009; Hsu and Yen 2007; Kang et al., 2007), fenugreek (Handa et al., 2005; Srichamroen et al., 2009; Jette et al., 2009), and ginseng (*Panax ginseng*) (Kim et al., 2002; Xie et al., 2002) have shown anti-obesity effects. The most effective herbal agents were perhaps ephedrine and caffeine, alone or in combinations. For several years, ephedrine and caffeine have been used as dietary supplements for weight loss until toxicity issues prompted the Food and Drug Administration (FDA) to halt their use for this purpose. Currently, there are no effective and safe herbal products for weight management despite numerous and tireless research efforts. Consequently, the search continues and prompts the investigation of this special folk medicine commonly known as the sweet leaf tea (*Rubus suavissimus*), one of the many *Rubus* species in the Rosaceae family.

Rubus species have long been used as traditional medicines for therapeutic purposes. For example, *Rubus ideaus* (Morimoto et al., 2005), *Rubus imperialis* (Novaes et al., 2001), *Rubus fruticosus* (Jouad et al., 2002), and *Rubus fruticosus* (Alonso et al., 1980; Swanson-Flatt et al., 1990) showed hypoglycemic effects through the reversal of insulin resistance. Moreover, the black raspberry extracts (*Rubus occidentalis*) displayed anti-angiogenesis (Liu et al., 2005) and anti-cancer (Wada et al., 2002) properties. As a traditional folk remedy, *R. suavissimus* has been widely applied in southwestern China to treat various diseases such as type 2 diabetes. Recent scientific investigations found that the leaf extract of *R. suavissimus* was highly potent against angiogenesis and gallic acid in the extract was partially responsible (Liu et al., 2006). Other studies also demonstrated that the sweet leaf tea extract was inhibitory of the activity of NF- κ B (Liu et al., 2005) and α -amylase (Li et al., 2007), which are closely related to glucose metabolism.

Recent scientific evidence has linked excessive blood vessel growth to diseases such as cancer, obesity, and asthma (Fan et al., 2006, Brakenhelm et al., 2008). Rupnick et al (2002)

showed that the use of an angiogenesis inhibitor caused weight loss whereas treatment withdrawal restored body weight gain, suggesting obesity or fat accumulation is dependent on angiogenesis. Studies also supported that antiangiogenic therapy has successfully inhibited the process of adipogenesis and the growth of pre-existing adipose tissues (Rupnick et al., 2002; Liu et al., 2003; Brakenhielm et al., 2004; Brakenhielm and Cao, 2008). Since the leaf extract of *R. suavissimus* was a potent angiogenesis inhibitor (Liu et al., 2006; Oner et al., 2007), a hypothesis emerged that it has the potential to cause significant weight loss. In addition to the angiogenesis inhibition, its identified ingredients, such as gallic acid and ellagic acid, were reported to reduce the α -amylase and bacterial activities, both of which could contribute to reduced energy absorption. The antiangiogenic effects, the inhibition of NF- κ B (Liu et al., 2005) and α -amylase (Li et al., 2007) activities, and anti-bacterial effects of the sweet leaf tea extract may work in concert to produce a significant overall weight loss effect. This study was therefore designed to determine the bioavailable components in the sweet leaf tea extract and whether these compounds could cause significant weight loss at a non-toxic dose.

4.2 Materials and Methods

4.2.1 Reference Standards

Gallic acid (GA), rutin (RUT), and ellagic acid (EGA) were purchased from Sigma Chemical Company (St. Louis, MO). The reference standards of rubusoside (RUB) and steviol monoside (STM) were isolated by our own lab and were identified by spectral data (UV, MS, ^1H NMR, ^{13}C NMR and 2D-NMR). Both RUB and SM have purities greater than 98% by HPLC–PDA analyses based on a peak area normalization method.

4.2.2 Extracts

The standardized Chinese sweet leaf tea extract (RUS; batch # XRUS0505) was processed by our own laboratory. The quality of the extract is monitored based on the yield of the five major

compounds (i.e., gallic acid, ellagic acid, rutin, rubusoside, and steviol monoside) via the analyses of HPLC.

The purified Chinese sweet leaf tea extract (GER) was composed of 1.38% w/w of gallic acid, 2.4% w/w of ellagic acid, and 23.41% w/w of rubusoside. These compositions were similar to those found in the standardized leaf extract (RUS) as shown in **Fig. 4.1**.

4.2.3 Animals

All Sprague-Dawley rats used in the experiments were purchased from Harlan (Harlan, Indianapolis, IN). Animals were housed individually in a stainless steel cage in an air-conditioned room at $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$, 50-60% relative humidity, and 12/12 h light/dark cycle. Prior to the euthanization, urine was collected from each rat through an individual metabolic cage (Lab Products Inc, Seaford, DW). Blood or serum was collected via cardiac puncture. All procedures were performed under the approved protocols by the Institutional Animal Care and Use Committee of Louisiana State University (LSU-IACUC), Louisiana, USA.

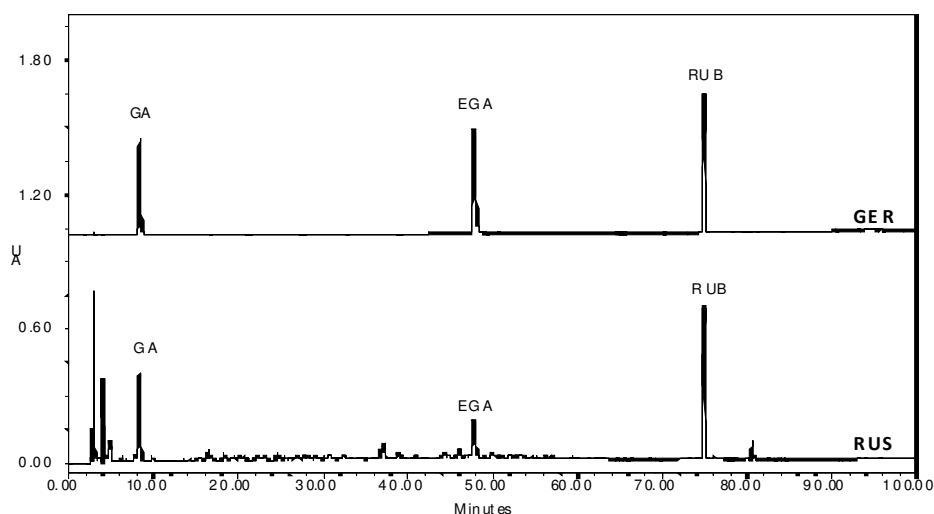


Figure 4.1. Chromatographic fingerprint of the purified Chinese sweet leaf tea (*Rubus suavisissimus*) extract (GER) composed of gallic acid (GA), ellagic acid (EGA), and rubusoside (RUB) in a composition equivalent to the total extract (RUS).

4.2.4 Diets

All rats were fed either with freshly prepared AIN-93G purified liquid diet (Dyets Inc, Bethlehem, PA) or standard chow (Purina 5001 Lab Diet, PMI Nutrition International, Brentwood, MO). The liquid diet was prepared at a concentration of 0.1755g/ml with tap water. The ingredients of the liquid diet are shown in **Table 4.1** where each mL of the liquid diet accounting for 1 calorie with 64% of total calories from carbohydrates, 19.3% from protein, and 16.7% from fat. On the other hand, the standard chow was composed of 58% carbohydrates, 28.5% protein, and 13.5% of fat in each calorie.

Table 4.1. Compositions of the AIN-93G purified liquid diet

Ingredients	grams/Liter of Diet
Casein	53.00
Sucrose	26.50
Cellulose	13.30
Maltose Dextrin	135.40
Soybean Oil	18.60
t-Butylhydroquinone (TBHQ)	0.004
AIN-93G Salt Mix	9.28
AIN-93G Vitamin Mix	2.65
L-Cystine	0.80
Choline Bitartrate	0.66
Xanthan Gum	3.00

4.2.5 Absorption of Marker Compounds in the Chinese Sweet Leaf Tea in Normal SD Rats via Oral Gavage

Thirty-two normal male Sprague-Dawley (SD) rats (3 to 6 week-old) were divided into two groups receiving either extract or blank diet. Prior to treatment, all rats were fasted overnight for 18 hours. The treated group (n=25) was administered with extract at a concentration of 0.1%

w/w (1g of extract/kg of body mass) via daily gavage for 3 consecutive days. The control group (n=8), on the other hand, was fed with standard chow (Purina 5001) and water as vehicles. Urine was collected cumulatively for 24 hours, a day before euthanization, through an individual metabolic cage.

4.2.6 Determination of Extract Delivery Methods

To ensure a non-stressful oral intake of extract, different extract delivery methods were examined using nine 6-8 week-old male SD rats. Delivery methods tested included the oral feeding method through water (as drink), powdered diet (Dyets Inc, Bethel, PA), liquid diet (Dyets Inc, Bethel, PA), and non-fat dairy yogurt (purchased from local grocery store). These methods were tested by mixing or dissolving the extract in the respective form of food or water. Prior to the feeding, each rat was fasted overnight for 18 hours. Extract was mixed at a concentration of 30mg/ml or 30mg/g and delivered either through a 15-ml tube (**Fig. 4.2**) or a feeder. Extract delivered by water was tested on all rats at Day 1 and Day 2. The same group of rats was again divided into 3 groups at day 3 and day 4 to receive extract-containing diet delivered through powdered diet, liquid diet, or non-fat dairy yogurt, respectively. Consumption of extract for each delivery method was recorded at 30 min and 60 min. Acceptance of delivery method was determined based on the consumption rate or amount of extract consumed.

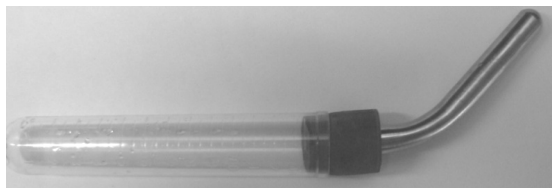


Figure 4.2. A 15-ml Falcon[™] tube used in the extract delivery.

4.2.7 Determination of Minimal Effective Dose for the Chinese Sweet Leaf Tea Extract

A 5-day absorption study was conducted on the same group of SD rats to determine the excretion of each marker compound (in their original structures) in the urine sample. After an overnight

fasting, all rats were fed with extract at a concentration determined earlier (30mg/ml) for 60 min. Amounts of extract consumed were recorded daily. Subsequently, the rats were fed with regular liquid food throughout the day cycle for 8 hours. A 24-hour urine was collected daily and stored at -20°C until analyzed. Concentrations of each marker component were analyzed with the HPLC and excretion rates of selected marker compounds were calculated to develop a dose-response curve within the range of dosages consumed by the animals during the 5 consecutive days.

4.2.8 The Anti-obesity Effect of the Chinese Sweet Leaf Tea Extract in Normal SD Rats

4.2.8.1 Experimental Design

To observe the weight loss effect resulted from the administration of Chinese sweet leaf tea extract, 25 normal SD rats were divided into 3 groups, which include a control group and 2 treated groups, with 8 rats in each treated group and 9 rats in the control group, as shown below.

- Group 1 (RUS): Treated group receiving the standardized Chinese sweet leaf tea extract
- Group 2 (GER): Treated group receiving the purified Chinese sweet leaf tea extract
- Group 3 (CTR): Control group receiving blank liquid food as vehicle

To ensure a non-stressful administration of extract, voluntary oral feeding method was selected as a delivery method in this study by dissolving the powdered extract in liquid food at a concentration of 30 mg/ml. The amount of extract was pre-determined and adjusted based on the body weight of each rat.

Prior to the initiation of treatment, one-week adaptation period was employed as an adaptation to the liquid food and extract. A single administration of crude extract was given at 0.5 g per kg of body weight for 74 days. The GER, which accounts for 27% of the extract by weight, was given at a dosage of 0.14g/kg of body weight (equivalent to the extract dosage of 0.5g/kg of body weight). All rats were given ad lib access to liquid food for 8 hours (day cycle) after the

consumption of extract or compounds mixture. Fasting period started at 6 pm each day until 9 am the next day. Food intake was measured daily and body weight was recorded every two days. Animals were observed daily for any abnormal physical and behavioral changes or signs of toxicity, including posture, rough hair coat, decreased responsiveness, and unusual breathing pattern, etc.

4.2.8.1.1 Adaptation Period

To reduce the effect of extract aversion, a one-week adaptation to the liquid food and extracts was employed. All rats were fasted overnight for approximately 18 hours prior to the feeding of liquid food or extracts. Replacement of liquid food with extracts began only until a constant rate or improvement of liquid food consumption was observed. Administration of extracts were conducted by dissolving the leaf extracts (in powder form) or the compounds mixture in the liquid food and then delivered through a 15-ml falcon[™] tube at a concentration of 30mg/ml. Extract volume was pre-determined based on the body weight. Concentration of extract was diluted initially to avoid aversion to the novel taste and was adjusted constantly as stable consumption rate was observed. Extract dose consumed was recorded daily for future references.

4.2.8.1.2 Treatment Period

4.2.8.1.2.1 Daily Administration of Extract

A single administration of extract started at 9 am daily. Extract-containing liquid food, at a pre-determined volume based on body weight (0.5g/kg of body mass), were given to the treated groups, corresponding to the treatment. Blank liquid food was given to the control group as vehicle. All tubes were pre-measured and final weight of the tubes was recorded to calculate the actual dosage consumed. Subsequently, all rats were given *ad libitum* access to the liquid food during the day cycle for 8 to 9 hours and then fasted overnight.

4.2.8.1.2.2 Measurement of Body Weight

Body weight of each rat was measured every two days prior to the feeding of extract or food.

Cumulative body weight (CBW) changes were calculated based on the initial fasting body weight as shown below:

$$CBW = [Current\ Body\ Weight\ (g) - Initial\ Body\ Weight\ (g)] / Initial\ Body\ Weight\ (g)$$

4.2.8.1.2.3 Measurement of Food Intake

Each rat was given *ad libitum* access to liquid food during the day cycle from 9.30 am (or upon completion of extract) to 6 pm daily via a glass water bottle. The weight of a water bottle with liquid food was pre-measured prior to the feeding and the final weight of the water bottle with liquid food was recorded to determine the total consumption of liquid food during the day cycle.

Relative daily food intake (%) was calculated to determine the effect of extract administration on daily food intake. Calculation of the relative daily food intake is shown below:

$$Relative\ Food\ Intake\ (\%) = Total\ Food\ Intake\ (g; \text{dry liquid food}) / Body\ Weight\ (g) * 100\%$$

4.2.8.1.2.4 24-hour Urine Collection

Urine was collected after 6 week of extract administration. At the day of urine collection, each rat was placed immediately in a metabolic cage (Lab Products, Inc. Seaford, DW) after administration of extract or liquid food to collect the 24-hour urine. Urine samples collected were stored in the refrigerator at -20°C until analysis.

4.2.8.1.3 Treatment Withdrawal

A four-week recovery period was conducted to further observe the body weight regain ability of the animals after the withdrawal of treatments. During this period, rats were fed with standard chow and water for 24 hours. Body weight was recorded weekly. The recovery period was divided into two phases, where each phase consisted of two weeks. During the first phase of the recovery period, rats had free access to food and water, without receiving any treatment, to

determine the ability of weight recovery. This phase also referred to as the blank period. At the second phase, rats received extract or compounds mixture daily with *ad libitum* access to food and water to further observe the phenotype effect after a two-week blank period or treatment withdrawal. Throughout the recovery period, all rats were not fasted overnight.

4.2.8.1.3.1 Blood Sampling and Clinical Pathology

At the end of the recovery period, rats were anesthetized with isoflurane and euthanized. Blood samples were collected from each group to examine any possible of toxicity or adverse effects that resulted from the consumption of the extracts. For the hematology analysis, blood samples were collected in an EDTA vacutainer (Beckin, Dickinson and Company, Franklin Lakes, NJ). The parameters tested included the RBCs, hemoglobin, hematocrit, red blood cell distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, mean platelet volume (MPV), and total WBCs. For the chemistry analysis, blood samples were collected in a vacutainer and were allowed to coagulate. Serum was separated via centrifugation at $2000 \times g$ for 10 min. Serum chemistry tested included serum glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (APH), urea nitrogen (BUN), and creatinine. Both blood and serum samples were stored at -20°C and immediately submitted to the Louisiana Animal Disease Diagnostic Laboratory (School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA) for analysis.

4.2.9 Statistical Analysis

Data were analyzed with Statistical Analysis System (SAS, Cary, NC). Regression models were fitted to determine the relationship between the concentration of marker compounds and dosage levels in the 24-hour urine sample. On the other hand, analysis of variance (ANOVA) with repeated measure was performed on the body weight changes and relative daily food intake.

Parameters resulted from serum and hematology tests were evaluated statistically for differences between the control and treated group using the one-way ANOVA. Tukey's post hoc test was performed on all tests to compare the group differences. Significance of all tests was set at $p \leq 0.05$. Outlier detection was performed and data was eliminated only when the R-student scores ≥ 2.50 and improvements were significantly observed in the coefficient of variation, R-square, and the test of normality. Power analysis was also conducted, with the variations resulted from current study, to further determine the sample size required to achieve significant results. All results reported were expressed as mean \pm SEM, unless otherwise stated.

4.3 Results

4.3.1 Absorption of Several Chinese Sweet Leaf Tea Components in Normal SD Rats via Oral Gavage

After a single administration of Chinese sweet leaf tea extract at the dose of 1g/kg of body weight, three out of five marker compounds were detected in their original structures (unmetabolized) in the 24-hour urine, namely gallic acid (GA), ellagic acid (EGA), and rubusoside (RUB) (**Fig. 4.3**). Neither RUT nor STM were detected in the 24-hour urine sample. Unmetabolized GA in the urine amounted to 91.7 μg , which was about 4.5% from orally administered 2028 μg . Unmetabolized EGA and RUB in the urine were 13.3 μg and 65.3 μg , accounting for 2.2% of orally administered 603 μg and 0.3% of orally administered 21808 μg , respectively. However, it was observed that oral gavage itself increased the distress level, which eventually causes removal of subjects. This phenomenon further increased the sample variation, suggested that this route of administration may have affected the accuracy of the results.

4.3.2 Determination of Extract Delivery Method

In order to reduce the variation arising from the oral gavage, a non-stressful voluntary oral feeding method was developed using nine previously untreated SD rats. **Table 4.2** demonstrated

the improved coefficients of variation (CV) as a result of administration method change. With the voluntary oral feeding method, the CV was reduced by 27%, 32.4%, and 6.6% for GA, EGA, and RUB, respectively. This new P.O. method consisted of mixing the extract with a liquid food at the concentration of 30 mg/ml. Within 30 minutes, all rats generally consumed 7.68 ± 1.11 g extract-containing liquid foods. By controlling the volume of the extract-containing liquid food, approximately 0.5g/kg extract dose was delivered to the rats at minimal stress. The consumptions of extract via drinking, yogurt, and powder diet were 0g, 0.84 ± 0.81 g, and 3.86 ± 0.57 g, respectively. Due to the convenience as well as the acceptance of the delivery method, the liquid diet was selected for further animal studies.

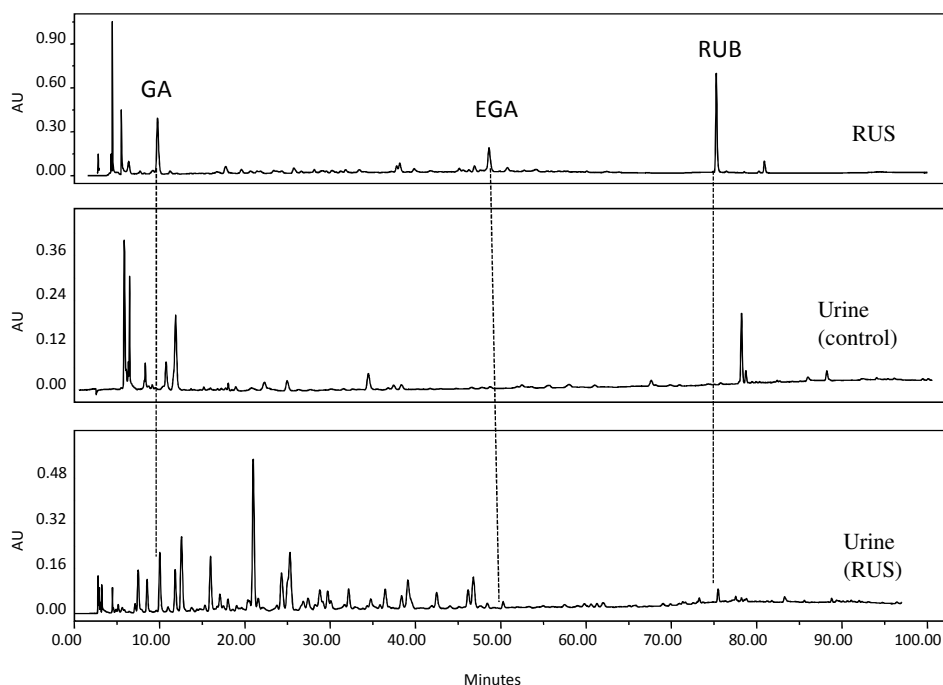


Figure 4.3. Chromatogram fingerprint of the standardized Chinese sweet leaf tea (*Rubus suavissimus*) extract (RUS), the control and treated (RUS) urine samples indicating the presence of the three major marker compounds, gallic acid (GA), ellagic acid (EGA), and rubusoside (RUB) obtained from the normal SD rats after a single oral administration of 0.5g/kg via oral gavage or blank diet.

Table 4.2. Comparison of the coefficient of variation of the recovery rate of gallic acid (GA), ellagic acid (EGA), and rubusoside (RUB) in the urine samples after a single administration of extract via oral gavage or voluntary oral uptake.

	Coefficient of Variation	
	Oral Gavage	Voluntary Oral Uptake
GA	63.85 (n=18)	46.15 (n=20)
EGA	82.46 (n=17)	56.53 (n=20)
RUB	67.62 (n=17)	63.16 (n=18)

4.3.3 Determination of the Minimal Effective Dose for the Chinese Sweet Leaf Tea Extract

Fig. 4.4 demonstrated the urinary excretion of the three main markers in the sweet leaf tea extract after a single oral administration to the normal SD rats. Twenty-four hour urine was collected from each rat for five consecutive days. The dosages consumed ranged from 0.3 g/kg to 1.4 g/kg of body weight. A quadratic regression was developed for each marker compound to determine the relationship between the dosage levels and the urinary excretion of the respective marker compound. Apparently, the excretion of total GA ($p = 0.0418$) and EGA ($p = 0.0003$) reached their maximum levels between the doses of 0.4 g/kg to 0.5 g/kg of body weight. While the level of GA decreased almost linearly with the oral dosage level, the EGA level decreased rapidly at lower dosage level (i.e., at the dose of 0.4 g/kg to 0.6g/kg) followed by a slow rate of decline as oral doses increased. The amount of RUB, on the other hand, appeared to be constant at 0.6% w/w even when oral doses increased to 1.4 g/kg ($p = 0.9861$). Current results revealed that the accumulative amounts of GA and EGA in the urine were dose-dependent but that of RUB in the urine was dose-independent. Overall, the highest amounts of GA, EGA, and RUB

were achieved by orally administering the sweet leaf tea extract at a 0.5g/kg dose. Based on this determination, the extract dose of 0.5g/kg body weight was chosen for the weight change study.

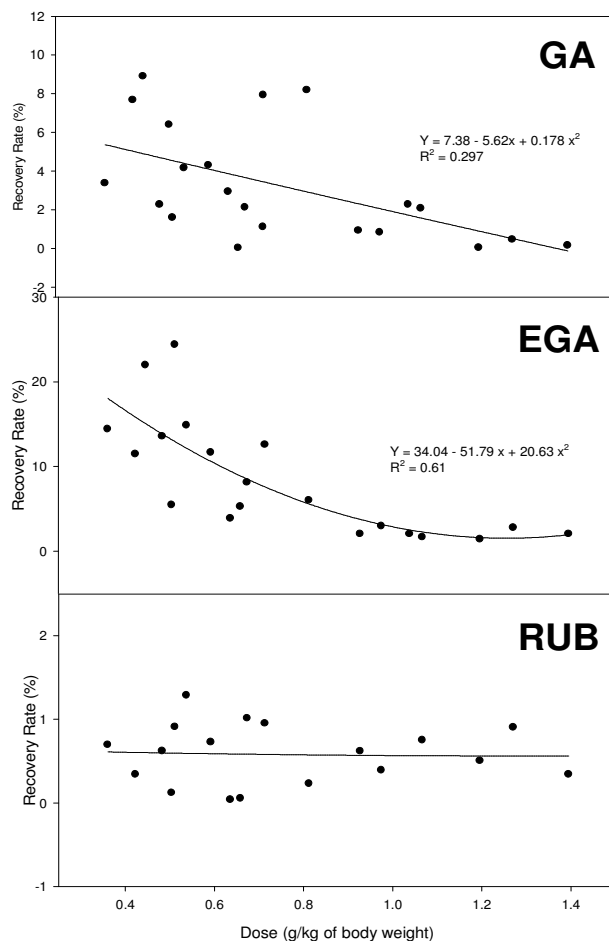


Figure 4.4. Recovery rates (%) of the unmetabolized gallic acid (GA), ellagic acid (EGA), and rubusoside (RUB) in the urine after a single oral administration of the Chinese sweet leaf tea extract (*R. suavissimus*) at random doses ranged from 0.3 to 1.5 g/kg of body weight. Quadratic regression was fitted for each compound (Y = recovery rate; x = dose).

4.3.4 Changes in Body Weight and Food Intake in Normal SD Rats Orally Administered the Sweet Leaf Tea Extract

Changes in the cumulative body weight gain of the treated groups in comparison with the control group were shown in **Fig. 4.5**. To best illustrate the response patterns, the overall body weight gain was divided into 3 phases based on the differences in the rate of body weight gain. Phase 1 was an accelerated growth period where there were no differences in body weight gain between

the treated and the control group. It was during the second phase that the rate of body weight gain started to deviate among the groups (**Table 4.3**). The deviation in body weight gain of both RUS and GER began at week 3 after a single administration of the extract at a dose of 0.5 g/kg of body weight and widen to an approximately 5%. The weight gain difference became stabilized in Phase 3 to 6.71% from the RUS group and 6.02% from the GER group. It is also interesting to show that the effect of GER on the body mass was comparable to the standardized Chinese sweet leaf tea extract. Although the treatments caused about 6% weight loss, it was not statistically different.

Food intake, overall, was not affected by the RUS or GER treatments and each animal consumed diet approximately 5% of their body weight. Despite the significant differences in the relative food intake caused by the initial exposures of rats to RUS and GER (**Fig. 4.6**), it was mostly attributed to the early phase taste adaptation.

4.3.5 Effect of the Treatment Withdrawal on Body Weight and Food Intake

Since the weight loss effect was stabilized to non-significant 6%, a spontaneous idea came to examine if the RUS and GER withdrawal from the diet for two weeks could result in the regaining of lost weight. As shown in **Fig.4.7**, body weight regained to the level comparable to the control group. More interesting, the resumption of RUS or GER treatment for the following two weeks had no effect, indicating a phased in effect as previously shown (**Fig. 4.5**).

4.3.6 Toxicology of the Chinese Sweet Leaf Tea Extract in Normal SD Rats

Oral administration of the extracts was well-tolerated for the entire 10-weeks experiment. There were no observed clinical signs of toxicity or any adverse effects. No significant results were observed between the treated and the control group in hematology (**Table 4.4**) or serum chemistry (**Table 4.5**) except that the BUN level in the RUS group was significantly lower than the control group. Yet, the variation was marginal and therefore may not be treatment-related.

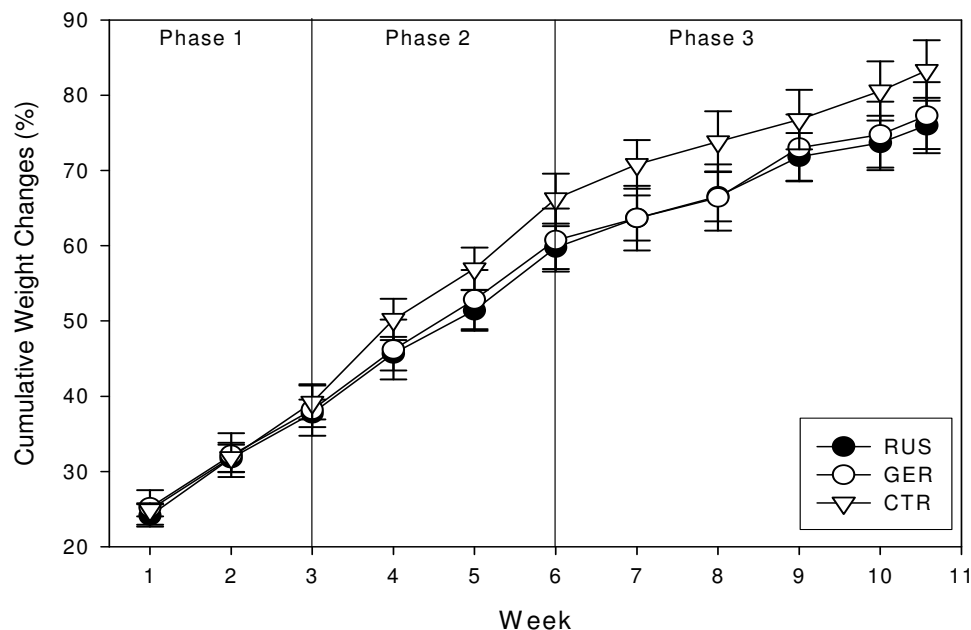


Figure 4.5. Effects of the standardized Chinese sweet leaf tea (*Rubus suavissimus*) extract (RUS) and the purified extract (GER) on cumulative body weight changes (%) in male SD rats fed with normal diet for 11 weeks. Vertical bars at each data point represent one unit of standard error of the mean (n=8 for RUS and GER group; n=9 for CTR group).

Table 4.3. Weekly differences in body weight changes between the treated groups (RUS and GER) and the control group at three different phases. At phase 3, differences between treated and control groups were averaged over time. All values were expressed as means or mean \pm standard error (n=8 for RUS and GER group; n=9 for CTR group).

Differences between	Phase 1			Phase 2			Phase 3				
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
CTR-RUS	0.78	0.11	1.47	4.54	5.54	6.49	6.71 \pm 0.99				
CTR-GER	-0.31	-0.30	1.01	4.00	4.01	5.51	6.02 \pm 1.44				

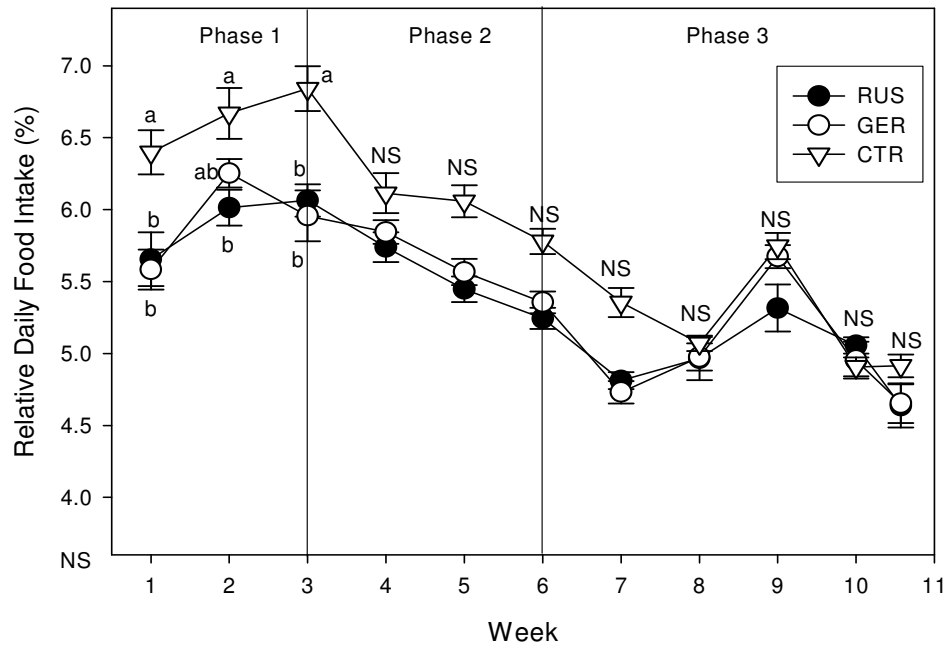


Figure 4.6. Effects of the standardized Chinese sweet leaf tea (*Rubus suavissimus*) extract (RUS) and the purified extract (GER) on the relative daily food intake (%) in male SD rats fed with normal diet for 11 weeks. Vertical bars at each data point represent one unit of standard error of the mean (n=8 for the RUS and GER groups; n=9 for the CTR group). Different letters at each week indicate a significant difference at $p \leq 0.05$. NS represents no significant differences.

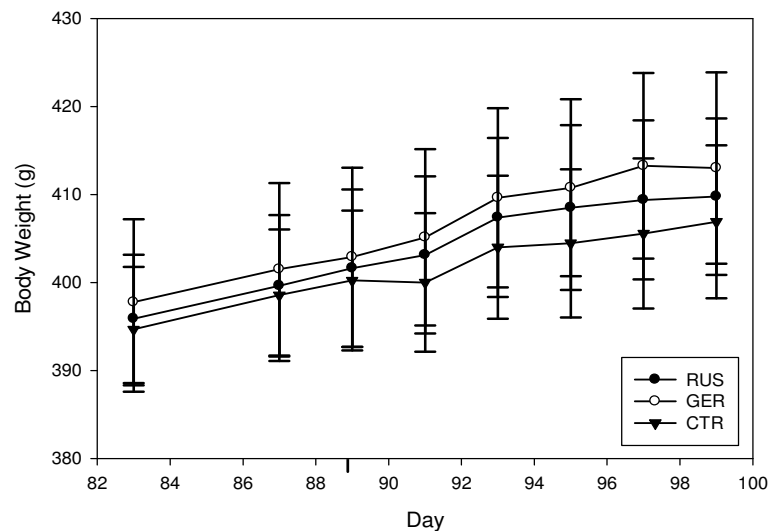


Figure 4.7. Effects of the standardized Chinese sweet leaf tea (*Rubus suavissimus*) extract (RUS) and the purified extract (GER) on body weight compared to the control group (CTR) after two weeks of treatment withdrawal.

Table 4.4. Effects of the standardized Chinese sweet leaf tea (*Rubus suavisissimus*) extract (RUS) and the purified extract (GER) on the hematology test values in male SD rats after the two weeks of treatment withdrawal.

	Groups		
	RUS (n=8)	GER (n=8)	CTR (n=9)
Glucose (mg/dL)	198.75 ± 26.56	250.13 ± 25.43	188.78 ± 17.23
AST (IU/L)	128 ± 14.66 ^a	86.86 ± 9.51 ^b	95.00 ± 7.88 ^c
ALT (IU/L)	43.75 ± 1.77	43.88 ± 2.39	44.8 ± 2.88 ^c
APH (IU/L)	137.00 ± 5.89	136 ± 8.52	150.67 ± 7.85
BUN (mg/dL)	19.00 ± 0.68*	21.63 ± 0.73	22.88 ± 0.72
Creatinine (mg/dL)	0.34 ± 0.02	0.37 ± 0.02	0.31 ± 0.02

Values were expressed as mean ± standard error where n=6 in the RUS group, n=5 in the GER group, and n=7 in the CTR group, unless otherwise specified. ^a n=5.

Table 4.5. Effects of the standardized Chinese sweet leaf tea (*Rubus suavissimus*) extract (RUS) and the purified extract (GER) on the serum chemistry values in male SD rats after two weeks of treatment withdrawal.

	Groups		
	RUS (n=6)	GER (n=5)	CTR (n=7)
RBC (10 ⁶ /μL)	8.13 ± 0.24	7.71 ± 0.15	7.33 ± 0.43
Hemoglobin (g/dL)	14.22 ± 0.44	14.48 ± 0.36	13.31 ± 0.74
Hematocrit (%)	42.32 ± 1.37	41.74 ± 0.90	38.31 ± 2.38
RDW (%)	11.98 ± 0.16	11.88 ± 0.04	12.00 ± 0.16
MCV (fL)	52.53 ± 0.68	54.16 ± 0.36	52.18 ± 0.57
MCH (pg)	18.18 ± 0.28 ^a	18.76 ± 0.20	18.20 ± 0.20
MCHC (g/dL)	34.36 ± 0.32 ^a	34.68 ± 0.16	34.87 ± 0.31
Platelets (10 ³ /μL)	689.67 ± 106.9	506.80 ± 96.35	595.71 ± 161.29
MPV (fL)	6.64 ± 0.23 ^a	8.34 ± 0.56	6.78 ± 0.72 ^a
WBC (10 ³ /μL)	4.32 ± 0.72	3.62 ± 0.53	3.94 ± 0.78

Values were expressed as mean ± standard error where n=8 in the RUS and GER groups, and n=9 in the CTR group, unless otherwise specified. *Value was significant ($p \leq 0.05$) as compared to the control group (CTR). ^a n=6. ^b n=7. ^c n=8

4.4 Discussion

Chinese sweet leaf tea has long been used as a traditional remedy to treat various diseases such as hypertension, atherosclerosis, diabetes, and to maintain healthy kidneys and relieve coughs (Huang and Jiang, 2002). Due to the intense sweetness, the leaves of *Rubus suavissimus* have been a popular beverage leaf tea in Southern China. Reports from recent studies also suggested that the leaf extract of the *Rubus suavissimus* contains anti-inflammatory, anti-allergic, and anti-angiogenic properties (Ono 2002; Liu et al., 2006; Fang et al., 2008). Although inhibiting angiogenesis could cause weight loss as shown by Rupnick et al. (2002), this is the first report

that the standardized Chinese sweet leaf tea extract could slow body weight gain in subjects who are on normal diet. This study revealed a potential weight loss effect in normal SD rats that were given a standard diet (low fat) but supplemented with the sweet leaf tea extract at the dose of 0.5g/kg of body weight. Liu et al (2006) has been reported that the Chinese sweet leaf tea extract contains anti-angiogenic properties, partially exerted by the presence of gallic acid. However, whether angiogenesis inhibition could totally explain the weight loss effect is unknown. It is possible that other mechanisms beyond the inhibition of angiogenesis may be responsible for the multiple components in the extract could target multiple organs and pathways and act in different locations.

Chou et al. (2009) revealed the presence of four major classes of components in the Chinese sweet leaf tea extract. They are gallotannins represented by gallic acid, ellagitannins represented by ellagic acid, flavonoids represented by rutin, and diterpene glycosides represented by rubusoside and steviol monoside. Since our preliminary absorption study showed that rutin and steviol monoside were not detected in the urine samples collected from the animals being fed with the Chinese sweet leaf tea extract (**Fig. 4.2**), it might be possible that they were not involved in the systemic functioning. The low absorption of rutin and steviol monoside might be the results of biotransformation in the gastrointestinal tract. Studies have shown that rutin (Manach et al., 1997; Jaganath et al., 2006) and steviol monoside (Geuns et al., 2007) were mainly metabolized and absorbed in the colon and thus was slowly absorbed into the plasma. Rutin was catabolized into 3-methoxy-4-hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid in the liver and then excreted through the urine (Jaganath et al., 2006). Since we did not measure the metabolites of rutin in the urine nor the serum, we could not totally exclude the systemic bioavailability of rutin. Steviol monoside, on the other hand, metabolized in the liver and excreted through the urine in the form of steviol glucuronide (Geuns et al., 2007). This may

possibly explain the phenomena in our current study where rutin and steviol monoside, either in the free or conjugated form, were not detected in the urine samples. Additional studies are still needed to further determine and verify the bioavailability of the marker components in the experimental subjects. However, this study was designed to answer a proof-of-concept question whether the sweet leaf tea had an effect on body weight gain and the data provided a positive answer. Therefore, it justifies additional investigations.

In the present absorption study, only gallic acid, ellagic acid, and rubusoside were detected in the urine of normal SD rats administered with 0.5g/kg of standardized Chinese sweet leaf tea extract, indicating they were bioavailable through the gastrointestinal (G.I.) tract absorption. In addition to the possibility of systemic functioning, components in the extract could have functioned locally inside the G.I. tract system. Gallic acid and ellagic acid have been shown to exhibit various pharmacological effects. For example, gallic acid was inhibitory of the alpha-glucosidase activity, an enzyme that break down carbohydrates into the absorbable form of glucose (Li et al., 2008) and suppressed high-fat diet-induced dyslipidemia in rodent (Hsu et al., 2007). Ellagic acid was found partially responsible for the anti-obesity effect in mice after the administration of ellagic acid-containing pomegranate leaf extract (Lei et al., 2007). Rubusoside has also been reported to have anti-microbial effect in the G.I. tract when it was given a high concentration based on a preliminary study (unpublished data). Because of the possible functions of these compounds in local and systemic ways, it is imperative to hypothesize that the observed weight loss effect of the Chinese sweet leaf tea might have been a collective effect of multiple components on multiple targets at multiple sites.

Since gallic acid, ellagic acid, and rubusoside are the major components and accounted for almost 27% by weight of the total extract, it was very intriguing to test if these tri-compounds combination (i.e., the purified extract) could explain the weight loss effect. This desire led to a

new formulation that consisted of a combination of gallic acid, ellagic acid, and rubusoside (GER) at a dose of 0.14g/kg of body weight that was equivalent to the total extract of 0.5g/kg. Surprisingly, our current results demonstrated that the tri-compound combination exhibited similar weight-suppressive effect as in the total extract. This shows that the tri-compounds combination may represent the majority of the responsible components in the Chinese sweet leaf tea extract in suppressing the body weight gain in normal rodents. This important finding shed directing light toward narrowing down the truly important components of the total extract. In the present study, GER was composed of similar amount of gallic acid and rubusoside but with higher amount of ellagic acid as in the total extract of the standardized Chinese sweet leaf tea, which was possibly caused by the extract's batch-to-batch variation. Free ellagic acid has been reported to have poor water solubility (Bala et al., 2006), which makes it even harder to recover during the process of purification or extraction. Our current study showed an increased of ellagic acid concentration in the GER by almost five-fold higher than the total extract while the concentration of gallic acid and rubusoside remained about the same. This may further suggest that the variation of ellagic acid between these two samples was highly due to batch-to-batch differences. Nonetheless, our results revealed interesting data where the GER could achieve similar effect as the standardized sweet leaf tea extract by suppressing 6% of weight gain compared to the control group. After all, the bottom line of the results clarified that the increased level of ellagic acid was independent from the phenotypic response and hence, ellagic acid, in this case, may not be a critical compound to the overall effect or was sufficient in the extract already. More detailed investigations are warranted to determine the contribution of ellagic acid alone or other bioactive components to the overall weight loss effect.

The combination of GER used only 27% by weight of the extract while the 70% was excluded. Since the GER demonstrated similar effect as the total standardized extract, we

predicted that these 70% of unknown components did not contribute to the overall weight loss effect. If GER is proven the majority of the effectors, the use of it in place of the total extract could be a very promising alternative as GER has an easier quality control, improved palatability, and reduced dose burden. Hence, GER, with the strength to avoid batch-to-batch variation often seen existent with the extract, should be considered for future studies as a standardized Chinese sweet leaf tea extract.

Our present results discovered a possible weight loss effect by the standardized Chinese sweet leaf tea extract. However, in addition to the extract quality control, the overall pharmacological response may be affected by the route of administration, dosages, and number of times administered as these factors may be critical to the metabolism and absorption of bioactive components. Kao et al (2006) stated that the pathways or mechanisms involved in the energy absorption may be highly dependent on the factors stated. In our first experiment, oral gavage was selected as the route of administration. Nevertheless, the distress level resulted from the method created a huge variation between the subjects, which yielded a coefficient of variation in the recovery of bioactive components of more than 50% (**Table 4.2**). Needless to say, complications arose from gavage led to removal of subjects from the study and ultimately reduced the overall sample size. Thus, for a long term study, oral gavage may be labor-intensive. To improve the variations and reduce the distress level, a voluntary oral uptake was selected for the study. Clearly, improvement of variation was observed with the selected extract delivery method via liquid food. Indeed, delivery of extract via liquid food or other form of diet was only limited to certain extracts due to novel food aversion and natural taste of the extract itself. Other methods tested on current study, such as delivery via powdered diet and yogurt, were some alternatives. Yet, major spillage had occurred with the powdered diet during delivery which further affected the intake of extract. The compositions in yogurt, however, may interact with the

extract thereby affect the accuracy of the experiment. Although other delivery methods, such as intraperitoneal or intravenous injection, may be preferred for accuracy of results, the most abundant compounds in the extract, such as gallic acid and ellagic acid, were metabolized and absorbed along the GI tract (Lei et al., 2003; Konishi et al., 2004). Hence, the currently used delivery method was still satisfied from the perspective of convenience and physiological effects.

For the first time, our study revealed a possible anti-obesity effect of the Chinese sweet leaf tea extract with an approximately 7% of weight difference compared to the control group. Unfortunately, we were unable to show a statistically difference between the treated and control groups ($P = 0.4776$). We predicted that inappropriate use of the model may have affected the investigation of anti-obesity effect in our current study. A statistical difference and significance may be achieved if obese model were used instead of the normal model. Secondly, a power analysis based on the variations from current study suggested a sample size increased ($n=14$) would have been needed to produce a statistically significant effect with 80% of power. In addition, the overnight fasting, which initially designed to encourage extract intake, also may affect the normal weight gain of all rats, thereby mask the effect of weight loss during the treatment period. More studies with detail and valid experimental design are warranted to obtain more valuable data in verifying the anti-obesity effect of the Chinese sweet leaf tea.

4.5 Summary

This is the first report that the Chinese sweet leaf tea extract had a possible weight loss effect on the normal SD rats. Based on the preliminary analyses of the 24-hour urine after a single oral administration of extract, gallic acid, ellagic acid, and rubusoside were likely functioned via systemic and/or local approaches. Overall, the single daily oral administration of standardized sweet leaf tea extract at 0.5 g/kg of body weight demonstrated a 6.71% weight difference compared with the control although the difference was not statistically significant. Because the

tri-compounds combination composed of gallic acid, ellagic acid, and rubusoside (GER) exhibited similar effect as the standardized Chinese sweet leaf tea extract, it is possible that these compounds may be the responsible components for the weight loss effect. However, the mechanisms remain unknown. No sign of toxicity was observed in current study which further verifies the safety of the Chinese sweet leaf tea extract. More studies and investigations are warranted to further verify the weight loss effect of the Chinese sweet leaf tea extract as well as the possible mechanism and pharmacokinetics.

CHAPTER 5. ANTI-OBESITY EFFECT OF THE CHINESE SWEET LEAF TEA EXTRACT (*RUBUS SUAVISSIMUS* S. LEE) IN DIET-INDUCED OBESE RATS

5.1 Background

Obesity is a common metabolic disease that is growing worldwide. With increased body weight gain, obesity is a common risk factor for diseases such as diabetes, hypertension, and cardiovascular disease due to the excessive deposition of adipose tissues (Haslam et al., 2005; Aronne et al., 2007; Shah et al., 2008). It is believed that the accumulation of adipose tissues may be due to an imbalance between energy intake and energy expenditure. Aronne et al. (2007) reported that a 5 to 10% of weight reduction highly improve lipid profiles, insulin sensitivity, and endothelial functions. Thus, lifestyle modifications, such as dietary management and increased physical activities, are obligatory to overcome the epidemic of obesity.

With the limited weight management programs and pharmacological options, the health-conscious population is now searching for treatment alternatives in weight maintenance with an emphasis on the application of natural products, especially functional foods. Natural products such as the green tea (Dulloo, 1999; Sayama et al., 2000; Zheng et al., 2004; Boschmann et al., 2007; Bose et al., 2008; Ito et al., 2008), ginger (Al-Amin et al., 2006; Goyal et al., 2006), and capsaicin (Hsu and Yen 2007; Kang et al., 2007; Snitker et al., 2009) have shown efficacies on reducing body weight of obese animal models. However, the scientific evidences of herbal medicines in the prevention or treatment of obesity is still unconvincing. The most effective herbal agents were perhaps ephedrine and caffeine, alone or in combinations. For several years, ephedrine and caffeine have been used as dietary supplements for weight loss until toxicity issues prompted the Food and Drug Administration (FDA) to halt their use for this purpose. Currently, there are no effective herbal products for weight management despite numerous and tireless research efforts. Consequently, the search continues and prompts this investigation of a

folk medicine commonly known as the sweet leaf tea (*Rubus suavissimus*), one of the many *Rubus* species in the Roseasae family.

As a traditional folk remedy, *R. suavissimus* has been widely applied in southwestern China to treat various diseases, which include the type 2 diabetes, hypertension, and to relieve cough. In the screening of medicinal plants, the leaf extract of the *R. suavissimus* was tested positive for the anti-angiogenic activity which has been shown to be partially exerted by the presence of gallic acid. Other studies demonstrated that sweet leaf tea extract was able to inhibit the activity of NF- κ B (Liu et al., 2005) and α -amylase activity (Li et al., 2007) where these factors are closely related in treating or regulating glucose metabolism. Because recent report showed a close relationship between angiogenesis and obesity (Brakenhielm 2008; Brakenhielm and Cao, 2008), a hypothesis was formed that the leaf extract of *R. suavissimus* may suppress the accumulation of adipose tissues thereby causing weight loss. In support of this hypothesis, gallic acid and rubusoside have been shown to reduce the activity of α -amylase and bacterial activities, which may further decrease energy absorption by blocking the nutrient breakdown in the colon. Therefore, in addition to angiogenesis inhibition, we believe that the multiple components obtained from the Chinese sweet leaf tea extract may work simultaneously in producing a significant weight loss effect via specific mechanisms.

Scientific research on the Chinese sweet leaf tea, especially on the focus of pharmacological function, has been scarce as the discovery of Chinese sweet leaf tea is novel. Unlike the Chinese sweet leaf tea, green tea has long been a popular ingredient in either food or the pharmacology industry, especially in countries such as Japan, China, Korea, and Morocco (Cabrera et al., 2006). For years, epidemiological and clinical studies have been conducted on green tea (*Camellia sinensis*) for various pharmacological functions such as improvement of insulin sensitivity and glucose tolerance (Wu et al., 2004; Li et al., 2006; Nagao et al., 2009),

protective functions on brain and pancreas (Unno et al., 2009), anti-cancer (Shirakami et al., 2009; Yang et al., 2009), anti-inflammatory (Navarro-Peran et al., 2008) activities, and immune system enhancement (Cabrera et al., 2006). In addition, green tea is one of the most extensively studied plants in the prevention of metabolic syndrome by stimulating fat oxidation and reducing body fat accumulation (Tokimitsu 2004; Nagao et al., 2007; Monteiro et al., 2008; Nagao et al., 2009). Studies also reported that the potential role of green tea in weight loss or weight maintenance is mainly attributed to the presence of catechins, such as the (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC) (Cabrera et al., 2006; Wolfram et al., 2006; Thielecke and Boschmann 2009). In addition to catechins, Zheng et al. (2004) showed that caffeine and theanine might be the compounds responsible for suppressing the weight of obese subjects via the regulation of lipid metabolism and thermogenesis. Results further demonstrated that the weight suppressive effect resulting from the combination of catechins and caffeine was actually stronger than the caffeine or catechins alone (Zheng et al., 2004). Dulloo et al. (1999) also reported a 4% increase of 24-hour energy expenditure in a green tea-treated group where the elevation of thermogenesis may be attributed to the synergistic effect of the green tea components (i.e., catechins and caffeine).

From previous studies, we know that gallic acid, ellagic acid, and rubusoside are the major compounds from Chinese sweet leaf tea extract that are responsible for weight loss. Since the presence of caffeine and catechins differentiate the Chinese sweet leaf tea from the green tea, it is questionable whether the Chinese sweet leaf tea extract would yield a similar or increased weight loss effect as in the green tea. Thus, in this study, green tea extract (as a positive control) and the purified Chinese sweet leaf tea extract composed of three major marker compounds, gallic acid, ellagic acid, and rubusoside, were fed to the obese prone Sprague-Dawley (SD) rats. Results from this study have provided further confirmation of the anti-obesity effect on the

Chinese sweet leaf tea extract and thus revealed a novel pharmacological function of the respective extract beyond its historic use as a natural sweetener or beverage leaf tea.

5.2 Materials and Methods

5.2.1 Reference Standards

Gallic acid (GA), rutin (RUT), and ellagic acid (EGA) were purchased from Sigma Chemical Company (St. Louis, MO). The reference standards of rubusoside (RUB) and steviol monoside (STM) were isolated by our own lab and were identified by spectral data (UV, MS, ^1H NMR, ^{13}C NMR and 2D-NMR). Both RUB and SM have purities greater than 98% by HPLC–PDA analyses based on a peak area normalization method.

5.2.2 Extracts

The purified Chinese sweet leaf tea extract (GER) is composed of 1.38% w/w of gallic acid, 2.4% w/w of ellagic acid, and 23.41% w/w of rubusoside. These compositions were similar to those found in the standardized sweet leaf tea (RUS) extract as shown in **Fig. 5.1**. Green tea extract, on the other hand, was provided by Amax Nutrasource (Eugene, OR). The major compositions in the green tea extract are shown in **Table 5.1** and the chromatographic fingerprint is shown in **Fig. 5.2**.

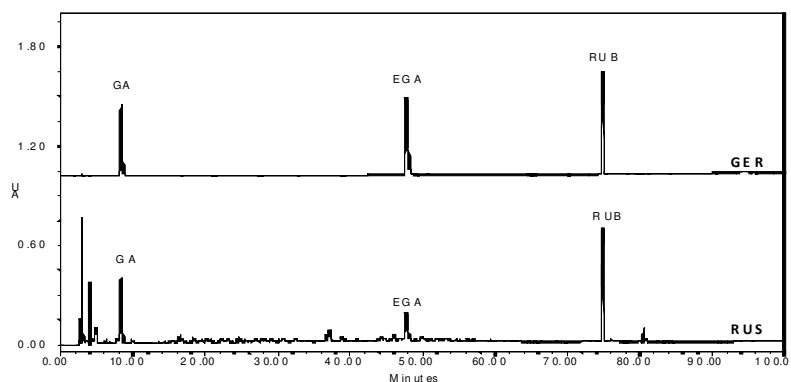


Figure 5.1. Chromatographic fingerprint of the purified Chinese sweet leaf tea (*Rubus suavissimus*) extract (GER) composed of gallic acid (GA), ellagic acid (EGA), and rubusoside (RUB) in a composition equivalent to the standardized extract (RUS).

Table 5.1. Major compositions of the green tea extract.

Content	Yield (%)
Polyphenols	50.78%
Tea Catechins	36.67%
EGC	8.57%
DL-C	3.24%
EGCG	16.51%
EC	1.87%
GCG	1.48%
ECG	5.00%
Caffeine	8.97%

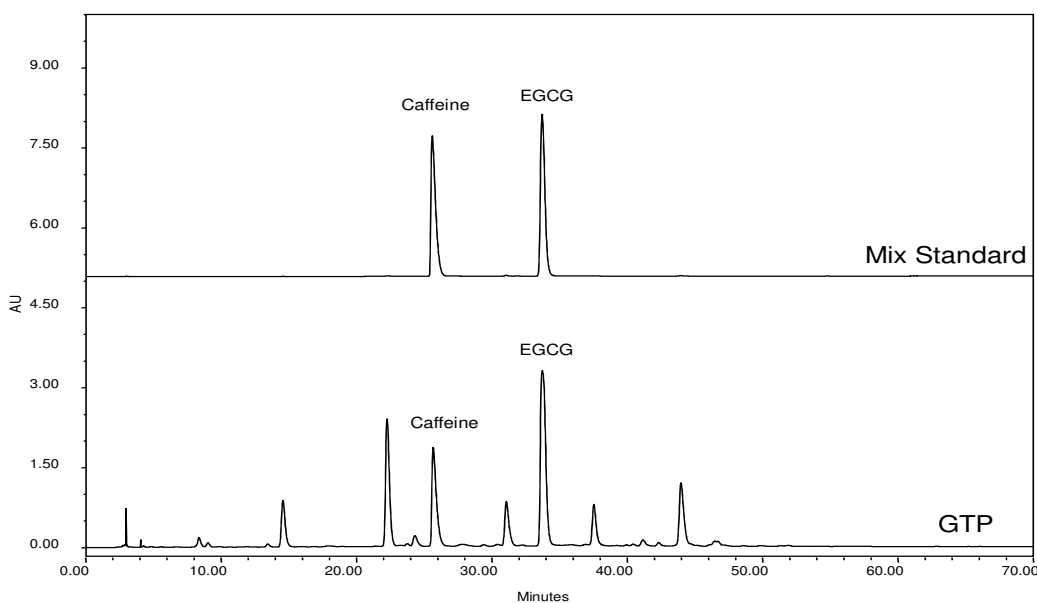


Figure 5.2. Chromatographic fingerprint of the green tea powder (GTP) and the mix standard solution composed of caffeine and EGCG.

5.2.3 Animals

Male Obese Prone Sprague-Dawley (OP-CD) rats (4-6 week old) used in the experiments were purchased from Charles River (Charles River Laboratories International Inc., Wilmington, MA, USA). Animals were housed individually in stainless steel cages in an air-conditioned room at

21°C ± 2°C, 50-60% relative humidity, and 12/12 h light/dark cycle. Prior to euthanization, urine was collected from each rat through an individual metabolic cage (Lab Products Inc, Seaford, DW, USA) and blood or serum was collected via cardiac puncture. In addition, liver, kidneys, gastrointestinal tract, cecum, epididymal, retroperitoneal, and perirenal adipose tissues were weighed individually and stored at -20°C for future analyses. Cecal and fecal contents were also collected and immediately stored at -20°C. All procedures were performed under the approved protocols by the Institutional Animal Care and Use Committee of Louisiana State University (LSU-IACUC), Louisiana, USA.

5.2.4 Experimental Design

Sixty rats were divided into 4 groups with 15 rats in each group. One group (n = 15) was fed with standard (Purina 5001, Nutrition International, Brentwood, MO, USA) and others (n = 45) with the high fat chow (Research Diets Inc, New Brunswick, NJ, USA). A small amount of extract was also given during phase 1 (**Fig. 5.3**) via liquid diet (Dyets Inc, Bethlehem, PA) to reduce the aversion to the extract. Prior to the extract administration, the obese group was again divided into three groups with two treated and one control group receiving either extract or vehicle during the treatment period, as shown below.

- Group 1 (GER): Treated group receiving the purified Chinese sweet leaf tea extract
- Group 2 (GTP): Treated group receiving the green tea (*Camellia sinensis*) extract
- Group 3 (HFD): Control group receiving high fat diet as vehicle
- Group 4 (STD): Control group receiving standard diet as vehicle

Gallic acid (GA), ellagic acid (EGA), and rubusoside (RUB) were incorporated into the high fat diet at a concentration of 1.38% w/w, 2.4% w/w, and 23.41% w/w, respectively (i.e., GER).

These concentrations were similar to the GA, EGA, and RUB found in a 3% standardized Chinese sweet leaf tea extract diet. The designated extract diet concentration was determined

based on the relative daily food intake as well as the acceptance of extract in order to reach the GER dosage of approximately 0.22g/kg of body weight, which is equivalent to the standardized extract dosage of 0.8g per kg of body weight. The GTP group, on the other hand, received green tea extract diet at a concentration of 3.5% to reach the dose of approximately 1.0 g per kg of body weight (0.01% w/w). All rats were provided *ad libitum* access to food and tap water throughout the treatment period for 9 weeks. Body weight and food intake were measured weekly. Fasting blood glucose level was measured at week 4 and week 8 of the treatment period, respectively. Animals were also observed daily for any abnormal physical and behavioral changes or signs of toxicity, including posture, rough hair coat, decreased responsiveness, and unusual breathing pattern, etc.

5.2.4.1 Measurement of Food Intake

All rats were given *ad libitum* access to either high fat or standard chow diet. Food intake was measured weekly and the relative daily food intake (%) was calculated to determine the effect of extract administration on daily food intake. Calculation of the relative daily food intake was shown below:

$$\text{Relative Food Intake (\%)} = \text{Total Food Intake (g)} / \text{Body Weight (g)} * 100\%$$

5.2.4.2 24-hour Urine Collection

At the day of urine collection, each rat was placed into a metabolic cage (Lab Products, Inc. Seaford, DW) to collect 24-hour urine and feces after the administration of extract or food. Urine and fecal samples collected were stored in the refrigerator at -20°C until analysis.

5.2.4.3 Blood Glucose Measurement

Fasting blood glucose was measured to monitor the improvement of blood glucose levels after the administration of the purified Chinese sweet leaf tea and green tea extract. Blood glucose was measured at week 4 and week 8. Measurements obtained prior to the initiation of treatment

served as a baseline (week 0). A small drop of blood was drawn from the tail-vein and blood glucose levels were measured with a commercial glucometer (Abott Laboratories, North Chicago, IL) after a 6-hour fasting during the day (8 am till 2 pm).

5.2.4.4 Blood Sampling and Clinical Pathology

At the end of the treatment, rats were anesthetized with isoflurane and euthanized. Blood samples were collected from each group to examine any possible toxicity or adverse effects that resulted from the consumption of the extracts. For the hematology analysis, blood samples were collected in an EDTA vacutainer (Beckin, Dickinson and Company, Franklin Lakes, NJ). The parameters tested included the RBCs, hemoglobin, hematocrit, red blood cell distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, mean platelet volume (MPV), and total WBCs. For the chemistry analysis, blood samples were collected in a vacutainer and were allowed to coagulate. Serum was separated via centrifugation at $2000 \times g$ for 10 min. Serum chemistry tested included serum glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (APH), creatine kinase (CK), total bilirubin, total protein, albumin, globulin, total cholesterol, urea nitrogen (BUN), creatinine, calcium, phosphorus, sodium, potassium, chloride, bicarbonate, and anion gap. Both blood and serum samples were stored at -20°C and immediately submitted to the Louisiana Animal Disease Diagnostic Laboratory (School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA) for analysis.

5.2.5 Statistical Analyses

All data was analyzed with the Statistical Analysis System (SAS, Cary, NC). Analysis of variance (ANOVA) with repeated measured was performed on the body weight changes, relative daily food intake, and blood glucose levels throughout the experimental period. The values of

serum chemistry and hematology tests were evaluated statistically for differences between the control and treated groups using the one-way ANOVA. Tukey's post hoc test was performed on all tests to compare the group differences. Significance of all tests was set at $p \leq 0.05$. Outlier detection was performed and data was eliminated only when the R-student scores ≥ 2.50 and improvements were significantly observed in the coefficient of variation, R-square, and the test of normality. All results reported were expressed as mean \pm SEM, unless otherwise stated.

5.3 Results

5.3.1 Effects on Body Weight and Food Intake of the Obese SD Rats

In the present study, obesity was induced in the obese-prone SD rats by feeding high-fat diet for 14 weeks. The experimental period was divided into three phases based on the level of extract dosages consumed. The first phase, lasted for approximately 5 weeks, was defined as an adaptation period where minimal or no extract was administered to the treated rats (**Fig. 5.3**). During the adaptation period, both the GER and GTP extracts were given to the animals as a supplementation to eliminate possible taste aversion. At this stage, the extract was mixed in the liquid food at a concentration of 10 mg/ml and all rats had free access to the extract during the 8-hour day cycle. **Fig. 5.3** showed that approximately 0.02% to 0.03% w/w of the extract consumption was attained at the end of this phase for both the GER and GTP groups. These levels of extract consumption affected neither the weight (**Fig 5.4**) nor food intake (**Fig 5.5**). Deviation of body weight changes started emerging towards the end of this phase, showing 1% and 7% less weight gain in the GER and GTP than the HFD group, respectively (**Fig. 5.4**). To increase the intake levels of extract, the delivery method was changed to incorporating the extracts into the chow diet (phase 2). A significant improvement in the extract consumption was observed during phase 2 which signified the beginning of the 9-week treatment period (**Fig 5.4**). As a result of increased extract consumption featured in the second phase, significant weight

gain reductions in the GER and GTP groups against the HFD group were observed after a week of extract administrations at the level of 0.05% w/w and 0.07% w/w, respectively, and the reductions were similar as the STD group. GTP caused further reduction in body weight gain as the dose escalated to approximately 0.08% w/w (**Fig. 5.3**). Furthermore, the food intake was seen to slightly fluctuate in this phase (**Fig. 5.5**) due to the change of the delivery method. Food intake became stabilized, thus consumption of extract, which symbolized the beginning of phase 3. The third phase of the experiment was the continuation of the treatment period that lasted for 6 weeks where the desired dosages (equivalent extract dose of 0.8 g/kg body weight for GER and 1 g/kg of body weight for GTP) were attained (**Fig. 5.3**). No difference in food intake was observed in this 6-week constant-dose treatment period among the obese groups (**Fig. 5.5**). The body weight gain rates in the GER and GTP groups were significantly lowered and maintained almost constantly at 20% and 28%, respectively. The body weight gain of the GER group was comparable with the standard control group (STD) both shown about 20% less weight gain than the HFD group. At week 5 of the 9-week treatment period, GTP already caused 27% ($p \leq 0.001$) and 12% ($p = 0.0081$) less body weight gain than the HFD and STD groups, respectively. The reductive effect of GER and GTP on body weight gain continued after week 5, albeit at slower rates. At the end of the treatment, a 22% ($p \leq 0.001$) and a 32% ($p \leq 0.001$) less body weight gains were observed in the GER and GTP group than the HFD group, respectively. No significant difference in body weight gain was observed between the GER and STD groups ($p = 0.2511$). Overall, the group on high-fat diet consumed about 4% of their body weight while that on standard diet consumed about 6% of their body weight during the treatment period (**Fig. 5.5**). Although there was a significant difference in the amount of food intake between the food types, the difference was mainly due to the diet composition and calorie intake.

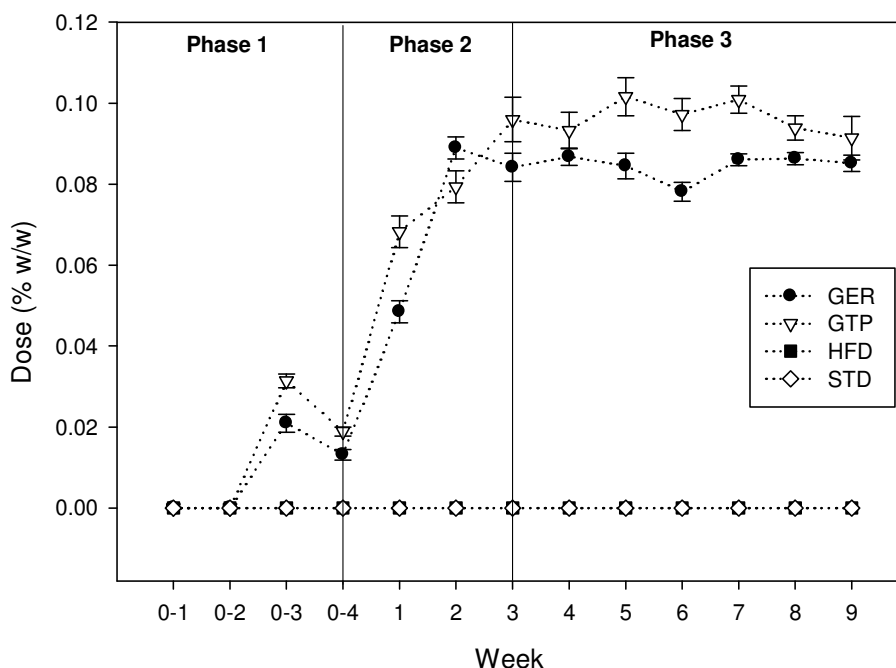


Figure 5.3. Average oral intake (% w/w) of the purified Chinese sweet leaf tea (*Rubus suavissimus*) extract (GER) and green tea (*Camellia sinensis*) powder (GTP) by the diet-induced obese SD rats. All data were expressed as mean \pm standard error where n=15. NOTE: The dose of GER is expressed as the equivalent dose to the standardized Chinese sweet leaf tea extract (RUS).

5.3.2 Effect on Organ Weight and Fat Accumulation of the Obese SD rats

Liver, kidneys, epididymal, retroperitoneal, and perirenal adipose pads were collected from the rats at the day of euthanization. The weights of liver, kidneys, mesentery with fat, and total abdominal fat are shown in **Table 5.2**. The distribution diagram of the adipose pads collected is shown in **Fig 5.6**. Total abdominal fat, which consisted of the epididymal, retroperitoneal, and perirenal fat, was remarkably reduced in the STD, GER and GTP groups by 57%, 48% and 70%, respectively, when compared to the HFD group (**Table 5.2**). The reduction of fat mass was mainly due to the reduction of retroperitoneal and perirenal adipose pads (**Fig. 5.6**). Mesentery fat in the STD, GER, and GTP group was also significantly reduced by 48%, 56%, and 74%, respectively, compared to the HFD control group. GTP-treated group, overall, had the lowest abdominal fat. GER and STD (low-fat diet) showed the same effect on fat accumulation. The

weight of liver and kidneys of the GER- or GTP-treated group was not significantly different from that of the HFD group. However, the weight of the liver and kidneys in the STD group (on low-fat diet) was much higher than the groups on high-fat diet.

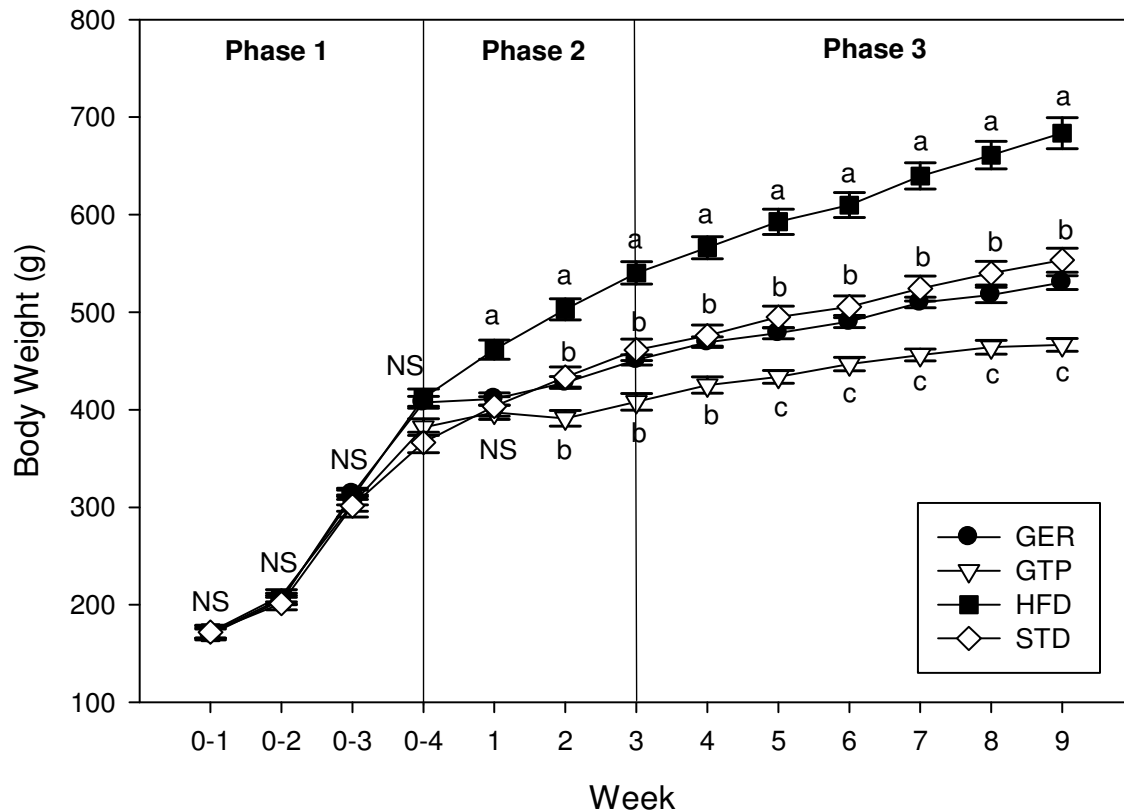


Figure 5.4. Effects of the purified Chinese sweet leaf tea (*Rubus suavissimus*) extract (GER) and green tea (*Camellia sinensis*) powder (GTP) on body weights in diet-induced obese SD rats compared to the control groups receiving high fat (HFD) and normal (STD) diet, respectively. All data were expressed as the mean \pm standard error where $n=15$. Different letters on each week indicate a significant difference at $p \leq 0.05$.

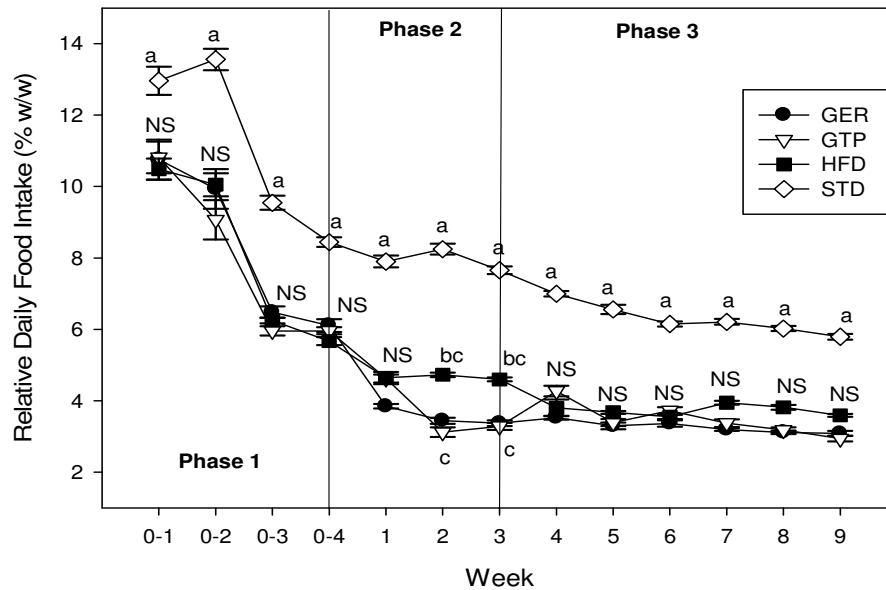


Figure 5.5. Effects of the purified Chinese sweet leaf tea (*Rubus suavissimus*) extract (GER) and green tea (*Camellia sinensis*) powder (GTP) on the relative daily food intake (% w/w) in diet-induced obese SD rats compared to the control groups receiving high fat (HFD) and normal (STD) diet, respectively. All data are expressed as the mean \pm standard error where $n=15$. Different letters on each week indicate a significant difference at $p \leq 0.05$. NS indicates no significant difference.

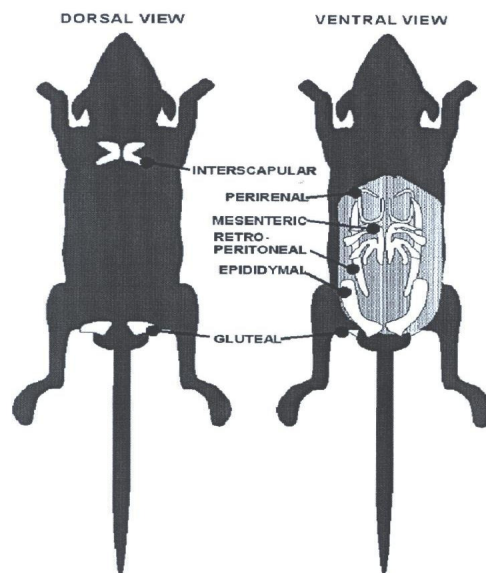


Figure 5.6. Distribution diagram of the perirenal, retroperitoneal, and epididymal fat in male rat. Reference: Remesar et al., 2002.

Table 5.2. Effects of the purified Chinese sweet leaf tea (*Rubus suavissimus*) extract (GER) and green tea (*Camellia sinensis*) powder (GTP) on the weight (% w/w of disemboweled body weight (DBW)) of organs and abdominal adipose tissues in diet-induced obese SD rats compared to the control groups receiving high fat (HFD) and normal diet (STD), respectively.

Relative Organs/Tissues Weight (% w/w)	Group			
	HFD	STD	GER	GTP
Liver	2.95 ± 0.17 ^b	3.52 ± 0.23 ^a	2.87 ± 0.06 ^b	2.81 ± 0.21 ^b
Kidneys	0.54 ± 0.04 ^c	0.71 ± 0.03 ^a	0.62 ± 0.01 ^b	0.65 ± 0.04 ^b
Mesentery with Fat	3.77 ± 0.71 ^a	1.95 ± 0.44 ^b	1.66 ± 0.07 ^b	0.99 ± 0.21 ^c
Total Abdominal Fat	10.07 ± 1.50 ^a	4.37 ± 0.88 ^b	5.28 ± 0.26 ^b	3.03 ± 0.73 ^c
Epididymal	3.90 ± 0.46 ^a	1.90 ± 0.40 ^b	2.18 ± 0.10 ^b	1.42 ± 0.25 ^c
Retroperitoneal	4.45 ± 0.93 ^a	1.66 ± 0.38 ^c	2.27 ± 0.13 ^b	1.15 ± 0.37 ^c
Perirenal	1.71 ± 0.30 ^a	0.81 ± 0.16 ^b	0.83 ± 0.04 ^b	0.46 ± 0.14 ^c

All values were expressed as mean ± standard error where n=15. Different letters on each row indicated a significant difference at $p \leq 0.05$.

5.3.3 Effect on Blood Glucose of the Obese SD rats

Fasting blood glucose was measured after a 6-hour fasting period during the day. **Fig. 5.7** showed the changes of blood glucose levels at week 0 (baseline), week 4, and week 8. With a comparable initial blood glucose level, the glucose level of the GER group was significantly lower ($p = 0.001$) than the HFD group after a 4-week administration of extract at 0.8 g/kg body weight and a 12% reduction from its baseline blood glucose level. GTP did not significantly lower the blood glucose level compared with the HFD group but has successfully maintained its stability. The blood glucose lowering effect of GER, however, was statistically insignificant at week 8. At the end of the treatment (week 8), all groups showed a significant decrease in blood

glucose levels compared to their baselines. The glucose levels of the untreated groups (i.e., HFD and STD groups) remained slightly higher than the treated groups. A systemic lower blood glucose level at week 8 was observed in all groups, possibly due to the variation in the blood drawing process.

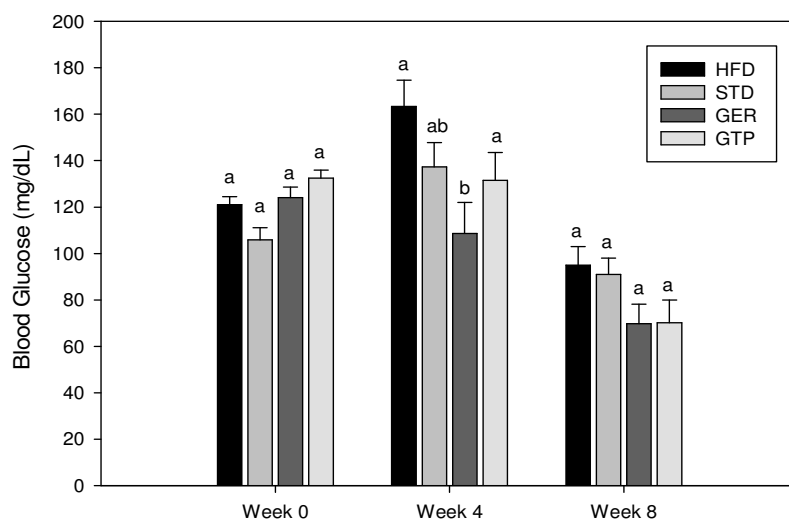


Figure 5.7. Effects of the purified Chinese sweet leaf tea (*Rubus suavissimus*) extract (GER) and green tea (*Camellia sinensis*) powder (GTP) on blood glucose levels (mg/dL) in diet-induced obese SD rats measured prior to the initiation of treatment (Wk 0), 4th (Wk 4), and 8th (Wk 8) week during the treatment period compared to the control groups receiving high fat (HFD) and normal (STD) diet, respectively. All data are expressed as the mean \pm standard error where n=15. Different letters on each week indicate a significant difference at $p \leq 0.05$.

5.3.4 Toxicology Related to Treatments

After 9 weeks of extract administration, no clinical sign of toxicity or abnormal behavior was observed in the treated animals. The GTP group appeared to be more physically active in general than others based on daily observations. There were no treatment-related adverse effects observed based on the hematology (**Table 5.3**) and blood chemistry results (**Table 5.4**). However, elevated values of CK were observed in all groups with excessively high variations. While the reasons were unknown, we predicted that it might be the result of instrumental error or fluctuation. In addition, the total cholesterol level was lower in the GER and GTP groups.

Although the BUN and creatinine levels in the GTP group were slightly different from the STD or HFD group, the values were still within normal ranges. No other abnormal values were noted based on existing results.

Table 5.3. Effects of the purified Chinese sweet leaf tea (*Rubus suavissimus*) extract (GER) and green tea (*Camellia sinensis*) powder (GTP) on the hematology test of diet-induced obese rats compared to the control groups receiving high fat (HFD) and normal (STD) diet, respectively.

	Groups			
	HFD	STD	RUS	GTP
Erythrocyte (10 ⁶ /μl)	8.09 ± 0.11 [¥]	8.03 ± 0.14	8.16 ± 0.16	8.06 ± 0.11
Hemoglobin (g/dL)	14.89 ± 0.34 [¥]	14.37 ± 0.30	14.13 ± 0.34	14.59 ± 0.29
Hematocrit (%)	42.06 ± 0.46 [¥]	41.69 ± 0.93	41.62 ± 0.84	43.07 ± 0.68
RDW (%)	11.93 ± 0.03 [¥]	11.89 ± 0.08 [¥]	11.90 ± 0.08 [¥]	11.86 ± 0.07
MCV (fL)	52.60 ± 0.54	52.64 ± 0.46	52.45 ± 0.58	53.62 ± 0.50
MCH (pg)	18.04 ± 0.44	17.96 ± 0.44	16.97 ± 0.58	18.48 ± 0.16
MCHC (g/dL)	34.66 ± 0.26	34.51 ± 0.20 [¥]	34.59 ± 0.22 [†]	34.29 ± 0.13
Platelets (10 ³ /μl)	573.70 ± 96.80	582.20 ± 74.25	617.40 ± 94.05	625.60 ± 57.29
MPV (fL)	7.07 ± 0.41 [¥]	7.28 ± 0.50	7.53 ± 0.54	7.37 ± 0.42
Total WBC (10 ³ /μl)	3.55 ± 0.32	3.73 ± 0.35 [¥]	4.08 ± 0.32	4.59 ± 0.35

All values were expressed as mean ± standard error where n=10, unless otherwise specified. Different letters on each row indicated a significant difference at $p \leq 0.05$. [†]n=8; [¥]n=9.

Table 5.4. Effects of the purified Chinese sweet leaf tea (*Rubus suavissimus*) extract (GER) and green tea (*Camellia sinensis*) powder (GTP) on the serum chemistry values of diet-induced obese rats compared to the control groups receiving high fat (HFD) and normal diet (STD), respectively.

	Groups			
	HFD	STD	GER	GTP
Glucose (mg/dL)	196.80 ± 13.36	216.10 ± 13.90	206.89 ± 16.31 [¥]	172.50 ± 10.89 [†]
AST(IU/L)	142.78 ± 16.08 [¥]	194.89 ± 41.82 [¥]	206.44 ± 32.64	168.57 ± 28.48 [*]
ALT(IU/L)	39.44 ± 2.14 [¥]	51.86 ± 3.84 [*]	45.22 ± 4.98	48.57 ± 2.83 [*]
AP (IU/L)	267.70 ± 15.35 ^a	227.90 ± 7.94 ^{ab}	205.40 ± 9.31 ^b	217.20 ± 10.83 ^b
CK (IU/L)	3785.33 ± 1399	2847.50 ± 1295 [¥]	5792.44 ± 2149 [¥]	4820.63 ± 1806 [†]
Bilirubin (mg/dL)	0.19 ± 0.04	0.22 ± 0.04	0.36 ± 0.05	0.25 ± 0.05
Protein (g/dL)	5.91 ± 0.08	5.76 ± 0.12	5.98 ± 0.12	5.80 ± 0.10
Albumin (g/dL)	3.12 ± 0.07	3.12 ± 0.07	3.31 ± 0.07	3.24 ± 0.06
Globulin (g/dL)	2.79 ± 0.06	2.64 ± 0.06	2.67 ± 0.06	2.56 ± 0.06
Cholesterol (mg/dL)	103.50 ± 3.20 ^{†a}	61.80 ± 3.30 ^c	79.90 ± 4.72 ^b	84.80 ± 4.05 ^b
BUN (mg/dL)	17.50 ± 0.48 ^{ab}	19.10 ± 0.43 ^a	16.40 ± 0.64 ^{bc}	14.40 ± 0.60 ^c
Creatinine (mg/dL)	0.30 ± 0.00 ^b	0.31 ± 0.01 ^b	0.33 ± 0.02 ^{ab}	0.38 ± 0.02 ^a
Calcium (mg/dL)	10.34 ± 0.15	10.26 ± 0.19	10.02 ± 0.24	9.93 ± 0.19
Phosphorus (mg/dL)	7.35 ± 0.22	8.00 ± 0.50	8.18 ± 0.60	8.45 ± 0.76
Sodium (mmol/L)	141.40 ± 0.48	141.30 ± 0.75	140.70 ± 0.96	140.60 ± 0.67
Potassium (mmol/L)	6.35 ± 0.24	6.51 ± 0.39	7.39 ± 0.63 [¥]	6.55 ± 0.42 [†]
Chloride (mmol/L)	100.30 ± 0.47	99.90 ± 0.64	101.00 ± 0.58	100.20 ± 0.59
Bicarbonate(mmol/L)	23.82 ± 0.45	24.28 ± 0.64	22.48 ± 0.86	22.36 ± 0.96
Anion Gap (mmol/L)	23.63 ± 0.70	23.63 ± 0.91	25.13 ± 1.31	25.77 ± 1.55

All values were expressed as mean ± standard error where n=10, unless otherwise specified. Different letters on each row indicated a significant difference at p ≤ 0.05. ^{*}n=7; [†]n=8; [¥]n=9.

5.4 Discussion

Chinese sweet leaf tea is a popular beverage leaf tea due to its natural sweet taste. In southern China, it has long been used as a traditional folk remedy. This folk medicine has been put under scientific investigation in recent years. Liu et al. (2006) reported that the anti-angiogenic properties of the Chinese sweet leaf tea extract are partially exerted by the presence of gallic acid. Chou et al. (2009) revealed five bioactive markers, gallic acid, rutin, ellagic acid, rubusoside, and steviol monoside in the leaf. However, preliminary studies demonstrated that only gallic acid, ellagic acid, and rubusoside were absorbed *in vivo*. Previously, our study found that the standardized Chinese sweet leaf tea extract at a 0.5g/kg dose caused a 6.7% cumulative weight loss in normal rodents (non-obesity-prone) against the control group. More surprisingly, the use of a combination of the three major marker compounds (i.e., gallic acid, ellagic acid, and rubusoside) had the same anti-obesity effect as the use of the whole crude extract itself. Those results indicate that gallic acid, ellagic acid, and rubusoside represent the majority, if not all, of the responsible components in the Chinese sweet leaf tea extract that causes significant weight loss through unknown mechanisms.

Although the mechanisms of action are unknown, it is suspected that the observed anti-obesity effect is likely caused by multiple components with possible synergistic interactions at multiple sites of actions. For example, gallic acid has been reported to be an inhibitor of alpha-glucosidase, which may further reduce the breakdown of carbohydrates to simple sugars such as glucose and fructose (Li et al., 2007). Hsu et al. (2007) also reported the anti-obesity effect after the administration of gallic acid at the dose of 100 mg/kg of body weight. Ellagic acid, indeed, may be partially responsible in the anti-obesity effect in mice after the administration of pomegranate leaf extract containing 10.6% w/w of ellagic acid (Lei et al., 2007). Rubusoside has shown some anti-bacterial activity (unpublished data) and could contribute to the digestion of

energy in the colon. Gallic acid could play a role systematically as an angiogenesis inhibitor and could cause reduced blood vessel formation and growth in the adipose tissues, thus leading to a reduced fat accumulation. These possible multiple mechanisms of action, multiple action sites, and synergism of different bioactive components warrant further investigations. Regardless, it is safe to conclude that the current results verified the hypothesis that the anti-obesity effect of the Chinese sweet leaf tea extract is due to the combination of gallic acid, ellagic acid, and rubusoside. It is clear that GER could be used to replace the standardized whole extract to produce the same anti-obesity effects.

This is the first report on the anti-obesity effect of the purified sweet leaf tea extract on obese-prone SD rats. Our present study clearly demonstrated that the combination of gallic acid, ellagic acid, and rubusoside (GER), in a similar composition to the standardized Chinese sweet leaf tea extract at a dosage of 0.8 g/kg of body weight (0.08% w/w), reduced significantly the body weight gain of the diet-induced obese rats by 22% and the total abdominal fat accumulation by 48%. Since abdominal fat accounts for the majority of weight gain in obese phenotypes, it is reasonable to state that the pronounced weight loss was in fact fat loss. One of the most interesting results was that the same anti-obesity effects produced by two drastically different methods. The first method put obesity-prone rats on high-fat diet (i.e., 60% of the total calories from fat) that was supplemented with GER. The other method put obesity-prone rats on standard low-fat diet (i.e., 14% of the total calories from fat) alone without any GER. Both methods caused approximately 22% less body weight gain. In addition, the total cholesterol in the GER group was significantly lower than the control group. This shows that GER has the ability to suppress body weight gain and improve lipid profiles when a fatty diet is consumed. Whether the same effects produced by GER on high-fat diet-fed rat are mechanistically similar to those produced in GER-free standard (low-fat) diet-fed rat, is subjected to further investigation.

However, it raises a very interesting question on how the extra fat in the diet is counter-balanced by the use of GER. Since the food intake was not affected in the GER group, it is imperative to predict that the administration of GER might have actually suppressed the energy absorption in the gastrointestinal tract during the treatment period. In addition to the observed anti-obesity effect, the blood glucose level was significantly improved after 4 weeks of extract administration and showed a trend of improvement after 8 weeks of treatment. This represents a new piece of scientific evidence to support the use of Chinese sweet leaf tea as a potential remedy to treat diabetes (Huang and Jiang, 2002). Obesity and insulin resistance are interrelated. Whether the anti-obesity effect of GER serves as a cause of the improvement of blood glucose levels is not understood.

Botanical extracts have been good sources of anti-obesity natural ingredients. One of the most extensively studied medicinal plants is probably the green tea. Green tea is a non-fermented tea possessing various health benefits. Currently, there are numerous reports on green tea and its anti-obesity effects by inhibiting the lipogenesis process (Dulloo et al., 1999; Hasegawa et al., 2003; Zheng et al., 2004; Wolfram et al., 2006; Kim et al., 2009; Nagao et al., 2009) and by increasing thermogenesis (Kovacs et al., 2004; Thielecke and Boschmann, 2009). With strong literature support, green tea extract was chosen in our study as a positive control. The use of green tea provides a benchmark to assess the effect of GER on the obesity-prone rats. Among the components found in the green tea, EGCG, which represents approximately 59% of the total catechins, and caffeine were likely to cause the effect of weight reduction. Zheng et al. (2004) reported that the administration of caffeine and catechins resulted in similar effects of weight reduction as the green tea extract by reducing the weight of intraperitoneal adipose tissues by 23%. The body weight reduction was even greater than those receiving caffeine, catechins, or theanine alone. Studies have argued that the anti-obesity effect of green tea was mainly due to

the presence of caffeine since its intake has been known to increase thermogenesis and fat oxidation (Cabrera et al., 2006; Thielecke and Boschmann, 2009). Dulloo et al. (1999) also suggested that although caffeine may not be effective alone, it may trigger the synergistic reaction with other compounds in the green tea extract, thereby inducing the thermogenesis and fat oxidation in obese subjects. This hypothesis was further supported by Zheng et al. (2004). Our results confirmed the findings by others and showed an effective weight reduction in the obese-prone SD rats after an administration of green tea extract containing 16.5% of EGCG and 8.97% of caffeine at a dosage of approximately 1 g/kg of body weight. The weight difference reached 32% at the end of the 9-week treatment period compared to the high-fat diet control group. The difference was even 10% higher than those receiving a slightly lower dose of sweet leaf tea extract equivalent GER. Despite the beneficial anti-obesity effect, caffeine has been known to increase the risk of coronary heart disease, diabetes, blood pressure, and mental alertness (Geleijnse 2008; Kennedy et al., 2008; Van Dam 2008) and may not be a good candidate for weight loss treatment. The toxicity issues related to caffeine and ephedrine consumption as a weight loss supplementation casted additional doubt on the use of caffeine as a safe weight loss agent (Haller et al., 2002; Dunnick et al., 2007). Although animal studies, including the current study, support the use of green tea as a weight management agent, human studies have found that the anti-obesity effect of green tea fell short of expectations (Kao et al., 2006; Wolfram et al., 2006; Hsu et al., 2008). Kao et al., (2006) reported that the controversial results from the green tea may possibly be due to the purity of extract used, experimental designs, the period of administration, physiological status of the subjects, as well as caffeine content in the green tea. In addition, based on daily observations, the GTP group was physically more active than all other groups. The caffeine administration in the present study, which was equivalent to the daily consumption of an average weight person (70 kg) of almost 35 cups of

green tea with an average caffeine content at 25 mg per cup, was way beyond the daily caffeine consumption of a normal healthy human (i.e., 50 mg daily). This may increase the possibility of side effects. Although the effect of green tea on weight suppression was shown to be effective, the long-term effects on human health, resulting from caffeine consumption, especially at high doses, are still unknown.

The qualitative fingerprints of the GER and the standardized Chinese sweet leaf tea extract (RUS) showed the existence of the three major components without the presence of EGCG or caffeine, which differentiate the Chinese sweet leaf tea from the green tea. Blood chemistry and hematological results showed no sign of toxicity of GER after a 9-week administration. Although the anti-obesity effect was stronger in the GTP group than in the GER, there are some major advantages of the latter. First, the habitual caffeine intake from the GTP may affect human health in the long term. In contrast, GER does not contain caffeine and thus has no predicted side effects. Second, the effective GER dose was 0.22g/kg of body weight (equivalent to the standardized extract dose of 0.8g/kg of body weight) comparing to the GTP at 1g/kg of body weight. Third, the GER can be better controlled with a fixed concentration of known compounds than the GTP or whole sweet leaf tea extract with hundreds of compounds. Thus, GER, in the same fashion as the combinational drug formulations, could avoid the common challenge of batch-to-batch variations associated with extract preparations. In addition, by removing almost 70% of inactive components from the crude extract, the palatability of GER has increased to levels for medicinal food or botanical drug development. Fourth, since the effect of GER was almost comparable to the STD group, it can be used by people who have difficulty in changing to a healthier diet and lifestyle towards better.

5.5 Summary

The current study revealed the anti-obesity effect of the purified Chinese sweet leaf tea extract (GER) on obese-prone rodents after the administration of extract, at an equivalent standardized extract dose of 0.8g/kg of body weight, for 9 weeks. The weight reduction reached 22% when compared to the high-fat diet group with 48% weight loss attributed by total abdominal fat loss. Green tea demonstrated an even stronger anti-obesity effect at 32% less weight gain than the high-fat diet control group but with higher a dose at 1g/kg of body weight. However, the adverse effects resulting from habitual caffeine intake from green tea are persistent and controversial results from clinical studies have shown that the anti-obesity effect of green tea is questionable. Instead, GER, without the presence of caffeine, is safe to consume with controlled quality as shown in current study. Moreover, the effective dose of GER was almost 5-fold lower than the green tea extract. With a similar effect as in the low-fat diet group, GER counter-balanced the effect of the high fat diet. Although the mechanisms remain unknown, they may involve multiple pathways or targets. Nevertheless, the current study raises a possibility that GER may be an alternative health supplement in treating obesity and that the mechanisms may be different than in GTP.

CHAPTER 6. CONCLUSIONS AND FUTURE RESEARCH

Current study discovered a new direction of the application of Chinese sweet leaf tea beyond its historical use as a beverage leaf tea or natural sweetener. Based on all preliminary and final results, we concluded that:

1. Based on the sweetness of extract, the traditional hot water extraction was satisfactory in extracting rubusoside considering the unnecessary use of ethanol. However, sequential extraction method is warranted to produce a new sweet leaf tea extract with maximized levels of all marker compounds.
2. Alcohol precipitation could be a simple purification tool to remove the polysaccharide from the water extract of Chinese sweet leaf tea.
3. The Chinese sweet leaf tea extract could be a useful functional ingredient in suppressing the weight gain of obese individual where gallic acid, ellagic acid, and rubusoside may be the three major compounds responsible for the anti-obesity effect. Because there were no difference in food intake, we believed that the 22% weight loss in obese animal model may possibly due to the decreased of fat accumulation either caused by increased in energy expenditure or decreased in energy absorption.

Future research will be focused on the mechanistic investigations of the Chinese sweet leaf tea or its purified extract on the suppression of fat accumulation.

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APPENDIX A. DIET COMPOSITIONS

Purified AIN-93G Liquid Rodent Diet (Use at 263.194g/L of cold water)

Calories (%) Provided by the Major Nutrients

Nutrient	Calories (%)
Carbohydrates	64.0
Proteins	19.3
Fat	16.7

Major Ingredients in the Liquid Diet

Ingredient	grams/Liter of Diet
Casein	53.00
Sucrose	26.50
Cellulose	13.30
Maltose Dextrin	135.40
Soybean Oil	18.6
t-Butylhydroquinone (TBHQ)	0.004
Mineral Mix (See table below for details)	9.28
Vitamin Mix (See table below for details)	2.65
L-Cystein	0.8
Choline Butartrate	0.66
Xanthan Gum	3.0

AIN-93G Mineral Mix for Liquid Diet (Use at 9.28g/L of Diet)

Ingredient	grams/kilogram
Calcium Carbonate	357.00
Potassium Phosphate, monobasic	196.00
Potassium Citrate H₂O	70.78
Sodium Chloride	74.00
Potassium Sulfate	46.60
Magnesium Oxide	24.00
Ferrous Sulfate 7H₂O	5.21
Zinc Carbonate	1.65
Manganous Carbonate	0.63
Cupric Carbonate	0.30
Potassium Iodate	0.01
Sodium Selenate	0.01025
Ammonium Paramolybdate 4H₂O	0.00795
Sodium Metasilicate 9H₂O	1.45
Chromium Potassium Sulfate 12H₂O	0.275

Lithium Chloride	0.0174
Boric Acid	0.0815
Sodium Fluoride	0.0635
Nickel Carbonate	0.0318
Ammonium Vanadate	0.0066
Sucrose, finely powdered	221.876

AIN-93VX Vitamin Mixture for Liquid Diet (Use at 10g/kg Diet)

Ingredient	grams/kilogram
Niacin	3.00
Calcium Pantothenate	1.60
Pyridoxine HCl	0.70
Thiamine HCl	0.60
Riboflavin	0.60
Folic acid	0.20
Biotin	0.02
Vitamin E Acetate (500 IU/g)	15.00
Vitamin B12 (0.1 %)	2.50
Vitamin A Palmitate (500,000 IU/g)	0.80
Vitamin D3 (400,000 IU/g)	0.25
Vitamin K1/Dextrose Mix (10 mg/g)	7.50
Sucrose	967.23

Standard Chow Diet (Diet # 5001) Ingredients (Provided by Laboratory Rodent Diet)

Calories (%) Provided by the Major Nutrients

Nutrient	Calories (%)
Carbohydrate	57.99
Protein	28.51
Fat	13.49

Nutrients Composition in the Standard Chow Diet

Nutrients	
Total Protein	23.9 %
Arginine	1.41 %
Cystine	0.31 %
Glycine	1.21 %
Histidine	0.57 %
Isoleucine	1.14 %
Leucine	1.83 %
Lysine	1.41 %

Methionine	0.67 %
Phenylalanine	1.04 %
Tyrosine	0.71 %
Threonine	0.91 %
Tryptophan	0.29 %
Valine	1.17 %
Serine	1.19 %
Aspartic Acid	2.81 %
Glutamic Acid	4.37 %
Alanine	1.43 %
Proline	1.49 %
Taurine	0.02 %
Fat (ether extract)	5.00 %
Fat (acid hydrolysis)	5.70 %
Cholesterol	200 ppm
Linoleic Acid	1.22 %
Linolenic Acid	0.10 %
Arachidonic Acid	<0.01 %
Omega-3 Fatty Acids	0.19 %
Total Saturated Fatty Acids	1.56 %
Total Monounsaturated Fatty Acids	1.60 %
Crude Fiber	5.10 %
Starch	31.90 %
Glucose	0.22 %
Fructose	0.30 %
Sucrose	3.70 %
Lactose	2.01 %

Minerals Composition in the Standard Chow Diet

Mineral	
Ash	7.00 %
Calcium	0.95 %
Phosphorus	0.66 %
Phosphorus (non-phytate)	0.39 %
Potassium	1.18 %
Magnesium	0.21 %
Sulfur	0.36 %
Sodium	0.40 %
Chlorine	0.67 %
Fluorine	16 ppm
Iron	270 ppm
Zinc	79 ppm
Manganese	70 ppm
Copper	13 ppm
Cobalt	0.90 ppm

Iodine	1.00 ppm
Chromium	1.20 ppm
Selenium	0.30 ppm

Vitamins Composition in the Standard Chow Diet

Vitamin	
Carotene	2.30 ppm
Vitamin K	1.30 ppm
Thiamin Hydrochloride	16 ppm
Riboflavin	4.5 ppm
Niacin	120 ppm
Pantothenic Acid	24 ppm
Choline Chloride	2250 ppm
Folic Acid	7.1 ppm
Pyridoxine	6.0 ppm
Biotin	0.30 ppm
Vitamin B12	50 mcg/kg
Vitamin A	15 IU/g
Vitamin D3 (added)	4.5 IU/g
Vitamin E	42 IU/kg
Ascorbic Acid	--

High Fat Diet (Provided by Research Diet, Inc. Product # D12492)

Calories (%) Provided by the Major Nutrients

Nutrient	Calories (%)
Carbohydrate	20.00
Protein	20.00
Fat	60.00

Major Ingredients in the High Fat Diet

Ingredient	grams/100 grams
Casein, 80 Mesh	200
L-Cystein	3
Corn Starch	0
Maltodextrin 10	125
Sucrose	68.8
Cellulose, BW200	50
Soybean Oil	25
Lard	245
Mineral Mix S10026	10

DiCalcium Phosphate	13
Calcium Carbonate	5.5
Potassium Citrate, 1 H₂O	16.5
Vitamin Mix V10001	10
Choline Bitartrate	2
FD&C Blue Dye	0.05

APPENDIX B. SAS PROGRAMS FOR STATISTICAL ANALYSES

SAS Programs for the analyses of the effects of methods and solvent systems on the chemical components of the Chinese sweet leaf tea extract

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.extraction
            DATAFILE= "F:\Lab\Crude Extraction\Data\Extraction data.xls"
            DBMS=EXCEL REPLACE;
    SHEET="SAS";
    GETNAMES=YES;
    MIXED=NO;
    SCANTEXT=YES;
    USEDATE=YES;
    SCANTIME=YES;
    RUN;
Data extraction;
set WORK.extraction;
run;
Title1'Crude Extraction';
PROC PRINT data=extraction;
run;
PROC Mixed;
Title2'GA Yield;
Classes Method Solvent;
Model GA = Method Solvent Method*Solvent;
LSMEANS Method Solvent Method*Solvent/Adjust=Tukey;
run;
PROC Mixed;
Title2'RUT Yield;
Classes Method Solvent;
Model RUT = Method Solvent Method*Solvent;
LSMEANS Method Solvent Method*Solvent/Adjust=Tukey;
run;
PROC Mixed;
Title2'EGA Yield;
Classes Method Solvent;
Model EGA = Method Solvent Method*Solvent;
LSMEANS Method Solvent Method*Solvent/Adjust=Tukey;
run;
PROC Mixed;
Title2'RUB Yield;
Classes Method Solvent;
Model RUB = Method Solvent Method*Solvent;
LSMEANS Method Solvent Method*Solvent/Adjust=Tukey;
run;
PROC Mixed;
Title2'STM Yield;
Classes Method Solvent;
Model STM = Method Solvent Method*Solvent;
LSMEANS Method Solvent Method*Solvent/Adjust=Tukey;
run;Quit;
```


SAS Programs for the Regression Analyses of the Supernatant, Precipitant, Polysaccharides, and Chemical Components Resulted from Alcohol Precipitation

```
dm 'log;clear;output;clear';
Title1 'Alcohol Precipitation';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.AP
            DATAFILE= "F:\ALcohol Precipitation_07\EtOH_prep_chart.xls"
            DBMS=EXCEL REPLACE;
    SHEET="SAS_YieldPSAC";
    GETNAMES=YES;
    MIXED=NO;
    SCANTEXT=YES;
    USEDATE=YES;
    SCANTIME=YES;
    RUN;
data AP_reg;
    set WORK.AP;
    RUN;
ODS RTF File = 'F:\AP_Regression Analysis_YieldPSAC';
PROC GLM data=AP_reg;
Title2 'Regression Analysis_Precipitant and Supernatant Yield;
model Ppercent Spercent= treatment treatment*treatment;
output out=AP r=resid_Ppercent resid_Spercent p=p_Ppercent p_Spercent;
run;

PROC GLM data=AP_reg;
Title2 'Regression Analysis_PSAC';
model PSAC= treatment treatment*treatment;
output out=AP r=resid_PSAC p= p_PSAC;
run;

PROC Sort data=AP_reg;
by treatment;
run;
PROC MEANS data=AP_reg mean std stderr n noprint;
Title2 'PROC MEAN';
by treatment;
var Spercent Ppercent PSAC;
output out=ap30 mean=m_Spercent m_Ppercent m_PSAC std=s_Spercent s_Ppercent
s_PSAC stderr=se_Spercent se_Ppercent se_PSAC;
run;
PROC PRINT data=ap30;
run;
PROC Univariate data=AP normal plot;
Title2 'PROC UNIVARIATE';
Var resid_Ppercent resid_Spercent resid_PSAC; run;
ODS RTF CLOSE;Quit;
```

```

PROC GLM data=AP_reg;
Title2 'Regression Analysis of the Chemical Components';
Model GA EGA RUB SM=treatment treatment*treatment;
Output out=AP_1 r= resid_GA resid_EGA resid_RUB resid_SM p=p_GA p_EGA p_RUB
p_SM;run;
PROC REG data=AP_reg;
Title2 'Regression Analysis RUT';
Model RUT =treatment;
output out=AP_2 r=resid_RUT p=p_RUT;run;

PROC SORT data=AP_reg;
by treatment;run;

PROC MEANS data=AP_reg mean std stderr n noprint;
by treatment;
var GA RUT EGA RUB SM ;
output out=ap30 mean=m_GA m_RUT m_EGA m_RUB m_SM std= s_GA s_RUT s_EGA
s_RUB s_SM stderr=se_GA se_RUT se_EGA se_RUB se_SM;run;

PROC PRINT data=ap30;run;

PROC Univariate data=AP_1 normal plot;
Var resid_GA resid_EGA resid_RUB resid_SM;run;

PROC Univariate data=AP_2 normal plot;
Var resid_RUT;run;
ODS RTF CLOSE;QUIT;

```

SAS Programs for the Regression Analyses of the Response of Chemical Components to the dosage levels

```

dm 'log;clear;output;clear';
Title1 'Animal Study_Absorption';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.AP
DATAFILE= "G:\Lab\Animal Study\Absorption
Study_Summer08\XRUS0505\Data\Absorption studyLFTrial_XRUS_20080421_dose
response.xls"
DBMS=EXCEL REPLACE;
SHEET="SAS";
GETNAMES=YES;
MIXED=NO;
SCANTEXT=YES;
USEDATE=YES;
SCANTIME=YES;
RUN;
data Dose;
set WORK.AP;RUN;
PROC GLM data=Dose;
Title2 'Dose Response';
model GA EGA RUB = Dose Dose*Dose;
Output out=response r=resid_GA resid_EGA resid_RUB;run;
Proc Univariate data=Dose normal plot;
var response;
run;

```

SAS Programs for the Analyses of the RUS and GER on the Body Weight Changes and Relative Daily Food Intake on Normal SD rats

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.Wtloss
    DATAFILE= "G:\Lab\Animal
Study\Rubus_WtLossSummer08\WtLoss_Rubus20080610\Data\Rubus_20080610.xls"
    DBMS=EXCEL REPLACE;
    SHEET="WtGain_SAS";
    GETNAMES=YES;
    MIXED=NO;
    SCANTEXT=YES;
    USEDATE=YES;
    SCANTIME=YES;
    RUN;

Data Weight (Drop=WDay7 WDay15 WDay21 WDay29 WDay35 WDay43 WDay49 WDay55
WDay61 WDay70 WDay74);
set Wtloss;

array W[11] WDay7 WDay15 WDay21 WDay29 WDay35 WDay43 WDay49 WDay55 WDay61
WDay70 WDay74;

do day= 1 to 11;
**1=Day7 2=Day 15 3=Day21 4=Day29 5=Day35 6=Day43 7=Day49 8=Day55 9=Day61
10=day70 11=day74;
Wt= W[Day];
output;end;run;

Proc Print data=weight;run;

PROC mixed data=weight;
Title2 'Body Weight Changes_Normalrat';
Class Rat Treatment day;
model Wt = treatment day treatment*day/ influence ddfm=Satterthwaite
outp=ResidWt;
repeated day/ type=ar(1) subject=rat(treatment);
LSMEANS treatment day treatment*day/ Adjust=Tukey;
Run;

PROC mixed data=weight;
Title2 'Relative Daily Food Intake_normal rat';
Class Rat Treatment day;
model FoodIntake = treatment day treatment*day/ influence
ddfm=Satterthwaite outp=ResidWt;
repeated day/ type=ar(1) subject=rat(treatment);
LSMEANS treatment day treatment*day/ Adjust=Tukey;
Run;

Proc Univariate data=ResidWt PLOT NORMAL;
Var Resid;
run;
```

SAS Programs for the Analyses of the Hematology and Blood Chemistry of the Normal SD rats after 10-week Administration of Extracts

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.Wtloss
            DATAFILE="H:\Lab\Animal Study_08\Weight
            Loss\WtLoss_Rubus20080610\SAS.xls"
            DBMS=EXCEL REPLACE;
SHEET="SAS_Blood";
GETNAMES=YES;
MIXED=NO;
SCANTEXT=YES;
USEDATE=YES;
SCANTIME=YES;
    RUN;
DATA Blood;
set Wtloss;
PROC GLM data=Blood;
Title2 'Hematology Test_Normal Rat';
Class group;
Model RBC HEM HET RDW MCV MCH MCHC PLA MPV WBC = group /influence Output
out=resid; LSMEANS group/ adjust= tukey; Run;
PROC UNIVARIATE data=resid PLOT NORMAL; Var resid;
run;

PROC GLM data=Blood;
Title2 'Blood Chemistry_Normal Rat';
Class group;
Model Glu ALT AST AP BUN CRE = group /influence Output out=resid; LSMEANS
group/ adjust= tukey; Run;

PROC UNIVARIATE data=resid PLOT NORMAL;
Var resid;
run;
```

SAS Programs for the Analyses of the Effects of GER and GTP on the Body Weight Changes and Relative Daily Food Intake in Diet-Induced Obese SD Rats

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.Wtloss
            DATAFILE= "G:\Lab\Animal Study\RUS_Wt
            Loss_09\RUS_Data20090428.xls"
            DBMS=EXCEL REPLACE;
SHEET="Wt_SAS";
GETNAMES=YES;
MIXED=NO;
SCANTEXT=YES;
USEDATE=YES;
SCANTIME=YES;
    RUN;
DATA Wt;
```

```

set Wtloss;
array W[13] WDay1 WDay2 WDay3 WDay4 WDay5 WDay6 WDay7 WDay8 WDay9 WDay10
      WDay11 WDay12 WDay13;
do day = 1 to 13;
Wt = W[Day];
**1=wk0-1 2=wk0-2 3=wk0-3 4=wk0-4 5=wk1 6=wk2 7=wk3 8=wk4 9=wk5 10=wk6 11=wk7
12=wk8 13=wk9;
output;end;run;

PROC Mixed data=Wt;
Class Group Day;
Title2 'Body Weight Changes_Obese Rat';
Model Wt = Group Day Group*Day / ddfm=Satterthwaite outp=ResidWt;
repeated Day/ type=ar(1) subject=rat(Group);
LSMEANS Group day Group*day/ Adjust=Tukey;
run;

PROC Mixed data=Wt;
Class Group Day;
Title2 'Relative Daily Food Intake_Obese Rat';
Model FoodIntake = Group Day Group*Day / ddfm=Satterthwaite outp=ResidWt;
repeated Day/ type=ar(1) subject=rat(Group);
LSMEANS Group day Group*day/ Adjust=Tukey;
run;

PROC UNIVARIATE data=ResidWt PLOT NORMAL;
Var Resid;run;

```

SAS Programs for the Analyses of the Effects of GER and GTP on the Weight of Organs and Tissues

```

dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.Wtloss
      DATAFILE= "G:\Lab\Animal Study\RUS_Wt
Loss_09\RUS_Data20090428.xls"
      DBMS=EXCEL REPLACE;
      SHEET="SAS_Organs_Contents";
      GETNAMES=YES;
      MIXED=NO;
      SCANTEXT=YES;
      USEDATE=YES;
      SCANTIME=YES;
      RUN;
DATA Organs;
set Wtloss;
Proc Print Data=Organs;
run;
PROC Mixed data=Organs;
Class Group;
Model Cecum = Group / ddfm=Satterthwaite outp=ResidWt;
LSMEANS Group / Adjust=Tukey;
run;
PROC UNIVARIATE data=ResidWt PLOT NORMAL;
Var Resid;
run;

```

SAS Programs for the Analyses of the Effects of GER and GTP on the Weight of Organs and Tissues

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.Wtloss
            DATAFILE= "G:\Lab\Animal Study\RUS_Wt
Loss_09\RUS_Data20090428.xls"
            DBMS=EXCEL REPLACE;
    SHEET="BG_SAS";
    GETNAMES=YES;
    MIXED=NO;
    SCANTEXT=YES;
    USEDATE=YES;
    SCANTIME=YES;
    RUN;
DATA BG;
set Wtloss;
array W[3] WDay1 Wday2 Wday3;
do day = 1 to 3;
BG = W[Day];
**1 = Wk0 2=Wk4 3=Wk8;
output;
end;
run;
Proc Print Data=BG;
run;

PROC Mixed data=BG;
Class Group Day;
Model BG = Group Day Group*Day / ddfm=Satterthwaite outp=ResidWt;
repeated Day/ type=ar(1) subject=rat(Group);
LSMEANS Group day Group*day/ Adjust=Tukey;
run;

PROC UNIVARIATE data=ResidWt PLOT NORMAL;
Var Resid;run;
```

SAS Programs for the Analyses of the Hematology and Blood Chemistry of the Diet-induced Obese Rats after 9-week Administration of Extracts

```
dm 'log;clear;output;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.Wtloss
    DATAFILE= "G:\Lab\Animal Study\RUS_Wt
Loss_09\RUS_Data20090428.xls"
    DBMS=EXCEL REPLACE;
    SHEET="Blood_SAS";
    GETNAMES=YES;
    MIXED=NO;
    SCANTEXT=YES;
    USEDATE=YES;
    SCANTIME=YES;
    RUN;
DATA Chem;
set Wtloss;
Proc Print Data=Chem;
run;


PROC GLM data=Chem;
Class Group;
Model RBC HMG HMT RDW MCV MCH MCHC PLA MPV WBC = Group /
ddfm=Satterthwaite outp=ResidWt influence;
LSMEANS Group / Adjust=Tukey;
run;

PROC GLM data=Chem;
Class Group;
Model GLU AST ALT AP CK BIL PRO ALB GLO CHO BUN CRE CAL PHOS SOD POT CHL
BIC ANION = Group / ddfm=Satterthwaite outp=ResidWt influence;
LSMEANS Group / Adjust=Tukey;
run;

PROC UNIVARIATE data=ResidWt PLOT NORMAL;
Var Resid;
run;
```

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**Purification of a Water Extract of Chinese Sweet Tea Plant
(*Rubus suavisissimus* S. Lee) by Alcohol Precipitation**GAR YEE KOH,[†] GUIXIN CHOU,[‡] AND ZHIJUN LIU^{*†}[†]School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70803, and [‡]Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

The aqueous extraction process of the leaves of *Rubus suavisissimus* often brings in a large amount of nonactive polysaccharides as part of the constituents. To purify this water extract for potential elevated bioactivity, an alcohol precipitation (AP) consisting of gradient regimens was applied, and its resultants were examined through colorimetric and HPLC analyses. AP was effective in partitioning the aqueous crude extract into a soluble supernatant and an insoluble precipitant, and its effect varied significantly with alcohol regimens. Generally, the higher the alcohol concentration, the purer was the resultant extract. At its maximum, approximately 36% (w/w) of the crude extract, of which 23% was polysaccharides, was precipitated and removed, resulting in a purified extract consisting of over 20% bioactive marker compounds (gallic acid, ellagic acid, rutin, rubusoside, and steviol monoside). The removal of 11% polysaccharides from the crude water extract by using alcohol precipitation was complete at 70% alcohol regimen. Higher alcohol levels resulted in even purer extracts, possibly by removing some compounds of uncertain bioactivity. Alcohol precipitation is an effective way of removing polysaccharides from the water extract of the sweet tea plant and could be used as an initial simple purification tool for many water plant extracts that contain large amounts of polysaccharides.

KEYWORDS: *Rubus suavisissimus*; Chinese sweet leaf tea; alcohol precipitation; purification**INTRODUCTION**

Rubus suavisissimus S. Lee (Rosaceae) is a perennial shrub widely abundant in Guangxi and Guizhou provinces of China. The leaf of *R. suavisissimus* is the material used to make beverage leaf tea by the local residents. Because of its intensely sweet flavor, it is better known as tiancha in Chinese or Chinese sweet tea. The sweet taste from the leaf is attributed to the presence of diterpene glucosides, dominated by the major sweet principle rubusoside (1). Rubusoside has a slightly bitter aftertaste, but it is about 115 times sweeter than sucrose at a concentration of 0.025%, making it a good candidate for a natural sweetener (2, 3). Other diterpene glucosides contributing to the sweetness and bitterness of the leaf include the sweet glycosides, suavisoside A (4), suavisosides B, G, H, I, and J as well as the bitter glycosides, suavisosides C1, D2, and F (3).

In addition to the use of rubusoside as a natural sweetener, Chinese sweet leaf has also been used as a folk medicine to treat various diseases. For example, in southern China, it is used as a traditional remedy for alleviating hypertension, diabetes, atherosclerosis, and maintaining healthy kidneys as well as to relieve coughs (5). Recent studies have also demonstrated that the sweet leaf exhibits anti-inflammatory, antiallergic (6, 7), and antiangiogenic activities (8). As a potential natural inhibitor of angiogenesis, the sweet leaf tea extract has been reported to be capable of

reducing corneal neovascularization in experimental rodents (9). Furthermore, the ability of sweet leaf to inhibit the transcription factor NF- κ B (10) and α -amylase activity (11) may also prevent certain metabolic diseases such as diabetes and obesity.

Traditionally, the sweet leaf as beverage or folk medicine was prepared using boiling water or decoction. This preparation recovered bioactive compounds such as gallic acid, rutin, ellagic acid, rubusoside, and steviol monoside as well as other yet-to-be identified compounds (unpublished data) in a water extract of the sweet leaf. These bioactive compounds may play an important role in the development of pharmaceutical and food products. For instance, gallic acid is one of the active compounds that has potent antiangiogenic (8) and α -glucosidase inhibitory (12) activities. Ellagic acid and rutin, however, are strong antioxidants (13, 14), and both compounds may also be responsible for anti-inflammatory activities (15, 16). In addition, ellagic acid also possesses potent α -amylase inhibitory (11) and anticancer (17, 18) properties. Although this preparation method may have extracted the majority of the bioactive components from the leaf material, it also pulls out a large amount of water-soluble polysaccharides and possibly other macromolecules such as proteins that are not bioactive, resulting in a crude leaf extract that has room for additional purification.

A purified extract with potentially improved bioactivity is highly desirable in many ways, including but not limited to reaching an effective dose range in a practical dosage. The first line of purification is often associated with the removal of

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APPENDIX D. ABBREVIATIONS

ALT: Alanine Aminotransferase

ANOVA: Analysis of Variance

AP: Alcohol Precipitation

APH: Alkanine Phosphatase

AST: Aspartate Aminotransferase

BUN: Blood Urea Nitrogen

CK: Creatinine Kinase

CV: Coefficient of Variation

EGA: Ellagic Acid

EtOH: Ethanol

GA: Gallic Acid

GER: Gallic acid, Ellagic acid, and Rubusoside; Purified Chinese Sweet Leaf Tea Extract

GI: Gastrointestinal

GTP: Green Tea Powder

HP: High-Pressurized Liquid Extraction

HPLC: High Performance Liquid Chromatography

HW: Hot Water Extraction

LP: Low-Pressurized Liquid Extraction

MA: Maceration

MCH: Mean Corpuscular Hemoglobin

MCHC: Mean Corpuscular Hemoglobin Concentration

MCV: Mean Corpuscular Volume

PLE: Pressurized Liquid Extraction

PSAC: Polysaccharides

RBC: Red Blood Cell

RDW: Red Blood Cell Distribution Width

RUB: Rubusoside

RUS: Standardized Leaves Extract of *Rubus suavissimus*

RUT: Rutin

SD: Sprague-Dawley Rat

STM: Steviol Monoside

WBC: White Blood Cell

VITA

Gar Yee Koh was born in November, 1985, in Selangor, Malaysia, and spent most of her childhood in Perak, Malaysia. In April 2003, she started her first year of college in Inti College Malaysia under the America University Transfer Program. Later in August 2004, she continued to pursue her undergraduate studies at Louisiana State University majoring in nutritional science and earned a Bachelor of Science degree in 2007. After receiving her bachelor's degree, she enrolled in the master's program in the School of Renewable Natural Resources at Louisiana State University in August 2007. She conducted research under the supervision of Dr. Zhijun Liu with the focus on botanical plant research. She is a candidate for the Master of Science in forestry in December 2009.