ANTIOXIDANT CAPACITY, TOTAL PHENOLICS AND SUGAR CONTENT OF SELECTED THAI HEALTH BEVERAGES

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ABSTRACT

The current evidences show an increasing trend of sugar intake paralleled to the rise of obesity and type 2 diabetes among the Thai population. Consumption of health beverages derived from fruits and herbs which contain high amounts of added sugar may play a causative role in development of these diseases. Furthermore, sugar fortification may affect antioxidant stability in these beverages, including shelf life of them.

This study was to determine antioxidant capacity, total phenolics and sugar content of selected Thai health beverages and then find an appropriate amount of fortified sugar in health beverages by evaluating the effect of varying added sugar on antioxidant capacity and total phenolic contents in selected pasteurized beverages with consumer acceptance in taste. The storage effects of pasteurized and sterilized beverages on antioxidant capacity and total phenolic compounds were also evaluated.

The beverage Brix in six pasteurized health beverages were increased by adding sugar (0, 10, 15, 20, and 25 °Brix), and they were tested for consumer acceptance at minimum amount of sugar fortification. They were also evaluated for antioxidant stability after 7 days of storage at 5°C in a refrigerator. Twelve sterilized beverages were selected to evaluate antioxidant stability after 1 and 3 months of storage at room temperature. The antioxidant capacities were determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and Photochemiluminescence (PCL) assay. Folin-Ciocalteu assay and Nelson's reducing sugar test were used to determine total phenolic compounds and total sugar contents, respectively.

There were decreasing trends in antioxidant capacity with increasing amounts of sugar content among the samples, with significant inverse correlation by DPPH assay ($r = -0.6$ to -0.9, $p < 0.01$), except sacred lotus root drink. With PCL assay, significant inverse correlation in lipid soluble antioxidant capacity (ACL) values was found only in ginger drink ($r = -0.5$, $p < 0.05$). No significant change in total phenolic compounds was found, except in ginger drink. Appropriately added sugar at 8 to 9 °Brix (18 – 24 g of sugar/serving) were well accepted in the sensory test by general consumers ($n = 30$). There were significant losses in antioxidant capacity measured by both assays and total phenolic compounds of pasteurized beverages by storage duration in some kinds of beverage. Most sterilized beverages showed a good retention of antioxidant capacity and total phenolic compounds during storage.

Even though adding sugars in the studied beverages may not have shown strong correlation with antioxidant capacities, general consumers should perceive that not only antioxidants but also excess caloric intake will be provided by fruit and herb beverages with added sugars.

KEY WORDS: BEVERAGE, ANTIOXIDANT CAPACITY, TOTAL PHENOLIC COMPOUNDS, SUGAR

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ความสามารถในการต้านออกซิเดชัน สารฟีโนลิก และปริมาณน้ำตาลของเครื่องดื่มสุขภาพไทยที่คัดเลือก
(ANTIOXIDANT CAPACITY, TOTAL PHENOLICS AND SUGAR CONTENT OF SELECTED THAI HEALTH BEVERAGES)

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บทคัดย่อ

สถานการณ์ในปัจจุบันได้ชี้ให้เห็นว่าคนไทยมีแนวโน้มในการบริโภคน้ำตาลเพิ่มมากขึ้นในขณะเดียวกับที่มีอัตราการเกิดโรคอ้วนและเบาหวานเพิ่มสูงขึ้นด้วย ซึ่งการพัฒนาไปสู่การเกิดโรคต่างๆเหล่านี้อาจเป็นผลสืบเนื่องมาจากที่มีการบริโภคเครื่องดื่มเพื่อสุขภาพที่มาจากผลไม้และสมุนไพรซึ่งมีการเติมน้ำตาลในปริมาณสูง นอกจากนี้ การเติมน้ำตาลยังอาจส่งผลถึงความคงตัวของคุณสมบัติต้านออกซิเดชันของสารพฤกษเคมีตามระยะเวลาในการเก็บของเครื่องดื่มเหล่านี้ได้

การวิจัยนี้จึงมีวัตถุประสงค์เพื่อวัดความสามารถในการต้านออกซิเดชัน สารฟีโนลิก และปริมาณน้ำตาลของเครื่องดื่มเพื่อสุขภาพไทยที่คัดเลือก จากนั้นหาปริมาณน้ำตาลที่เหมาะสมต่อการเติมในเครื่องดื่มเพื่อสุขภาพโดยประเมินผลของการเติมน้ำตาลในปริมาณต่างๆที่มีต่อคุณสมบัติต้านออกซิเดชันและปริมาณสารประกอบฟีโนลิกในเครื่องดื่มผลไม้และสมุนไพรชนิดพาสเจอร์ไรส์บางชนิดและทำการประเมินการยอมรับของผู้บริโภคด้วย นอกจากนี้ยังได้ทำการศึกษาถึงผลของการเก็บเครื่องดื่มพาสเจอร์ไรส์และสเตอริไรส์ที่มีต่อคุณสมบัติต้านออกซิเดชันและปริมาณสารประกอบฟีโนลิกอีกด้วย

ผู้วิจัยได้เลือกตัวอย่างเครื่องดื่มเพื่อสุขภาพพาสเจอร์ไรส์จำนวน 6 ชนิดเพื่อทำการเติมน้ำตาลโดยการเพิ่มความหวานของเครื่องดื่มด้วยค่าบริกซ์ต่างๆกัน ได้แก่ 0, 10, 15, 20 และ 25 องศาบริกซ์ และได้ทำการทดสอบการยอมรับของผู้บริโภคด้วยการเติมน้ำตาลในปริมาณที่น้อยที่สุด นอกจากนี้ยังได้ทำการประเมินความคงตัวของคุณสมบัติต้านออกซิเดชันในระยะเวลาการเก็บ 7 วันที่อุณหภูมิ 5 องศาเซลเซียสในเครื่องดื่มพาสเจอร์ไรส์ และที่ระยะเวลาการเก็บ 1 และ 3 เดือนที่อุณหภูมิห้องในเครื่องดื่มสเตอริไรส์จำนวน 12 ชนิด ในการศึกษานี้ใช้วิธีวิเคราะห์คุณสมบัติต้านอนุมูลอิสระ 2 วิธีคือ 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay และ Photochemiluminescence (PCL) assay การวิเคราะห์ปริมาณสารประกอบฟีโนลิกและปริมาณน้ำตาลใช้ Folin-Ciocalteu assay และ Nelson's reducing sugar test ตามลำดับ

ผลการศึกษาพบว่า คุณสมบัติต้านออกซิเดชันมีแนวโน้มการลดลงเมื่อปริมาณน้ำตาลที่เติมในเครื่องดื่มเพิ่มขึ้น (5 ใน 6 ชนิด ยกเว้นน้ำรากบัว) ซึ่งพบความสัมพันธ์แบบผกผันอย่างมีนัยสำคัญด้วยวิธี DPPH ($r = -0.6$ ถึง -0.9, $p < 0.01$) สำหรับวิธี PCL พบความสัมพันธ์แบบผกผันอย่างมีนัยสำคัญในน้ำขิงเท่านั้น ($r = -0.5$, $p < 0.05$) และไม่พบการเปลี่ยนแปลงอย่างมีนัยสำคัญของปริมาณสารประกอบฟีโนลิกในเครื่องดื่มที่ทำการศึกษานอกจากน้ำขิง สำหรับการเติมน้ำตาลที่ปริมาณเหมาะสม 8 ถึง 9 องศาบริกซ์ (มีน้ำตาลเป็นส่วนประกอบ 18 ถึง 24 กรัมต่อหนึ่งหน่วยบริโภค) ได้รับการยอมรับจากผู้บริโภคทั่วๆไป (จำนวน 30 คน) โดยผลการทดสอบทางประสาทสัมผัสอยู่ในเกณฑ์ดี เครื่องดื่มพาสเจอร์ไรส์บางชนิดมีการสูญเสียคุณสมบัติต้านออกซิเดชัน และปริมาณสารประกอบฟีโนลิกในระหว่างการเก็บ ในขณะที่เครื่องดื่มสเตอริไรส์ส่วนใหญ่มีความคงตัวของคุณสมบัติต้านอนุมูลอิสระในระหว่างการเก็บที่ดีกว่า

แม้ว่าการเพิ่มปริมาณการเติมน้ำตาลในเครื่องดื่มที่ทำการศึกษามีความสัมพันธ์กับคุณสมบัติต้านออกซิเดชันไม่อยู่ในเกณฑ์ที่สูงมากนัก แต่อย่างไรก็ตามผู้บริโภคโดยทั่วไปควรจะมีการรับรู้ว่าไม่เพียงแต่สารต้านอนุมูลอิสระที่จะได้รับจากเครื่องดื่มจากผลไม้และสมุนไพรเท่านั้นแต่ยังจะได้รับพลังงานส่วนเกินจากเครื่องดื่มเหล่านี้อีกด้วย

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LIST OF ABBREVIATIONS

kg	Kilogram
g	Gram
tsp	Teaspoon
kcal	Kilocalories
HAT	Hydrogen atom transfer
SET	Single electron transfer
DPPH	2,2-diphenyl-1-picrylhydrazyl
IC_{50}	50 % inhibitory concentration
PCL	Photochemiluminescence
ACL	Antioxidant capacity in lipid soluble substance
ACW	Antioxidant capacity in water soluble substance
DM	Diabetes
BMI	Body mass index
CHD	Coronary heart disease
WHO	World Health Organization
HDL	High density lipoproteins
VLDL	Very low density lipoproteins
lb	pound
fl oz.	Fluid ounce
hr	Hour
min	Minute
mo	Month
d	Day
wk	Week

LIST OF ABBREVIATIONS (cont)

AAE	Ascorbic acid equivalent
TE	Trolox equivalent
GAE	Gallic acid equivalent
n	Number
mL	Milliliter
µmol	Micromole
µL	Microliter
µM	Micromolar
nm	Nanometer

CHAPTER I
BACKGROUND AND RATIONALE

Many researches have shown that free radicals are involved in development of a variety of diseases, including cancer, cardiovascular disease, and diabetes, which cause of mortality in Thai and worldwide population (1-3). Antioxidants have been studied and proven to decrease the likelihood of developing those diseases (1,4-6). These health benefits have been attributed to the presence of antioxidants (tocopherols, cathechins, flavonoids, carotenoids, vitamin C and etc.) in foods and beverages from fruits and vegetables (7-10). Based on scientific evidences, there have been increasing interests in recent years about healthy life styles as well as interest in food supplements with antioxidants. Moreover, life style of Thai people have changed from the past, most of them are in a hurry and spend lot of times outdoors. Convenient food supplements or commercially available health beverages have been growing up in the market and it is the first choice for consumers who health concern.

Most of health beverages in the markets are produced from fruits, vegetables and herbs such as green tea, herbal drink, and fruit and vegetable juices that become popular among general consumers. This observation was supported by the report from Research Center of Siam Commercial Bank showed that total sales of fruit juices in the markets grew by 10 % (275 million liters) in the year 2000 compared to 4.2 % in the year 1999 (250 million liters) (11). The Thai Farmers Research Center (TFRC) reported that an annual domestic turnover for herbal drinks in Thailand reached 2.7 billion baht, indicating a 10 – 15 % growth in the year 2000 and stated that preferred drinks includes chrysanthemum (kek-huai), ginger (khing), tiger's herb (bai-bua-bok), ringworm bush (chum-hed-thed), roselle (kra-jeab), and bael fruit (ma-toom) (12). Recently, they also showed the increased market turnover of fruit and vegetable juices that reached 3.8 billion baht, a year-on-year increase of 20 %. Notably, high growth has been evident in pure fruit and vegetable juices, particularly for pasteurized product that now account for 10 % of the total (13).

However, the development of food industrial processing and marketing mostly emphasized the acceptance of consumers by fortification of color, aroma or flavor to enhance the taste of products. Sweeteners such as sucrose, high fructose corn syrup, and honey are the most popular flavor additives for many foods and beverages. Moreover, commercial health beverages are the most popular drinking in all age and gender of Thai consumers, especially in children who trend to eat more sweet foods and drinks. The data from the national campaign, **"Thai kids don't eat sweets" (Dek Thai Mai Kin Wan)** showed that average sugar intake of Thai population in the year 2001 equal to 29.0 kg/mo. or 16 tsp/d which increase 2.3 times compared to the year 1985 (12.7 kg/y or 7 tsp/d) (14). Beside, sweetener usage in food and beverages processing is to high increase, up to 600,000 metric tons (15). This data indicated that Thai people consume excessive amount of sugar according to the Thai kids don't eat sweets campaign which recommends that sugar daily intake should not be more than 6 tsp or 24 g (16).

In our preliminary survey, the sweetened beverage from common fruits, herbs and tea such as black tea, roselle drink, bael fruit drink, and chrysanthemum drink were collected from several cafeterias around the Ramathibodi Hospital, we have found that 1 serving size (200 ml) of these beverages can supply up to 10 % of the energy requirement for adult females and males [female and male, age ≥ 19 y require energy intake 1,750 and 2,150 kcal/d, respectively (Dietary Reference Energy Intake for Thais 2003)] (17). It is estimated that the largest source of added sugar comes from processed food, especially from beverages such as soft drink and fruit juices, which linked with an increase risk of type 2 diabetes as recently reported by Schulze et al. (2004) (18). Thus the sweet consumption of Thai people may increase risk in many chronic diseases such as diabetes, obesity, cancer, and heart disease (19,20). Aekplakorn et al. (2004) showed that prevalence of overweight and obesity in Thai aged 20 – 59 y from the second National Health Examination Survey are 28.3 % and 6.8 %, respectively (21). In addition, the estimated national prevalence of diabetes in Thai adults is 9.6 % (2.4 million people) in the year 2003 (22). The prevalence of Type 2 DM in Thai children and adolescents also remarkably increased from 5 % during 1987 – 1996 to 17.9 % during 1997-1999 (mean age = 11.6 ± 2.1 y) (23). Moreover, World Health Organization (WHO) reported that prevalence of obesity in

Thai children aged between 5 – 12 y is 16 % and they trend to have obesity in their adulthood by 42 – 63 % (14,24). Dental caries of Thai children both in rural and urban area, aged between 5 – 9 y, dramatically increase from 74 % in the year 1974 to 87 % in the year 2001 (14). Furthermore, excessive amount of sugar intake can stimulate free radicals formation and reduced vitamin E level in the blood stream (25,26) as well as an experimental study in rats fed with high sucrose diets, results in significantly decrease α-tocopherol and increase triglycerides in the blood circulation (27).

Not only sugar can be harmful to human health but also may affect the stability of bioactive components and antioxidant capacity in beverages. Recently, the study of Tolcott et al. (2003) found that pasteurized yellow passion fruit juice fortified sucrose 10 % lowered antioxidant capacity values in the first 14 days of storage at 37°C (day 0, 17.2 ± 0.25 μM Trolox equivalent (TE)/ml VS day 14, 2 ± 0.25 μM TE/ml) (28). Chen et al. (2001) added green tea catechins (GTC) in commercial available soft drinks (Coca-Cola, 7-up, and Pepsi) and found that GTC degraded up to 45 % in 7-up for 6 months and completely degraded in Pepsi and Coca-Cola only for 4 months (29). Both studies showed that sugar might affect the stability of antioxidant capacity and phenolic compounds in health beverages. With little evidence to support this correlation, it is interesting to prove and it will be useful for manufacturers to improve nutritional quality of their products and educate consumers choosing suitable beverages with less sugar and more nutritive values for their health.

Besides, the storage condition of beverages may affect the stability of antioxidant capacity. Many studies showed a storage effect of some beverages with different storage times and durations. For instance, Kabasakalis et al. (2000) found that in commercial fruit juices stored in open containers in the refrigerator for 31 days ascorbic acid lost by about 60 – 67 % while ascorbic acid in fresh orange juice lost at the much slower rate of 7 – 13 % (30). Turker et al. (2004) studied the effect of temperature on the stability of Shalgam anthocyanins in the pasteurized fermented black carrot beverage stored at 4, 25 and 40°C for 90 days. They found that the highest anthocyanins retention was observed at 4°C storage temperatures with half-life between 231 and 239 days (31). And Piga et al. (2002) found that mandarin juice showed good retention of initial antioxidant activity with a slight decrease of ascorbic acid at the end of storage at 4°C for 15 days (32). However, it has been little known

about the changes of bioactive components and antioxidant capacity of pasteurized and sterilized in Thai health beverages during their shelf life with suitable storage condition.

Therefore, the goal of this study is to determine the relationship between fortified sugar and antioxidant capacity and total phenolic contents, and to find out the appropriated amount of fortified sugar in Thai health beverages and also evaluate the stability of antioxidant capacity and total phenolic compounds during shelf life storage both pasteurized and sterilized Thai health beverages. This study is done in collaboration with Research and Development Unit, The Royal Chitralada Projects whose activities is the model for Thai small and medium enterprises (SME) and aims to research and secure benefits of utilizing agricultural products and leftovers through value added industrial processes in order to publicize, recommend, and demonstrate the research results to farmers and fruit and vegetable growers as well as to the general public.

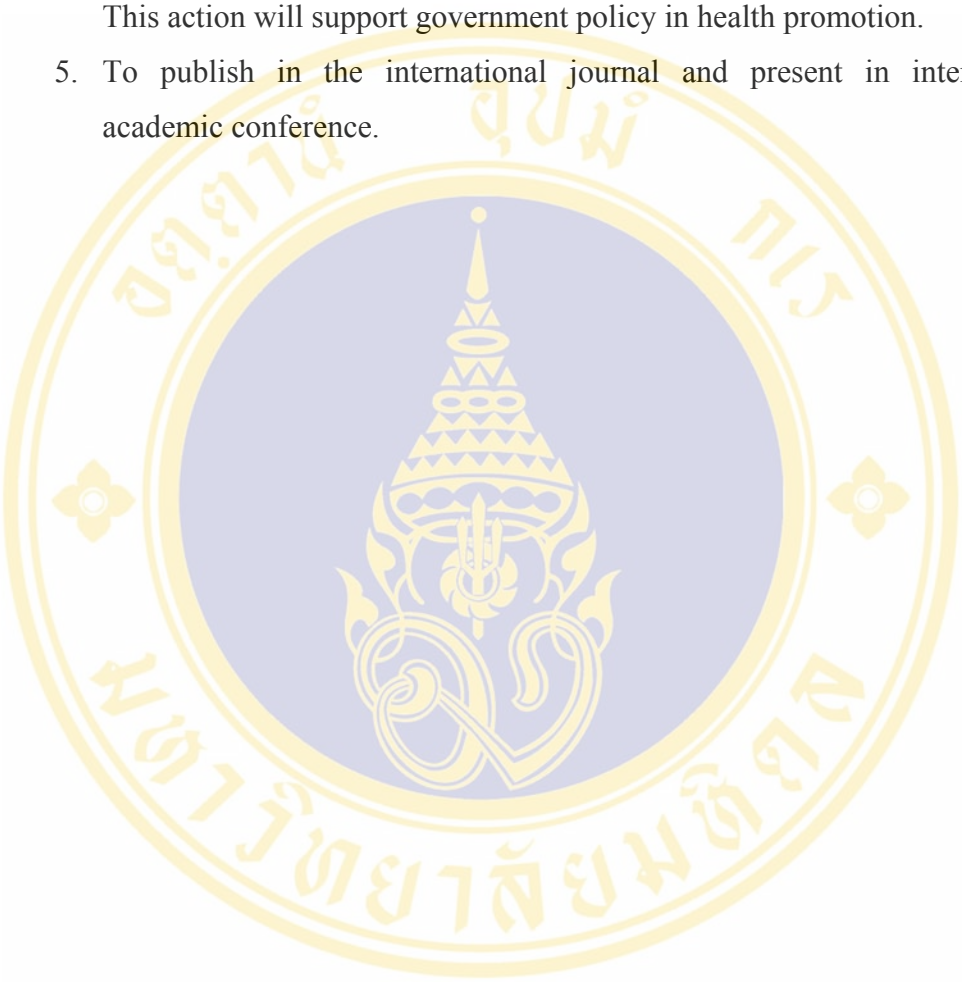
Objectives

1. To determine antioxidant capacity, total phenolic compounds and sugar content of selected pasteurized and sterilized Thai health beverages.
2. To investigate the relationship between fortified sugar and antioxidant capacity and total phenolic compounds of selected Thai health beverages.
3. To find out an acceptance of consumers on selected Thai health beverages with minimum amount of fortified sugar in appropriated sweetness.
4. To evaluate the effect of storage on stability of antioxidant capacity and total phenolic compounds in selected pasteurized and sterilized Thai health beverages.

Expected outcome

1. The scientific evidence based data prove whether fortification of sugar in Thai health beverage can correlate the stability of antioxidant capacity and total phenolic contents or not.
2. The appropriate amount of fortified sugar in Thai health beverages which have consumer's acceptable for taste.

3. To improve nutritional value of health beverages and to apply for industrial food processing.
4. To apply the outcome of this study to educate and stimulate Thai consumers to eat less sweets especially those who have health concern. This action will support government policy in health promotion.
5. To publish in the international journal and present in international academic conference.



CHAPTER II
LITERATURE REVIEW

2.1 Free radicals and human health

Free radicals are defined as atoms, molecules or compounds that contain an unpaired electron in outer orbit. They are generally unstable, short-lived, and highly reactive electrophilic. Free radicals are produced in living cells by normal metabolism and by exogenous sources such as carcinogenic compounds and ionizing radiations (1,33). Because oxygen is necessary to generate energy in living cells, it has been found to be a general source of free radical formation and potentially toxic to humans. Oxygen-derived radicals, often collectively referred to as reactive oxygen species (ROS), are frequently initiated by the addition of electron (e) to molecular oxygen. The superoxide radical ($O_2^{\bullet -}$) is the most well-known oxygen-derived free radical. Unlike the other oxygen derived intermediates, they can lead to the formation of addition reactive species. The complete reaction of oxygen is summarized in the following equations:

$O_2 + e \longrightarrow O_2^{\bullet -}$	Superoxide ion radical	(1)
$O_2^{\bullet -} + H_2O \longrightarrow H_2O^{\bullet} + OH^-$	Hydroperoxyl radical	(2)
$H_2O^{\bullet} + e^- + H \longrightarrow H_2O_2$	Hydrogen peroxide	(3)
$H_2O_2 + e^- \longrightarrow OH^{\bullet} + OH^-$	Hydroxyl radical	(4)

This chain reaction can attack biomolecules, including protein in tissues or enzymes, lipids in cell membranes, carbohydrates, and DNA, causing cellular injury and death as shown in **Figure 2-1.** (1,34,35)

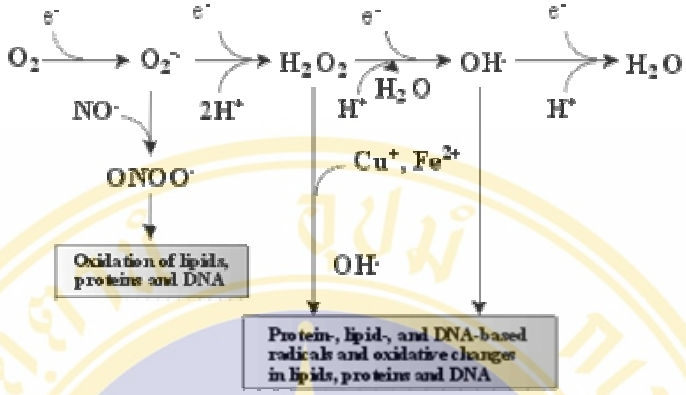


Figure 2-1. Effects of reactive oxygen species on living cells (from reference 36)

An over-production of these reactive species can occur, due to oxidative stress brought about by the imbalance of the bodily natural defense system and free radical formation. Data accumulated over many years clearly show that oxidative stress is considered to play a causative role in a number of disease processes, e.g., aging and several degenerative diseases such as cancers, cardiovascular and cerebrovascular disease, cataracts and cognitive dysfunction etc. as shown in **Figure 2-2** (1,33,37).

Figure 2-2. Oxidative stress and disease processes (from reference 38)

There was some evidence to support this association; Dandona et al. (1996) measured 8-hydroxydeoxyguanosine (8-OHdG), a product of hydroxyl radical reaction with guanosine, in mononuclear cells. They found that there was a greater concentration of 8-OHdG, as well as reactive oxygen species in both type 1 and type 2 diabetic patients, than in nondiabetic controls. They suggested that these changes may contribute to the accelerated aging, atheroclerated aging, atherosclerosis, and microangiopathic complications commonly found in the disease (39). Collins et al. (1998) measured DNA damage to lymphocytes in patients with type 1 diabetes. They found significantly higher DNA strand breaks and oxidized pyrimidines in the diabetics compared with matched controls; strand breaks also correlated with body mass index in the diabetics. Moreover, a strong correlation was present between for mamidopyrimidine glycosylase-sensitive sites and serum glucose levels. They concluded that DNA damage in lymphocytes is a useful marker of oxidative stress (40).

2.2 Antioxidants

2.2.1 Natural antioxidant defense system

Although free radicals are abundantly produced in abundance in all cells, there are numerous natural defenses to either prevent their formation or to neutralize them after they are formed as shown in **Table 1-1** (1). Natural antioxidant defense systems could be divided into two groups based on their sources, into endogenous and other exogenous antioxidants as described follow:

(1) *Endogenous antioxidants* are some antioxidants produced in the body, includes; (a) enzymatic defense, such as Se-glutathione peroxidase, catalase, and superoxide dismutase, which metabolize superoxide, hydrogen peroxide, and lipid peroxides, thus preventing most of the formulation of toxic $OH^{\bullet}$, and (b) nonenzymatic defense, such as glutathione, histidine-peptide, the iron-binding proteins tranferrin, urate, and plasma protein thiols, with the last two accounting for the major contribution to the radical-trapping capacity of plasma.

(2) *Exogenous antioxidants* are obtained from the diet. Dietary antioxidant are defined as any substances that which are present at low concentrations compared with those of oxidizable substrates, these antioxidants significantly delay or prevent

oxidation of the substrate. The term oxidizable substrate encompasses almost everything (except H_2O) found in foods and in living tissues and includes proteins, lipids, carbohydrates, and DNA (1,5). Well-established antioxidants derived from the diet are vitamins C, E, A and carotenoids such as beta-carotene and lycopene. Besides, these antioxidant vitamins, plant polyphenols are an important class of defense antioxidants; they include phenols, phenolic acid, flavonoids, tannins, and lignan. Flavonoids are mostly abundant in diet which are commonly found in fruits, vegetables, wines, tea and other beverages as shown in **Table 2-2** (1,5, 7,41,42).

When the endogenous defense systems are incompletely efficient and existence of some physiopathological situations (cigarette smoke, air pollutants, UV radiation, high polyunsaturated fatty acid diet occur, inflammation, ischemia/reperfusion, etc) in which ROS will be produced in excess and at the wrong time and place, dietary antioxidants are therefore needed for diminishing the cumulative effects of oxidative damage over the life span (5, 7,41)

Table 2-1. Natural antioxidant defense free radicals[†]

A. Antioxidant enzymes
Catalase
Glutathione peroxidase
Glutathione reductase
Superoxide dismutase (Both co-factors: Cu – Zn and Mn)
B. Metal – binding protein
Ceruloplasmin
Ferritin
Lactoferrin
Metallotheinein
Transferrin
Hemoglobin
Myoglobin
C. Common antioxidants (“Scavengers”) /chain–breaking antioxidants
Bilirubin
Uric acid
Thiols (R – SH)
Carotenoids (beta-carotene, lycopene, etc)
Flavonoids (quercetin, rutin, catechin, etc)
Vitamins A, C, E
D. Other antioxidants
Metal ions in antioxidant enzyme (Cu, Mn, Zn, Se)
Glutathione (GSH)

[†] from reference 1

Table 2-2. Major biological active antioxidants in diets[†]

Antioxidants	Sources
Vitamin A/carotenoids	Orange-colored fruits/vegetables, spinach, peas, peppers, broccoli
Vitamin C (ascorbic acid)	Citrus fruits, cruiferous vegetables, potatoes, various other vegetables
Vitamin E (tocopherols, tocotrienols)	Nuts, whole grains, vegetable oils, seeds, butter, egg yolk, sweet potatoes
Polyphenol; Flavonoids	Colored fruits/vegetables, onions, apples, red grapes, tea, chocolate, potatoes

[†]From reference 1

2.2.2 Dietary antioxidants and health

Several epidemiological studies reported protective effects of high consumption of fresh fruits and vegetables on some types of cancers, diabetes, cardiovascular disease, stroke, and other chronic diseases. Scientific evidences showed that vitamins, minerals, fibers, and biologically active phytochemicals contained in fruits and vegetables have beneficial effects through mechanisms of antioxidant activity, stimulation of the immune system, decrease in platelet aggregation, alteration in cholesterol metabolism, modulation of steroid hormone concentrations and hormone metabolism, blood pressure reduction, antibacterial and antiviral activity (5,8,43).

It has been reported that total dietary intake of polyphenols (about 1g/d), especially flavonoid is much higher than that vitamin C (about 10 times), and vitamin E and carotenoids (about 100 times), respectively. The rich sources of polyphenols are fruits, plant-derived beverage, vegetable, cereals, chocolate, and dry legumes (40,45). For general consumers, the major source of polyphenols is beverages such as red wine, coffee, tea, and fruit juices in regularly consuming depending on dietary habits and preferences of each individual (44,46,47). There were clinical studies showing health benefits on antioxidant status after consumption of fruit juices and beverages such as mixed fruit juices, pomegranate juice, orange juice which provided different kinds of

polyphenols (48-51). Hence, researchers and food manufactures are interested in researches and development of plant polyphenols as health products for public consumers.

Based on scientific evidences, there has been increasing consumption of commercially available health beverages made from fruits, vegetables and herbs. They have been growing up in the markets and are the first choice in general consumers who concern their health. Report from the Thai Farmers Research Center (TFRC) showed that an annual domestic turnover in the year 2000 for herbal drinks in Thailand was reached 2.7 billion baht, stated that preferred drinks includes chrysanthemum (kek-huai), ginger (khing), tiger's herb (bai-bua-bok), ringworm bush (chum-hed-thed), roselle (kra-jeab), and bael fruit (ma-toom) (12). Recently, the new report from this Center (2006) showed the increased market turnover of fruit and vegetable juices reached 3.8 billion baht (about 1.4 times). Notably, the high growth has been evident in pure fruit and vegetable juice, particularly, for pasteurized products that now account for some 10 % of the total market turnover (13).

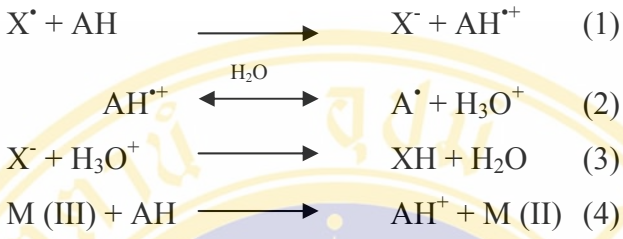
2.2.3 Measurement of antioxidant capacity

The antioxidant capacity is related to the total amount of radical which are reduced by a defined amount of antioxidants. Thus it will be a quantitative parameter. Many analytical methods have been developed to determine the antioxidant capacity in all kind of matrices such as plasma, beverages, vegetables, and fruits. On the basis of the chemical reaction involved, methods to examine antioxidant capacity of a sample can be roughly divided in principle into two major categories; assay based on hydrogen atom transfer (HAT) and single electron transfer (SET) reactions (52,53) as shown in **Table 2-3**.

(a) HAT-based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation (AH = any H donor). Most of HAT-based assays monitor of competitive reaction kinetics, and the quantitation is derived from kinetic curves. They generally are composed of a synthetic free radical generator, an oxidizable molecule probe, and antioxidant.

$$X^{\bullet} + AH \longrightarrow XH + A^{\bullet}$$

(b) SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals. The assay involves one redox reaction with the oxidant as indicator of the reaction endpoint.



SET and HAT mechanisms almost always occur together in all samples, with the balance determined by antioxidant structure and pH. HAT reactions are solvent and pH independent and are usually quite rapid, typically completed in seconds to minutes. While SET reactions are pH dependent and are usually slow and can require long times to reach completion.

Due to multiple reaction characteristic and mechanisms as well as different phase localizations are usually involved, no single assay will accurately reflect all of the radical sources or all antioxidants in a mixed or complex system (52,53,54).

So, in this study we used two different methods; photochemiluminescence (PCL) as HAT mechanism and 2,2-diphenyl-1-picrylhydrazyl (DPPH) as SET mechanism to measure antioxidant capacity of beverages.

Table 2-3. In vitro antioxidant capacity assay[†]

Principle	Antioxidant capacity assay
Assays involving hydrogen atom transfer reaction (HAT-based methods)	ORAC (oxygen radical absorbance capacity)
	TRAP (total radical trapping antioxidant parameter)
	Crocin bleaching assay
	IOU (inhibited oxygen uptake)
	Inhibition of linoleic acid oxidation
	Inhibition of LDL oxidation
	TOSC (total oxidant scavenging capacity)
	Chemiluminescense (CL)
	Photochemiluminescense (PCL)
Assays by single electron-transfer reaction (SET-based methods)	TEAC (trolox equivalent antioxidant capacity)
	FRAP (ferric ion reducing antioxidant parameter)
	DPPH (2,2-diphenyl-1-picrylhydrazyl)
	DMPH (*N*,*N*-dimethyl-*p*-phenylenediamine)
	Copper (II) reduction capacity
	Total phenols assayed by Folin-Ciocalteu reagent

[†]Modified from reference 52,53

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay

The DPPH radical is one of a few stable and commercially available radicals' organic nitrogen radicals as shown in **Figure 2-3**, which gives a deep purple color. Any substance that can donate a hydrogen atom (Antioxidant; AH) to the solution of $DPPH^{\bullet}$ can reduce the stable free radical and change the color of solution from deep purple to pale yellow according to equation below:

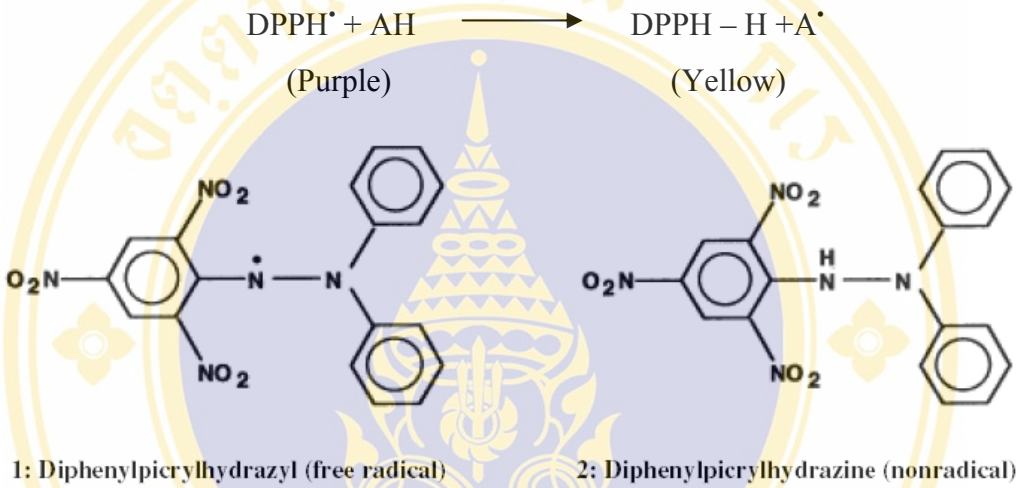


Figure 2-3. Structures of DPPH radical and its nonradical reduced form (from refence 58)

Non-reactive radical form of DPPH can be spectroscopically measured by its color intensity at 515 nm. The antioxidant capacity is defined as IC_{50} or EC_{50} value, the concentration of the tested antioxidant that causes a decrease in the initial $DPPH^{\bullet}$ concentration (loss in absorbance) by 50% or expressed as values which DPPH is directly determined from the calibration curve with a suitable standard antioxidant. (52,53,55-58).

Photochemiluminescense (PCL) assay

This assay as described by Popov and Lewin (59-62), involves the phochemical generation of superoxide ($O_2^{\bullet-}$) free radical with chemiluminescense detection. The assay is initiated by optical excitation of a suitable photosensitizer (S), resulting in the generation of the superoxide radical anion as equation below.

$$S + h\nu + O_2 \longrightarrow [S^* O_2] \longrightarrow S^{\bullet+} + O_2^{\bullet-}$$

Luminol plays a double role as photosensitizer and also as oxygen radical detecing reagent. This reaction takes place in a complete system under the name PHOTOCHEM®. This assay can be separated to measure water (ACW) and lipid soluble (ACL) antioxidant capacity using ACW and ACL kits provided by the manufacturer. The antioxidant capacity is expressed as by means of the lag phase (ACW) or by means of the area under the curve (ACL). Ascorbic acid and trolox are used as standard for ACW and ACL, respectively.

Total phenolic assay by Folin-Ciocalteu method

This assay was improved by Singleton and Rossi (63,64). It actually measures a reducing capacity of phenolic compounds which react with Folin-Ciocalteu reagent (a molybdotungstophosphoric heteropolyanion reagent; Mo (VI)) only under basic conditions (adjusted by a sodium carbonate solution to pH~10).

$$\underset{\text{(Yellow)}}{\text{Mo (VI)}} + e^- \longrightarrow \underset{\text{(Blue)}}{\text{Mo (V)}}$$

The reaction occurs through electron transfer mechanism, phenolate anion reduced Folin-Ciocalteu reagent. The blue compounds formed betweent phenolate anion and Folin-Ciocalteu reagent are independent of the structure of phenolic compounds, and monitored of optical density at 765 nm at optimum reaction time and temperature for color development. This assay is convenient, simple, and reproducible, therefore it has become a routine assay in determining phenolic antioxidants (52,53).

3.2.4 Storage effects on antioxidant stability

There are numerous factors other than variety of plant products may affect the polyphenol content of plant, including ripeness at time of harvest, environmental factors, processing, and storage (46,65). Storage condition may affect the content of polyphenols that are easily oxidized. Oxidation reactions result in the formaton of more or less polymerized substances, which lead to changes in the quality of foods and beverages, particularly in color and organoleptic characteristics. Such a changes may be beneficial (as in the case with black tea) or harmful (browning of fruits) to

consumer acceptability (44,46,65). Several scientific evidences support the storage effects of phenolic compounds, antioxidant capacity and food quality in processed fruit juices and beverages. Kabasakalis et al. (2000) observed that ascorbic acid in commercial fruit juices stored in open containers in the refrigerator for 31 days lost by about 60 – 67 % while ascorbic acid in fresh orange juice lost at the much slower rate of 7 – 13 % (30). Johnson et al. (2002) reported the decrease of ascorbic acid in commercially available orange juices after 4 weeks during storage at 4°C (66). Choi et al. (2002) found the complete degradation of ascorbic acid contents in pasteurized blood orange (*Citrus sinensis*) juice after 5 weeks of refrigerated storage at 4.5°C and total anthocyanin also had 25 % decrease with storage time (67). Piga et al. (2002) also found that mandarin juice showed a loss of initial antioxidant activity with a maximum decrease of ascorbic acid ranging from 37 % to 45 % at the end of storage at 4°C for 15 days (32). Recently, Esteve et al. (2005) reported that pasteurized refrigerated Spanish orange juices were found the lost more over 50% of ascorbic acid within 3 weeks of refrigerated storage at 4°C (68).

In contrast, Miguel et al. (2004) reported that no significant changes in anthocyanins levels, the content of sugars and organic acids during storage at 4°C for 72 hours after extraction of pomegranate juices (69). Van der Sluis et al. (2005) found that no significant changes in polyphenolic antioxidants during storage at 4°C or at 20°C for 1 month in apple juice (70).

In contradiction, Talcott et al. (2003) observed that ascorbic acid in pasteurized yellow passion fruit juice completely lost after 14 days of storage, polyphenolic compounds declined during storage but increase in lipophilic antioxidant capacity throughout 28 days of storage at 37°C. Besides, increasing brown pigments during storage, affected the perceived quality of the juice in odor and color (28). Meanwhile, Del Caro et al. (2004) reported that minimal processed citrus juices showed a decrease in flavonoid and ascorbic acid contents, whereas the trolox equivalent antioxidant capacity (TEAC values) of “red blush” grape juice significantly increased during storage at 4°C for 10 and 15 days (71).

Nevertheless, Turker et al. (2004) studied the effect of temperature on the stability of Shalgam anthocyanins in a pasteurized fermented black carrot beverages

stored at 4, 25 and 40°C for 90 days; they found that the highest anthocyanins retention was observed at 4°C storage temperature with half-life between 231 and 239 days (31). However, there are a few studies to evaluate the effect of storage condition and storage duration in shelf life on antioxidant capacity and total phenolic compounds of pasteurized and sterilized Thai health beverages.

2.3 Sugar

Sugar is a generic term used to identify simple forms of carbohydrates, which includes monosaccharide (fructose, glucose, and, galactose) and disaccharides (maltose, lactose, and sucrose). Sugar can be divided into two groups based on their origin. There were intrinsic or naturally occurring sugars refer to the sugar that is an integral constituent or whole fruit, vegetable, and milk products and extrinsic or added sugars refer to source or other refined sugar in soft drinks and incorporated into food, fruit drinks, and other beverages (20,72).

Sugar is the predominant caloric sweeteners, including refined sugar, corn syrup, fructose, high fructose corn syrup, and maltose. These sweeteners have used as the primary sweetener in processed foods and beverages. Although sugars have been several important roles as an ingredient in foods or food processing (as shown in **Table 2-4**), they also provide energy without nutrients (72,73).

Table 2-4. Sugar roles in foods[1]

- Contributes sweetness
- Enhances flavors
- Improves appearance (browning, color preservation)
- acts as a preservative
- keeps food moist
- tenderizes
- provides a base for yeast fermentation
- keeps sauces smooth
- keeps cooked fruits and vegetables firm
- increases volume of cakes and other baked goods
- contributes to texture of baked goods and confectioneries
- lowers the freezing point (prevents coarse ice crystals)

[1]From reference 73

2.3.1 Sugar and health

There are evidences that high consumption of adding sugar from processed foods and beverages contributes importantly to excess caloric intake and is an important factor underlying the development of obesity, diabetes, coronary heart disease and other health effects in human. Obesity is basically a consequence of higher energy intake than energy expenditure, where excess calories are stored as fat (74). Diabetes is becoming a serious problem in most of the region as dietary energy intake and the prevalence of overweight and obesity increase (75). Meanwhile, overweight and obesity have additional impact on cardiovascular outcomes independent of their strong associations with established coronary risk factors, e.g., high blood pressure and high cholesterol level. As recently reported of the 17,640 participants who had survived to age 65 and older, those who were overweight, and particularly those who were obese earlier in life, had significantly higher risks of hospitalizations and death from heart disease and diabetes in older age compared with persons of normal weight with similar other cardiovascular risk factors at the beginning of the study (76).

Over the past several decades, there were trend to increase levels of overweight and the prevalence of obesity among population. World Health Organization (WHO)

reported in the year 2003 that prevalence of Thai children aged between 5 – 12 years is 16% and they trend to have obesity in their adulthood by 42 – 63% (14,77). Recent report (2004) of the second National Health Examination Survey (NHES II) conducted in 1997 showed that the prevalence of overweight (BMI ≥ 25 kg/m^2) and obesity (BMI ≥ 30 kg/m^2) in 3,220 Thai adults aged 20 to 59 y are 28.3% and 6.8%, respectively (21). Moreover, according to WHO, there will be an almost 70% increase in the number of cases of diabetes in developing countries by the year 2025. Especially in the Southeast Asia Region, it is estimated that there will be almost 80 million diabetics in 2025, the highest among all six of the WHO Regions (78). Anyhow, the estimated national prevalence of diabetes in Thai adults is 9.6 % (2.4 million people) in the year 2003 (22). The prevalence of Type 2 DM in Thai children and adolescents also remarkably increased from 5 % during 1987 – 1996 to 17.9 % during 1997-1999 (mean age = 11.6 ± 2.1 y) (23). Recently reported by the International Collaborative Study of Cardiovascular Disease in Asia (Inter Asia) in the year 2004, the prevalence of self-reported cardiovascular disease in Thai adults was 1.5 % in men and 1.7 % in women (representing 184,000 male and 226,000 female cases) (79). So, obesity, diabetes, and coronary heart disease are the major health problem of Thai people.

There were numerous studies to support that high intake of sugar, especially from sugar-sweetened beverages is associated with the development of obesity, diabetes, and coronary heart disease. It should be noted that calorically sweetened beverages are referred to any beverage to which a caloric sweetener has been added, including carbonated or noncarbonated soft drinks, fruit drinks, fruit punch, lemonade, sweetened powder drinks, or any other nonartificially sweetened beverages. Excluded from this definition are sugars naturally present in fluids and that are not added in processing, in preparation, or at the table. Added caloric sweetener has been added, including sucrose, high fructose corn syrup, honey, molasses, and other syrup (80).

Roben et al. (2002) designed a randomized, double-blind study to compare the effect of calorically sweetened (152 g sucrose/d) or artificially sweetened (0 g sucrose/d) beverages (soft drinks and flavored fruit juices) on weight gain in moderately overweight men and women for 10 wk. This study found that drinking calorically sweetened beverages (consumed large amount of sucrose by 28 % of energy intakes) resulted in greater weight gain and fat mass over (increase by 1.6 and

1.3 kg, respectively) than did drinking diet beverages (decrease by 1.0 and 0.3 kg, respectively). Moreover, systolic and diastolic blood pressure also increased in the sucrose group and decrease in the sweetener group (81). Sørensen et al. (2005) found that during intervention (with daily food and drink supplement sucrose or artificial sweeteners from soft drink for 10 wk), sucrose intake increase by 151 %, results in a 1.6-kg weight gain in the sucrose group. Meanwhile, sucrose intake decrease by 42 %, results in a 1.2-kg weight loss in the sweetener group (82). Because, those who consumed calorically sweetened beverages did not compensate for this consumption by reducing the intake of other beverages and foods and thus gained weight.

Lugwid et al. (2001) showed that in 548 adolescents (age 11.7 ± 0.8 y) participating in the Planet Health projects with changing in consumption of sugar-sweetened drinks and difference in measurement of obesity for 19 months, for each additional serving of sugar-sweetened drink consumed, both body mass index (BMI) and the odds ratio of becoming obese increased 1.6 times for each additional sugar-sweetened drink consumed everyday (83). Berkey et al. (2004) studied in 16,771 children of both boy and girl aged between 9 to 14 y participating in the U.S. Growing Up Today Study and completed food frequency questionnaires assessing typical past year intakes in 1996, 1997, and 1998 and analyzed change in BMI corresponding to beverage intakes over two 1-year periods among children, found that consumption of sugar-added beverages was associated with small BMI gains during the corresponding year, girl who drank 1 serving/d of sugar-added beverages gained more weight than girls drinking none, as did girls drinking 2 or 3 serving/d. Boy who increased consumption of sugar-added beverages from the prior year experienced weight gain and children who increase intakes by 2 or more serving/d from the prior year gained weight (84).

A perspective study of 65,000 US women conducted from 1991 to 1999 among women in the Nurse' Health Study II, was recently analyzed by Schulze et al. (2004). The diabetes analysis included 91,249 women free of diabetes and other major chronic disease at baseline in 1991. The weight change analysis included 51,603 women for whom complete dietary information and body weight were ascertained in 1991, 1995, and 1999. During 716,300 person-years of follow-up were identified 741 incident cases of confirmed type 2 diabetes. Over a 4 year period of weight gain was highest

among women who increased their sugar-sweetened soft drink consumption from 1 or fewer drinks/wk or more drinks/d and was lowest among women who decreased their intake after adjusting for lifestyle and dietary confounders. Increased consumption of fruit punch was also associated with greater weight gain compared with decreased consumption. Moreover, women consuming 1 or more sugar-sweetened soft drinks/d had relative risk of type 2 diabetes of 1.83 (95 % confidence interval 1.42 – 2.36; $p <$ 0.001) compared with those who consumed less than 1 of these beverages per month and consumption of fruit punch was associated with increased diabetes risk (18).

Besides, a high sugar intake is also linked to increased risk of heart disease. Because simple sugars are the primary source of high triglycerides, and very low-density lipoproteins (VLDL), which are independent risk factor for atherosclerosis. With a diet high in sucrose (>20 % of energy intake) is associated with an increase of plasma triglyceride concentrations. This increase is due to both increased hepatic secretion and impaired clearance of VLDL (20,85). Epidemiological studies confirm that in population who have high sugar intake, there is a correspondingly higher risk of developing heart disease. As the study of the Scottish Heart Health Study (1994) of 10,359 men and women found that neither extrinsic nor intrinsic sugars were significant independent correlates of prevalent CHD after adjustment for other dietary variables (86). Data from the Coronary Artery Risk Development in Young Adults (CARDIA) study in 1998 show a consistent inverse association between increased dietary sucrose intake and HDL cholesterol concentrations, in both cross-sectional and longitudinal analyses in blacks and whites, in both men and women, and after adjustment for other covariates (87).

2.3.2 Dietary recommendation of sugar

Due to the evidence that excess intake of sugars contributes significantly high caloric intake and associates with increase risk in various diseases. The dietary guidelines for Americans recommendations are to choose beverages and foods to moderate intake of sugar. The Food Guide Pyramid recommends that total added sugars should not exceed 6 tsp or 24 g for a 1,600 calories diet, 12 tsp or 48 g for a 2,200 calories diet and 18 tsp or 72 g for 2,800 calories diets (88). For Thai population Thai kids don’t eat sweets campaign recommends that daily sugar intake should not

more than 6 tsp or 24 g (16). While several national food intake surveys have reported that added sugar intake has increased and also exceeded the recommendations. As the average intake of added sweeteners for the U.S. population, age two and older, is 20.5 tsp or 82 g/d (89). Concurrently, an increased daily intake of 150 – 300 kcal (for different age – sex groups) has occurred with approximately 50 % of increased calories comes from the consumption of calorically sweetened beverages among the U.S. population, especially the consumption of high-fructose corn syrup (HFCS) which used as caloric sweetener > 40 % in almost soft drinks and fruit drinks (80,90). Nielson et al. (2004) also pointed out that energy intake from sweetened beverages increased 135 % whereas it reduced from milk consumption by 38 % (91).

This phenomenon is also concurrence in Thailand. For example, the average sugar intake in the year 2001 (29.0 kg/mo. or 16 tsp./d) was increased about 2.3 times compared to the year 1985 (12.7 kg/y. or 7 tsp./d) (14). The consumption of calorically sweetened beverages among Thai children as recently reported in 2005 that Thai children aged between 6 to 14 y consumed soft drink and other sweetened beverages in Bangkok and all regions by about 62 – 82 % (92). It should be noted that 1 canned sugar-sweetened beverage as soda (12 oz) contained 150 kcal or about 40 – 50 g of sugar. If these calories are added to a typical diet daily with no offsetting reduction in other caloric sources, it could leads to a 15-lb (6.75 kg) weight gain in 1 year (93).

Therefore, one of the solution to reduce caloric intakes, should be decreased intake of those sugar-sweetened beverages, as recently reported a randomized, controlled pilot study of Ebbeling et al. (2006) studied in 103 adolescents aged 13 to 18 y who regularly consumed sugar-sweetened beverages (SSBs) such as soft drinks, and juice drinks at least 1 serving (ie, 360 mL or 12 fl oz) per day assigned to intervention and control group. The intervention group relied on home deliveries of non-caloric beverages to displace SSBs for 25 weeks and thereby decreased consumption by 82 %. BMI change difference was strongly linked with baseline BMI, found significantly between the intervention and control groups, net BMI change was -0.75 ± 0.34 kg/m^2 (94).

With the current situation, Popkin et al. (2006) have developed the Beverage Guidance System and aimed to recommend the population older than 6 y. This Beverage Guidance System ranks beverages in 6 levels from the least preferred to the

most preferred by the panel (Level 6 – beverages that should be consumed in limited quantities to Level 1 – those that should be consumed as the major beverages, ie, water). As recommendation of the panel which suggests the distribution of beverage intake (14 % of energy from beverage) for a person with 2,200-kcal daily energy requirement following range below and as showed in **Figure 2-4**.

Level 1: water, 20 – 50 fl oz/d

Level 2: tea and coffee (unsweetened), 0 – 40 fl oz/d (can replace water; caffeine is a limiting factor up to 400 mg/d, ≈ 32 fl oz coffee/d)

Level 3: Low – fat and skim milk and soy beverages, 0 – 16 fl oz/d

Level 4: noncalorically sweetened beverages, 0 – 32 fl oz/d (could substitute for tea and coffee with the same limitations as for caffeine)

Level 5: Caloric beverages with some nutrients, 0 – 8 fl oz/d 100% fruit juice/d, 0 – 1 alcoholic drink/d for women and 0 – 2 drinks/d for men (one drink = 12 fl oz beer, 5 fl oz wine or 1.5 fl oz distilled spirits), and 0 fl oz whole milk/d

Level 6: calorically sweetened beverages, 0 – 8 fl oz/d

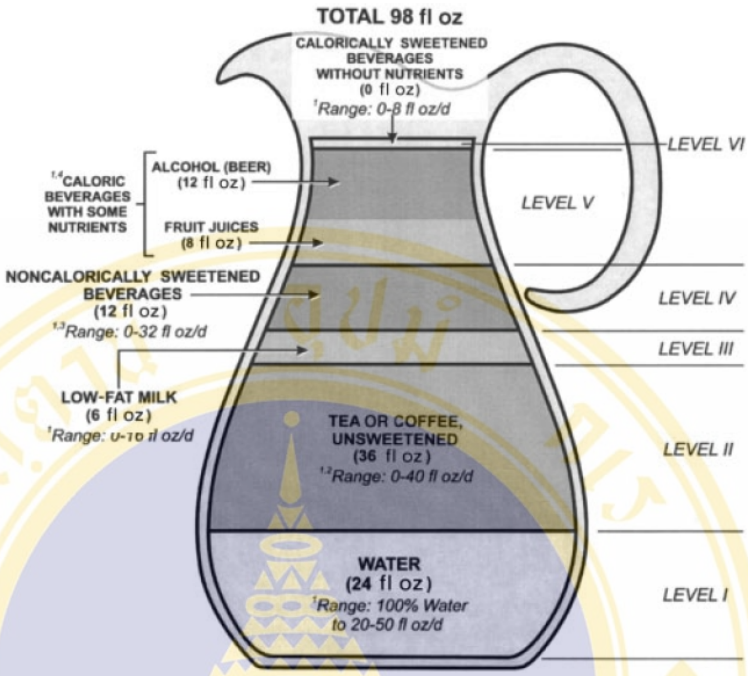


Figure 2-4. Acceptable beverage consumption patterns (14% of energy from beverages) for a person with a 2200-kcal daily energy requirement. The total sum of 98 fl oz, as shown at the top of the figure, 1 fl oz = 29.57 mL (from reference 80).

The goal of this guidance system is getting beverage intake from very-low-calorie beverage ≥ 80 %, which should be consist of water, unsweetened tea, and coffee and only about 20 % of low-fat milk, juice, alcohol, and calorically sweetened beverages (80). Therefore, it is suitable to find out an appropriated amount of fortified sugar in calorically sweetened Thai beverages with the lowest adding sugar in processing and well-consumer acceptance in taste.

2.4 Sugar-added beverages and antioxidant stability

With strong evidence of dietary sugar and development of many diseases in human, there was little known about the effect of sugar-added in beverages on stability of antioxidants and bioactive components. The study of Tolcott et al. (2003) found that pasteurized yellow passion fruit juice fortified with sucrose 10 % antioxidant capacity values unexpectedly lowered using the oxygen radical absorbance capacity (ORAC) assay (day 0, 17.2 ± 0.25 μM Trolox equivalent (TE)/ml VS day 14, 2 ± 0.25 μM

TE/ml). They also found the greatest loss in total carotenoids at all quantification wavelengths in the first 14 days of storage at 37°C (28). Chen et al. (2001) studied the stability of green tea catechins (GTC) as a mixture under various processing conditions by added GTC in commercial available soft drinks (Coca-Cola, 7-up, and Pepsi). They found that GCT (analyzed by HPLC methods) degraded up to 45 % in 7-up for 6 months and completely degraded in Pepsi and Coca-Cola drinks for only 4 months. This study suggested that other ingredients used in production of tea drinks might interact with GCT and affect its stability (29). Both studies showed that sugar might affect the stability of antioxidant capacity and phenolic compounds in sugar-added beverages.

From the above studies, if we can show that high fortified sugar in beverages have potency to reduce antioxidant capacity, this information would be useful to motivate general consumer who expects to get antioxidants from fruit and herb beverages to choose low calorically sweetened beverages. On the other hand the demand of consumer would motivate the manufacturer to reduce sugar adding in their production of beverages.

From all the above reasons, this study was conducted to determine the relationship between fortified sugar, antioxidant capacity and total phenolic contents, and to find out the appropriated amount of fortified sugar in Thai health beverages and also to evaluate the stability of antioxidant capacity and total phenolic compounds during shelf life storage of both pasteurized and sterilized Thai health beverages.

CHAPTER III
MATERIALS AND METHODS

3.1 Study Design

The study samples included pasteurized and sterilized Thai health beverages, produced from the pasteurized fruit juice plant and the fruit juice cannery under The Royal Chitralada Projects. Firstly, some of pasteurized and sterilized beverages were screened for antioxidant capacities, total phenolic compounds and total sugar contents. Secondly, selected pasteurized beverages were fortified sugar in different level of sweetness, and then determined antioxidant capacity, total phenolic compounds, and total sugar contents and they were also determined the changes of antioxidant capacity and total phenolic compounds after 7 days of storage. Thirdly, some of them were tested for sensory evaluation to find out a minimum amount of fortified sugar which accepted in taste from general consumers. Fourthly, sterilized or canned beverages were determined antioxidant capacity, total phenolic compounds, and total sugar contents after processing and determined the changes of antioxidant capacity and total phenolic compounds after 1 and 3 months of storage (**Figure 3-1**).

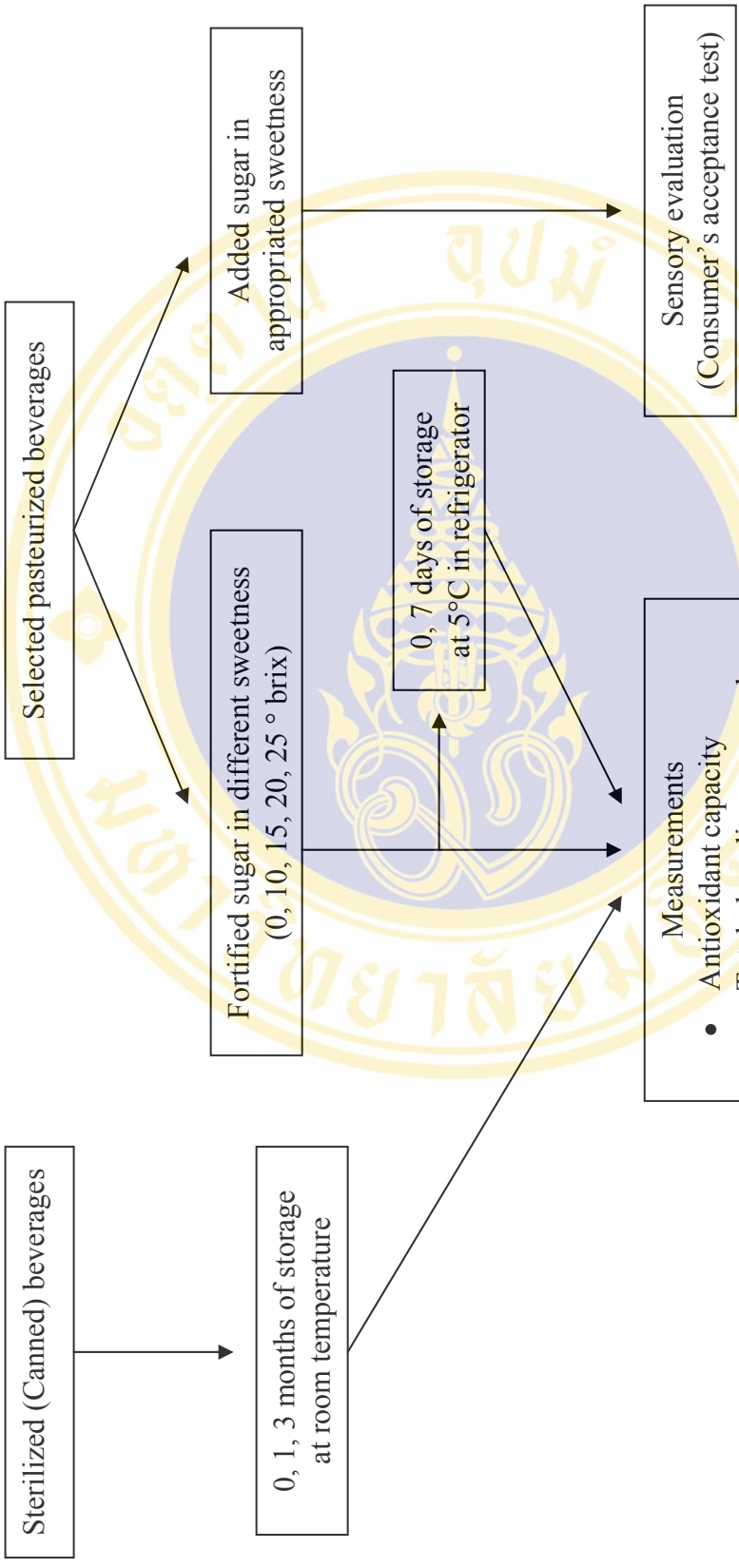


Figure 3-1. Experimental design

3.2 Research Methodology

3.2.1 Sample selections

Selection criteria:

- Well known or popular beverages
- Produced from common Thai fruits and herbs known as herbal medicine or known as containing many bioactive compounds in their composition (**Table 5**)

Studied samples

Studied samples were 15 pasteurized (P) and sterilized (S) Thai health beverages of The Royal Chitralada Projects as follow:

1. Mango juice (Both of P and S)
2. Roselle juice (Both of P and S)
3. Ginger juice (Both of P and S)
4. Bael fruit juice (Both of P and S)
5. Chrysanthemum drink (Both of P and S)
6. Lemongrass drink (Both of P and S)
7. Lemon juice (P)
8. Longan drink (P)
9. Sacred lotus root drink (P)
10. Orange juice (S)
11. Pineapple juice (S)
12. Pineapple with passion fruit juice (S)
13. Ganoderma with honey drink (S)
14. Ganoderma with chrysanthemum drink (S)
15. Tamarind juice (S)

Table 3-1. Bioactive compounds and therapeutic uses in selected fruits and herbs[1]

Common name	Scientific name	Bioactive components	Therapeutic uses
1. Mango	*Mangifera indica Linn.*	β –cryptoxanthin, ascorbic-acid, β –carotene, gallic acid	Anti-asthmatic, antiseptic, antiviral, cardiotonic, emetic, expectorant, hypotensive, laxative
2. Orange	*Citrus reticulata*	β -cryptoxanthin, flavanones.	Tonic, combat digestive upsets, anti-constipation
3. Lemon	*Citrus limon*	Flavanones, hesperidin, ascorbic acid	Appetizer, anti-inflammatory, antitussive, antiflu, stomachic, antiscorbutic properties.
4. Passion fruit	*Passiflora edulis*	β –cryptoxanthin, ascorbic-acid	Nutritive, sedative, diuretic, heart tonic
5. Ganoderma (Reishi mushroom)	*Ganoderma lucidum*	sterols, coumarin, mannitol, polysaccharides, triterpenoids	Anti-microbial, stimulate immune response, lower blood pressure and cholesterol level
6. Pineapple	*Ananas comosus (Linn.) Merr.*	β-sitosterol, campesterol	Diuretics, antitussive, expectorant
7. Sacred lotus root (Rakbua)	*Nelumbo nucifera*	pyrocatechol, d-gallic-catechin, leucocyanidin, vitamins B and C	Tonic, antidiarrhoeal, antidysentry
8. Ginger (Khing)	*Zingiber officinale Rosc.*	Gingerol, zingiberene	Carminative, antinauseant and antiflatulence agent.
9. Bael fruit (Matoom)	*Aegle marmelos Corr.*	Tannin, mucilage, β-sitosterol	Carminative, digestive, tonic and laxative
10. Tamarind (Makham)	*Tamarindus indica Linn.*	Ascorbic acid, tartaric acid	Antipyretic, antitussive, expectorant, anti-constipation
11. Roselle (Krajeab)	*Hibiscus sabdariffa Linn.*	Anthocyanin, protocatechuic acid	Diuretic, expectorant, lithagogue
12. Chrysanthemum (Kekhuai)	*Chrysanthemum indicum Linn.*	Glycoside chrysanthimin	Antibacterial action, Lower blood pressure, rejuvenating effect
13. Lemongrass (Takhrai)	*Cymbopogon citrates (DC.) Stapt*	Citral, geraniol, methylheptenone,	Diuretics, antiflatulence, antiflu, antimicrobial agent.
14. Longan (Lumyai)	*Dimocarpus longan Lour.*	Ascorbic acid, vitamin B, niacin	Stomachic, febrifuge, vermifuge

[1] Data from reference 95-104

3.2.2 Sample collection and preparation:

Each type of selected pasteurized beverages was collected three separate of productions (n = 3) after boiling in time limit of each raw materials before step of sugar adding in the processing of pasteurization. After that they were divided in equal amount (by volume) and fortified sugar (42% High Fructose Syrup) from pasteurized fruit juice plant of The Royal Chitralada Projects in different level of beverage Brix, using handheld refractometer (Model Brix 0 – 32 %), represented in brix scale (°Brix), there were 4 levels of sweetness and control as followed: 0, 10, 15, 20, and 25 °Brix, respectively. All of them were cooled immediately before filling in the dark polyethylene tubes and stored at -20°C until analysis. In addition these pasteurized beverages were also analyzed the changes of antioxidant capacity and total phenolic compounds after 7 days of storage in the refrigerator.

For sterilized or canned beverages, They were collected three separate of productions (n = 3) after the step of sterilization and cooling and then they were stored at room temperature until analysis at each time point of storage (0, 1, and 3 months) for antioxidant capacity, total phenolic contents, and total sugar contents.

3.3 Sensory evaluation for consumer's acceptance

Chrysanthemum drink, roselle drink, ginger drink, and bael fruit drink were selected for sensory evaluation of appropriated amount of fortified sugar. First, The beverages were fortified at minimum sweetness (6 – 7 °Brix) according to the lowest beverage Brix of juice of the pasteurized fruit juice plant under The Royal Chitralada Projects and then they were increased level of beverage Brix to 8 – 9 °Brix. The process of pasteurization used in this study in each beverage followed the process of the research and development unit under The Royal Chitralada Projects presented in **Appendix A**. An informal panel of thirty untrained assessors (n=30) had evaluated the samples on the basis of degree of acceptability for color, odor, taste, texture and overall using a nine – point hedonic scale (106) ranging from "dislike extremely, 1" to "neither like nor dislike, 5" to "like extremely, 9". The questionnaire used in the sensory test is shown in **Appendix B.**

3.4 Laboratory assessment

3.4.1 Determination of antioxidant capacity

3.4.1.1 The DPPH radical scavenging assay

This method is based on the hydrogen donating or radical scavenging ability of DPPH (2,2-diphenyl-1-picrylhydrazyl), a stable free radical which is reduced in presence of the antioxidant active substances (52,53,58). For the analysis, the 3 mL of aliquot of 5×10^{-4} M DPPH radical solution dissolved in methanol (1:4) was mixed with 0.2 mL of appropriately diluted samples or standard solution at various concentrations and deionized water was used as control and diluent. The mixture was then shaken vigorously and left for 2 min at room temperature, at which time the decrease in absorbance at 515 nm was recorded using a spectrophotometer (UV-160A, SHIMADZU). Trolox and ascorbic acid (1mM) were used as standard. All samples were measured in duplicate. Percent relative standard deviation was less than 10 %. Results were expressed in micromole per 100 ml of trolox or ascorbic acid equivalents (μmol TE or μmol AAE/100ml) and also calculated the IC_{50} values (%v/v), the 50% inhibitory concentration, which represent the concentration of antioxidant samples that caused 50 % loss of DPPH radical (fall in absorbance), the lower the IC_{50}, the higher the antioxidant capacity.

3.4.1.2 The Photochemiluminescence (PCL) assay

This assay involves the photochemical generation of superoxide ($O_2^{\bullet-}$) free radical combined with chemiluminescence detection, luminol, which acts as photosensitizer as well as oxygen radical detection reagent. This reaction takes place in the PHOTOCHEM®, by Analytik Jena AG (Jena Germany). The ACW and ACL kits provided by manufacturer were used to measure hydrophilic and lipophilic antioxidant capacity, respectively (59-62). The ACW kit, 1.5 mL reagent 1 (water), 1 mL reagent 2 (buffer solution pH 10.5), 25 μL reagent 3 (luminol), and 10 – 30 μL standard solution or appropriately diluted antioxidant samples were mixed and measured. The ACL kit, 2.3 mL reagent 1 (methanol), 200 μL reagent 2 (buffer solution), 25 μL reagent 3 (luminol), and 10 – 30 μL standard solution or appropriately diluted antioxidant samples were mixed and measured. These are standardized conditions. All samples were appropriately dissolved and diluted in

reagent 1 of ACL and ACW kits before analysis in each measurement. Ascorbic acid and trolox were used as calibration reagents for ACW and ACL, respectively, at measuring concentration ranges of 1 – 3 nmol. All samples were measured in duplicate. Percent relative standard deviation was less than 10 %. The antioxidant capacity was determined by means of lag phase (L) in seconds; $L = L_0 - L_1$ (where L_0 and L_1 are the respective parameters of the blank and sample) or by means of the degree of PCL inhibition (I), $I = 1 - S/S_0$ (where S_0 is the integral under the blank curve and S is the integral under the sample curve) for ACW or ACL values, respectively. The results were expressed as nanomolar range and calculated them to micromole per 100 mL of ascorbic acid or trolox equivalent (μmol TE or μmol AAE/100mL).

3.4.2 Determination of total phenolic compounds

Total phenolic compounds in the study samples were measured using the Folin – Ciocalteu methods with some modification (63,64). For the analysis, 50 μL of appropriately diluted sample or standard solution at various concentrations were mixed with 100 μL of Folin – Ciocalteu reagent and deionized water was used as control and diluent. The solution was then diluted to a total volume of 1150 μL with deionized water and mixed thoroughly. After incubation for 10 min at room temperature, 500 μL of 20% Na_2CO_3 solution was added with mixing immediately and further incubated at room temperature for 2 hr. The mixture was then recorded the absorbance at 765 nm using a spectrophotometer (UV-160A, SHIMADZU). All samples were measured in duplicate. Percent relative standard deviation was less than 10 %.Gallic acid (1mg/mL) was used as standard and the total phenolic compounds of the samples were expressed in milligrams per 100 mL of gallic acid equivalents (mg GAE/100mL).

3.4.3 Determination of total sugar contents

Sample preparation: 1 mL of appropriately diluted samples or standard solution at various concentrations was mixed with 0.5 mL of 0.1N HCl. The mixture was heated in 100°C water bath for 15 min, and then soaked in cooling water. After cooling, 0.5 mL of 0.1N NaOH was added with the mixing. The extracted sample solution was used for analysis of total sugar content using Nelson's reducing sugar

test. (107). For the analysis, 1 mL of the extracted sample or standard solution was mixed with 1 mL of Alkaric copper reagent and heated in 100°C water bath for 15 min and then soaked in flow water. After cooling, the mixture was added 1 mL of Arsenomolybdic reagent with mixing immediately to dissolve solve the sediments and then diluted to a total volume of 12.5 mL with deionized water and mixing thoroughly. After incubation for 30 min at room temperature, the absorbance at 500 nm was recorded using spectrophotometer (UV-160A, SHIMADZU). Deionized water was used as control. 1 % of D-glucose solution was used as calibration curve at measuring ranges of 0.01 – 0.04 %. All samples were measured in duplicate. Percent relative standard deviation was less than 10 %.The total sugar contents of the samples were expressed in grams per serving of D-glucose and also calculated the estimated total calories from sugar by 1 gram of glucose provides 4 kcal.

The reagents preparation used in this method are presented in **Appendix C**.

3.5 Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed with the used of Statistical Package for the Social Sciences (SPSS) for Windows solfware, version 11.5 (SPSS Inc., Chicago, Illinois, USA). The mean values were analyzed one-way analysis of variance (one-way ANOVA); and the Bonferroni multiple comparisons was used to detect a significant difference between the means. Independent paired t-test was used for significant difference of sensory evaluation. One-way repeated-measures analysis of variance was used to test the change of the components at each time points of storage. Correlations of any parameters were analyzed by Pearson's correlation coefficient in bivariate correlation and linear regression analysis. Differences were considered significant if $p\text{-value} < 0.05$.

CHAPTER IV
RESULTS

4.1 Antioxidant capacity and total phenolic compounds of pasteurized and sterilized beverages

4.1.1 Antioxidant capacity and total phenolic compounds of pasteurized and sterilized beverages

The results of antioxidant capacity of 12 pasteurized and sterilized beverages measured by DPPH and PCL methods are shown in **Table 4-1**. By DPPH assay, sacred lotus root drink showed the significantly highest antioxidant capacity in both equivalent to trolox (TE) and ascorbic acid (AAE) and also showed the significantly lowest IC_{50} values **(Table 4-2)**. With the PCL method, chrysanthemum drink and roselle drink showed the significantly highest antioxidant capacity of both ACL and ACW values. Lemon juice had the lowest values of antioxidant capacity by both DPPH and PCL assays. It was noticed that ginger drink was not assessable for ACW value whereas lemon juice was not assessable IC_{50} value.

Figure 4-1 showed that bael fruit drink had the significantly highest total phenolic compounds (83.83 ± 37.59 mg GAE/100mL). There were no significant differences in phenolic contents among the other beverages. Interestingly, lemon juice had the lowest total phenolic compounds (0.93 ± 0.43 mg GAE/100mL).

Table 4-3 showed total sugar content and estimated calories of pasteurized and sterilized beverages in this study. Sacred lotus root drink and Lemongrass drink had the significantly lowest beverage Brix as well as the total sugar contents and estimated calories among the samples. There was linear regression between beverage Brix and total sugar contents with significant correlation at $R^2 = 0.566$ **(Figure 4-2)**.

Table 4-1. Antioxidant capacity of pasteurized and sterilized beverages by using DPPH and PCL assays[1]

Type of beverage	DPPH assay		PCL assay	
	µmol TE/100ml	µmol AAE/100ml	ACL values (µmol TE/100ml)	ACW values (µmol AAE/100ml)
1. Sacred lotus root drink	99.03 ± 2.76 ^{e}	92.47 ± 8.85 ^{d}	77.81 ± 7.48 ^{b}	131.67 ± 7.41 ^{bc}
2. Chrysanthemum drink	75.79 ± 23.37 ^{de}	68.72 ± 22.54 ^{cd}	324.75 ± 40.13 ^{c}	656.17 ± 95.07 ^{c}
3. Bael fruit drink	68.38 ± 8.74 ^{d}	59.95 ± 3.14 ^{bcd}	165.42 ± 20.86 ^{d}	298.66 ± 71.18 ^{d}
4. Roselle drink	59.38 ± 5.30 ^{cd}	68.94 ± 37.09 ^{cd}	341.22 ± 54.68 ^{c}	671.18 ± 70.68 ^{e}
5. Lemongrass drink	37.04 ± 0.62 ^{c}	36.97 ± 10.95 ^{acd}	79.05 ± 12.62 ^{b}	227.16 ± 23.91 ^{cd}
6. Longan drink	20.35 ± 8.81 ^{ab}	19.50 ± 8.05 ^{ab}	13.16 ± 0.43 ^{ab}	20.81 ± 0.20 ^{ab}
7. Mango juice	18.91 ± 4.31 ^{ab}	17.82 ± 4.18 ^{ab}	14.11 ± 0.20 ^{ab}	51.09 ± 1.83 ^{ab}
8. Orange juice	12.32 ± 2.00 ^{ab}	12.48 ± 0.79 ^{a}	64.11 ± 8.54 ^{ab}	53.35 ± 6.33 ^{ab}
9. Pineapple juice	9.21 ± 0.43 ^{a}	11.05 ± 2.12 ^{a}	57.02 ± 5.74 ^{ab}	53.35 ± 6.82 ^{ab}
10. Tamarind juice	7.81 ± 1.65 ^{a}	8.32 ± 3.06 ^{a}	24.68 ± 1.43 ^{ab}	48.91 ± 3.59 ^{ab}
11. Ginger drink	7.65 ± 0.92 ^{a}	8.97 ± 1.19 ^{a}	21.45 ± 2.43 ^{ab}	NA
12. Lemon juice	1.33 ± 0.55 ^{a}	3.62 ± 0.55 ^{a}	7.41 ± 1.18 ^{a}	18.15 ± 1.82 ^{ab}

[1]All analytical data are mean values of three independent samples (n=3) ± SD
Values with different letters (a – e) within column of each sample are significantly different at $p < 0.05$
TE: Trolox equivalent, AAE: Ascorbic acid equivalent, ACL: Antioxidant capacity of lipid soluble substances, ACW: antioxidant capacity of water soluble substances, NA: Not assessable

Table 4-2. IC_{50} values of pasteurized and sterilized beverages by using DPPH assay[1]

Type of beverage	IC_{50} (%v/v)[2]
1. Sacred lotus root drink	2.04 ± 0.18 [a]
2. Chrysanthemum drink	2.68 ± 0.87 [a]
3. Bael fruit drink	3.34 ± 0.23 [a]
4. Roselle drink	4.56 ± 0.86 [a]
5. Orange juice	5.59 ± 0.19 [ab]
6. Lemongrass drink	5.74 ± 0.62 [ab]
7. Mango juice	11.16 ± 1.64 [bc]
8. Longan drink	12.21 ± 3.80 [cd]
9. Pineapple juice	17.99 ± 2.57 [de]
10.Ginger drink	20.28 ± 3.94 [ef]
11.Tamarind juice	26.39 ± 1.51 [f]
12.Lemon juice	NA

[1] All analytical data are mean values of three independent samples (n=3) ± SD
[2] 50% inhibitory concentrations: the concentration of antioxidant that causes 50% loss of the DPPH radical
Values with different letters (a – f) within column of each sample are significantly different at $p < 0.05$
NA: Not assessable

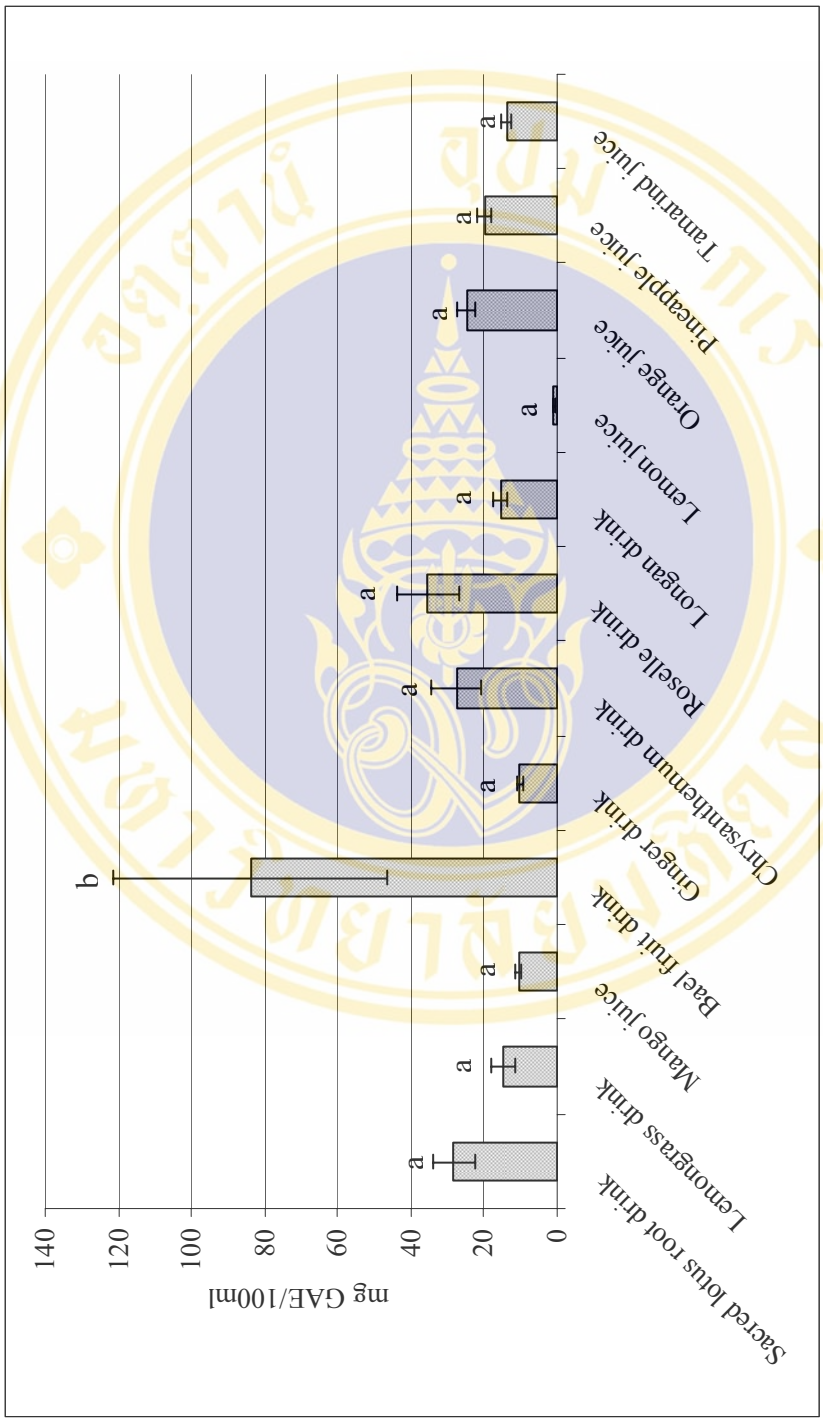


Figure 4-1. Contents of total phenolic compounds in pasteurized and sterilized beverages. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars. Values with the same letters in each sample are not significantly different at $p < 0.05$. GAE: Gallic acid equivalent

Table 4-3. Total sugar contents, and estimated calories of pasteurized and sterilized beverages[1]

Type of beverage	Beverage Brix	Total sugar contents (g/serving) [2]	Estimated calories (kcal/serving)[3]
1. Sacred lotus root drink	10.20 ± 0.20	23.80 ± 2.44 [c]	95.20 ± 9.76
2. Lemongrass drink	11.20 ± 0.20	22.66 ± 2.34 [c]	90.65 ± 9.35
3. Mango juice	12.47 ± 0.42	29.01 ± 0.70 [abc]	116.06 ± 2.78
4. Bael fruit drink	13.20 ± 0.20	32.47 ± 6.76 [abc]	129.89 ± 27.06
5. Ginger drink	13.23 ± 0.25	36.75 ± 3.54 [a]	147.01 ± 14.18
6. Chrysanthemum drink	13.33 ± 0.42	36.41 ± 2.35 [a]	145.63 ± 9.42
7. Roselle drink	13.50 ± 0.30	35.03 ± 5.05 [ab]	140.10 ± 20.20
8. Longan drink	13.60 ± 0.20	34.70 ± 5.14 [ab]	138.82 ± 20.55
9. Lemon juice	13.60 ± 0.20	35.83 ± 0.86 [ab]	143.31 ± 3.42
10. Orange juice	14.27 ± 0.31	32.08 ± 2.27 [abc]	128.31 ± 9.09
11. Pineapple juice	14.27 ± 0.31	37.44 ± 5.02 [a]	149.74 ± 20.08
12. Tamarind juice	14.47 ± 0.42	37.96 ± 1.07 [a]	151.86 ± 4.29

[1]All analytical data are mean values of three independent samples (n=3) ± SD [2] Analyzed by using Nelson's reducing sugar test

[3]Estimated total calories calculated from 1 gram glucose provides 4 kcal, 1 serving = 240 ml

Values with different letters (a – c) within column of each sample are significantly different at $p < 0.05$

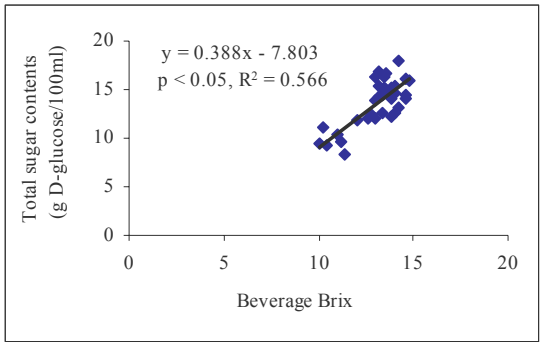


Figure 4-2. Linear regression between Beverage Brix in pasteurized and sterilized beverages and total sugar contents in Nelson's reducing sugar test

4.1.2 Correlation between total phenolic compounds and antioxidant capacity of pasteurized and sterilized beverages

The correlation between the total phenolic compounds and the values of TE, and AAE in DPPH assay were found with significant correlation at r = 0.537 and 0.574, respectively **(Figure 4-3A and 4-3B).** The negative correlation (r = -0.451) between the total phenolic compounds and IC_{50} values in DPPH assay was also found with significant correlation **(Figure 4-3C).** For the PCL assay, the linear correlation between the total phenolic compounds and the ACL and ACW values were also found with significant correlation at r = 0.459 and 0.417, respectively (**Figure 4-4A and 4-4B**).

The correlation between the values of TE in DPPH assay and ACL in PCL assay as well as the values of AAE and ACW were found with significant correlation at r = 0.654 and 0.661, respectively (**Figure 4-5A and 4-5B**). Significant inverse correlation between IC_{50} values and ACL or ACW values were also found with r = -0.562 and -0.569, respectively (**Figure 4-6A and 4-6B**).

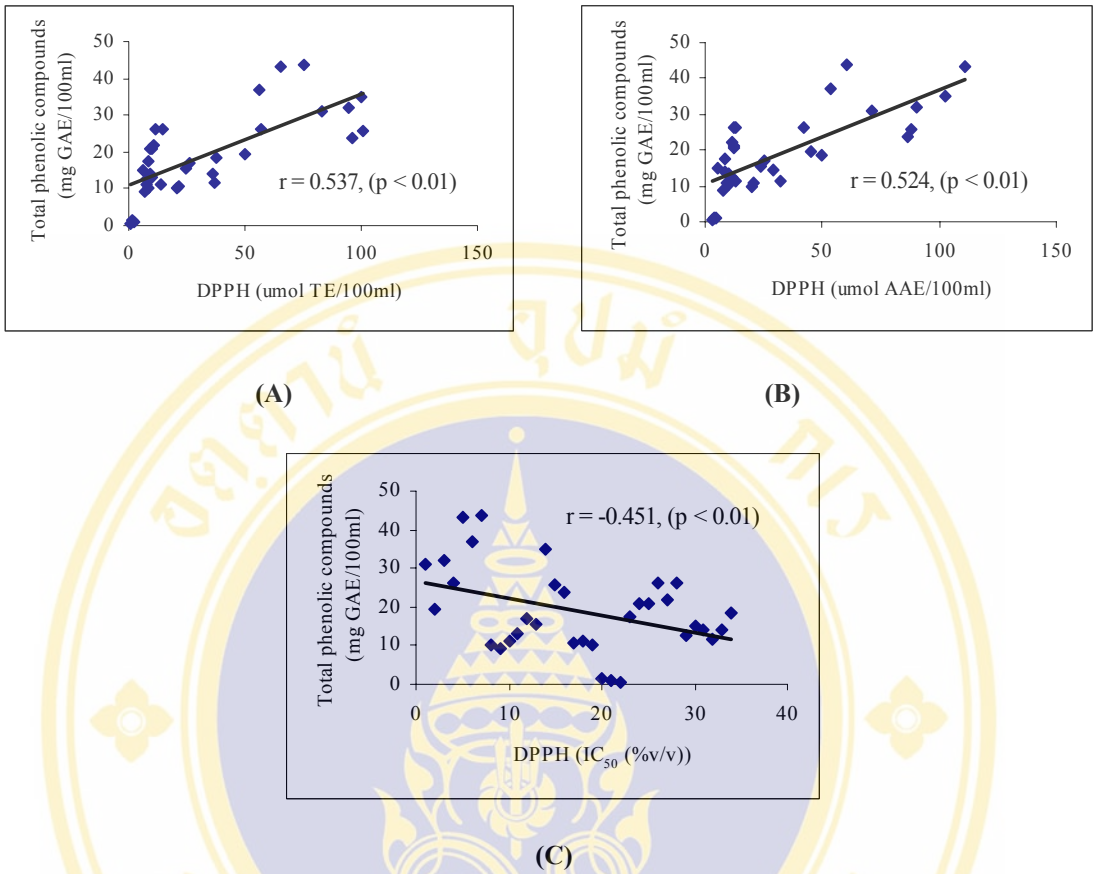


Figure 4-3. The correlation between antioxidant capacity equivalents to trolox (A), ascorbic acid (B), and IC_{50} values (C) of pasteurized and sterilized beverages in DPPH assay and total phenolic compounds

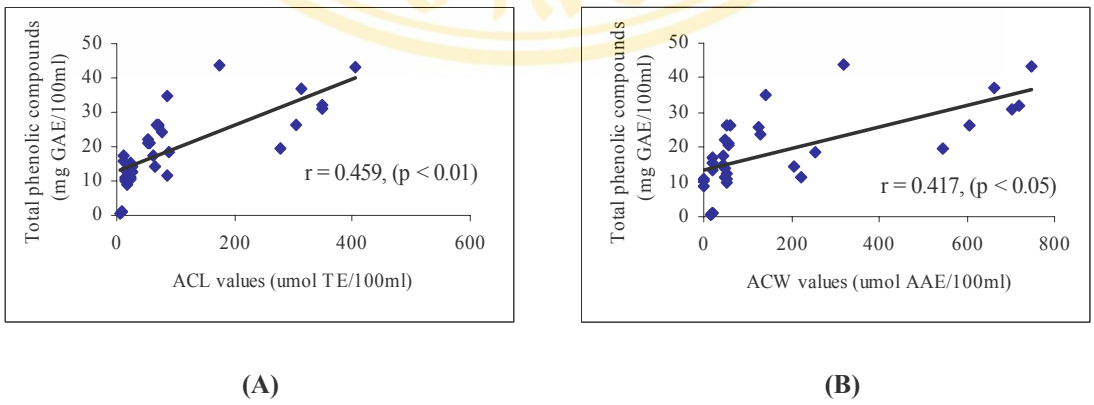
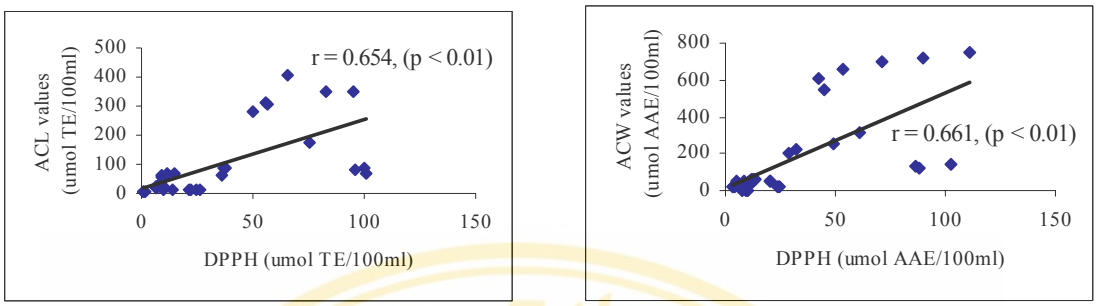
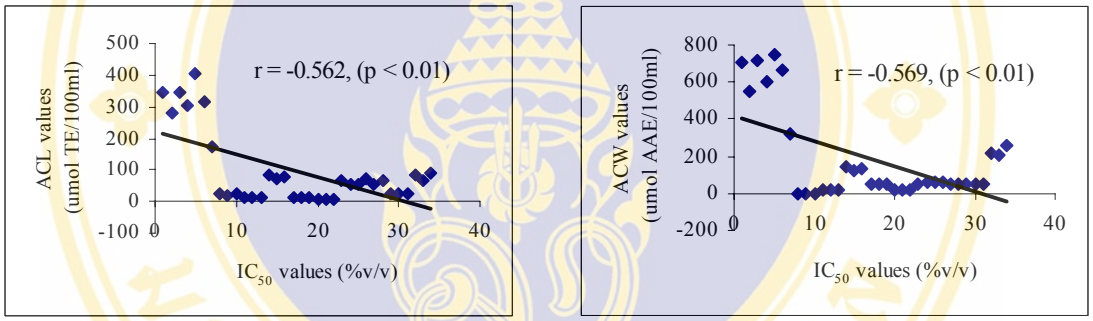


Figure 4-4. The correlation between ACL (A) and ACW (B) values of pasteurized and sterilized beverages in PCL assay and total phenolic compounds



(A) (B)

Figure 4-5. The correlation between antioxidant capacity equivalent to trolox (A) and ascorbic acid (B) of pasteurized and sterilized beverage in DPPH and in PCL assay



(A) (B)

Figure 4-6. The correlation between IC_{50} values of pasteurized and sterilized beverages in DPPH assay and ACL (A) and ACW (B) values in PCL assay

4.2 The correlation between fortified sugar and antioxidant capacity and total phenolic compounds of selected pasteurized beverages

As a result in part one, antioxidant capacity equivalent to trolox and ascorbic acid in DPPH assay were found similar values, because on basis of mechanism of DPPH assay is the single electron transfer (SET) reaction which are sensitive to ascorbic acid and uric acid (51,36). In our study, an IC50 value of standard ascorbic acid (18.5 ± 1.2 μmole) was lower than trolox (21.7 ± 2.3 μmole). Therefore the data in this and next part have shown only ascorbic acid equivalent.

There were trends of decreasing in the antioxidant capacity of selected pasteurized beverages including trends of increasing in the IC_{50} values as the sugar contents increased (**Table 4-4**). However, only the antioxidant capacity equivalent to ascorbic acid in DPPH assay had significant inverse correlation with the amount of sugar contents among the studied beverages as followed; bael fruit drink, chrysanthemum drink, ginger drink, lemongrass drink, and roselle drink with r = -0.880, -0.703, -0.656, -0.644, and -0.611, respectively (**Figure 4-7**). While, the significant correlation between IC_{50} values in DPPH assay and the amount of sugar contents was found in some beverages as followed; beal fruit, chrysanthemum, and roselle drink with r = 0.847, 0.640, and 0.703, respectively (**Figure 4-8)**. For the PCL assay, significant inverse correlation between ACL value and the amount of sugar contents was found only in Ginger drink with r = -0.536 (**Figure 4-9C**).

Although, there were no significant changes in the total phenolic compounds as the sugar contents of selected beverage increased by adding sugar (**Figure 4-11**) but significant correlation ($r = 0.646$, $p < 0.01$) between the total phenolic compounds and the amount of sugar contents of Ginger drink was seen (**Figure 4-11C**).

Table 4-4. Antioxidant capacity of fortified sugar with different beverage Brix in selected beverages by using DPPH and PCL assays

Type of beverage	Beverage Brix	Sugar contents[1] (g/100 ml)	Antioxidant capacity2			
			DPPH assay		PCL assay	
			µmol AAE/100ml	IC_{50} values (%v/v)[3]	ACL values (µmol TE/100ml)	ACW values (µmol AAE/100ml)
1. Chrysanthemum drink	0.47 ± 0.12	5.00 ± 0.49	103.81 ± 10.10	1.95 ± 0.12	455.64 ± 150.56	624.36 ± 382.46
	10	13.55 ± 1.52	97.70 ± 13.49	2.01 ± 0.19	404.06 ± 198.01	629.23 ± 297.43
	15	19.13 ± 0.82	88.30 ± 11.96	2.26 ± 0.36	336.06 ± 132.25	587.38 ± 262.89
	20	24.66 ± 1.37	81.93 ± 9.92	2.27 ± 0.26	302.59 ± 136.80	530.22 ± 233.64
	25	29.37 ± 2.54	77.04 ± 13.54	2.44 ± 0.35	265.70 ± 231.83	504.84 ± 222.90
2. Bael fruit drink	0.87 ± 0.12	4.55 ± 0.49	81.62 ± 0.68	2.50 ± 0.06	241.65 ± 129.43	109.73 ± 34.88
	10	11.62 ± 1.78	71.11 ± 10.35	2.90 ± 0.43	237.45 ± 138.61	101.43 ± 27.71
	15	16.04 ± 2.06	59.05 ± 11.05	3.03 ± 0.24	213.41 ± 143.83	99.88 ± 39.29
	20	23.61 ± 0.68	58.47 ± 9.28	3.26 ± 0.39	196.13 ± 112.91	91.62 ± 43.31
	25	28.35 ± 1.12	49.00 ± 0.82	3.66 ± 0.33	193.09 ± 126.39	90.70 ± 49.22

[1] Analyzed by using Nelson's reducing sugar test
[2] All analytical data are mean values of three independent samples (n=3) ± SD
[3] 50% inhibitory concentrations: the concentration of antioxidant that causes 50% loss of the DPPH radical
AAE: Ascorbic acid equivalent, ACL: Antioxidant capacity of lipid soluble substances, ACW: antioxidant capacity of water soluble substances

Table 4-4. Antioxidant capacity of fortified sugar with different beverage Brix in selected beverages by using DPPH and PCL assays (cont.)

Type of beverage	Beverage Brix	Sugar contents[1] (g/100 ml)	Antioxidant capacity[2]			
			DPPH assay		PCL assay	
			µmol AAE/100ml	IC_{50} values (%v/v)[3]	ACL values (µmol TE/100ml)	ACW values (µmol AAE/100ml)
3. Roselle drink	1.00 ± 0.00	4.68 ± 0.28	79.53 ± 10.26	4.40 ± 0.22	563.22 ± 128.65	503.15 ± 130.02
	10	12.58 ± 0.74	75.16 ± 12.22	4.71 ± 0.47	518.81 ± 67.55	459.43 ± 20.66
	15	18.53 ± 0.32	73.42 ± 4.28	4.92 ± 0.45	502.69 ± 86.99	458.72 ± 85.77
	20	24.82 ± 0.52	69.89 ± 9.90	4.94 ± 0.56	491.69 ± 83.10	479.68 ± 166.21
	25	27.98 ± 1.59	63.11 ± 5.94	5.71 ± 0.20	464.80 ± 79.97	428.81 ± 121.62
4. Sacred lotus root drink	0.53 ± 0.12	4.78 ± 0.10	100.06 ± 28.57	2.19 ± 0.40	96.02 ± 26.79	80.10 ± 16.05
	10	12.48 ± 0.01	112.53 ± 18.26	1.88 ± 0.15	69.68 ± 14.84	145.01 ± 4.58
	15	18.30 ± 0.63	100.70 ± 11.02	1.97 ± 0.11	76.61 ± 7.29	122.55 ± 18.91
	20	23.87 ± 0.13	102.07 ± 10.98	1.97 ± 0.15	92.27 ± 12.62	121.64 ± 9.06
	25	23.87 ± 0.13	90.93 ± 7.31	2.09 ± 0.18	71.49 ± 14.08	102.05 ± 8.61

[1] Analyzed by using Nelson's reducing sugar test
[2] All analytical data are mean values of three independent samples (n=3) ± SD
[3] 50% inhibitory concentrations: the concentration of antioxidant that causes 50% loss of the DPPH radical
AAE: Ascorbic acid equivalent, ACL: Antioxidant capacity of lipid soluble substances, ACW: antioxidant capacity of water soluble substances

Table 4-4. Antioxidant capacity of fortified sugar with different beverage Brix in selected beverages by using DPPH and PCL assays (cont.)

Type of beverage	Beverage Brix	Sugar contents[1] (g/100 ml)	Antioxidant capacity[2]			
			DPPH assay		PCL assay	
			µmol AAE/100ml	IC_{50} values (%v/v)[3]	ACL values (µmol TE/100ml)	ACW values (µmol AAE/100ml)
5. Lemongrass drink	0.47 ± 0.12	4.90 ± 0.20	48.51 ± 5.77	3.55 ± 1.46	118.41 ± 33.55	159.93 ± 42.89
	10	12.83 ± 0.38	43.73 ± 4.59	3.86 ± 1.54	108.58 ± 29.44	161.90 ± 55.11
	15	17.81 ± 0.99	41.71 ± 4.20	5.17 ± 0.98	86.56 ± 33.72	141.57 ± 41.32
	20	23.68 ± 1.23	40.40 ± 2.92	4.86 ± 0.57	81.63 ± 36.07	143.00 ± 46.68
	25	29.38 ± 4.08	37.85 ± 4.25	5.36 ± 0.86	77.01 ± 35.91	131.62 ± 53.19
6. Ginger drink	0.13 ± 0.23	5.33 ± 0.32	7.62 ± 1.86	23.74 ± 2.53	21.09 ± 3.35	NA
	10	13.65 ± 0.98	7.59 ± 1.83	22.37 ± 1.40	21.36 ± 3.59	NA
	15	17.28 ± 0.14	6.81 ± 1.05	23.64 ± 2.64	18.67 ± 2.59	NA
	20	22.42 ± 0.28	5.45 ± 0.66	26.01 ± 4.89	18.97 ± 3.95	NA
	25	26.96 ± 0.83	5.28 ± 0.69	26.20 ± 0.52	15.03 ± 2.60	NA

[1] Analyzed by using Nelson's reducing sugar test
[2] All analytical data are mean values of three independent samples (n=3) ± SD
[3] 50% inhibitory concentrations: the concentration of antioxidant that causes 50% loss of the DPPH radical
AAE: Ascorbic acid equivalent, ACL: Antioxidant capacity of lipid soluble substances, ACW: antioxidant capacity of water soluble substances, NA; Not assessable

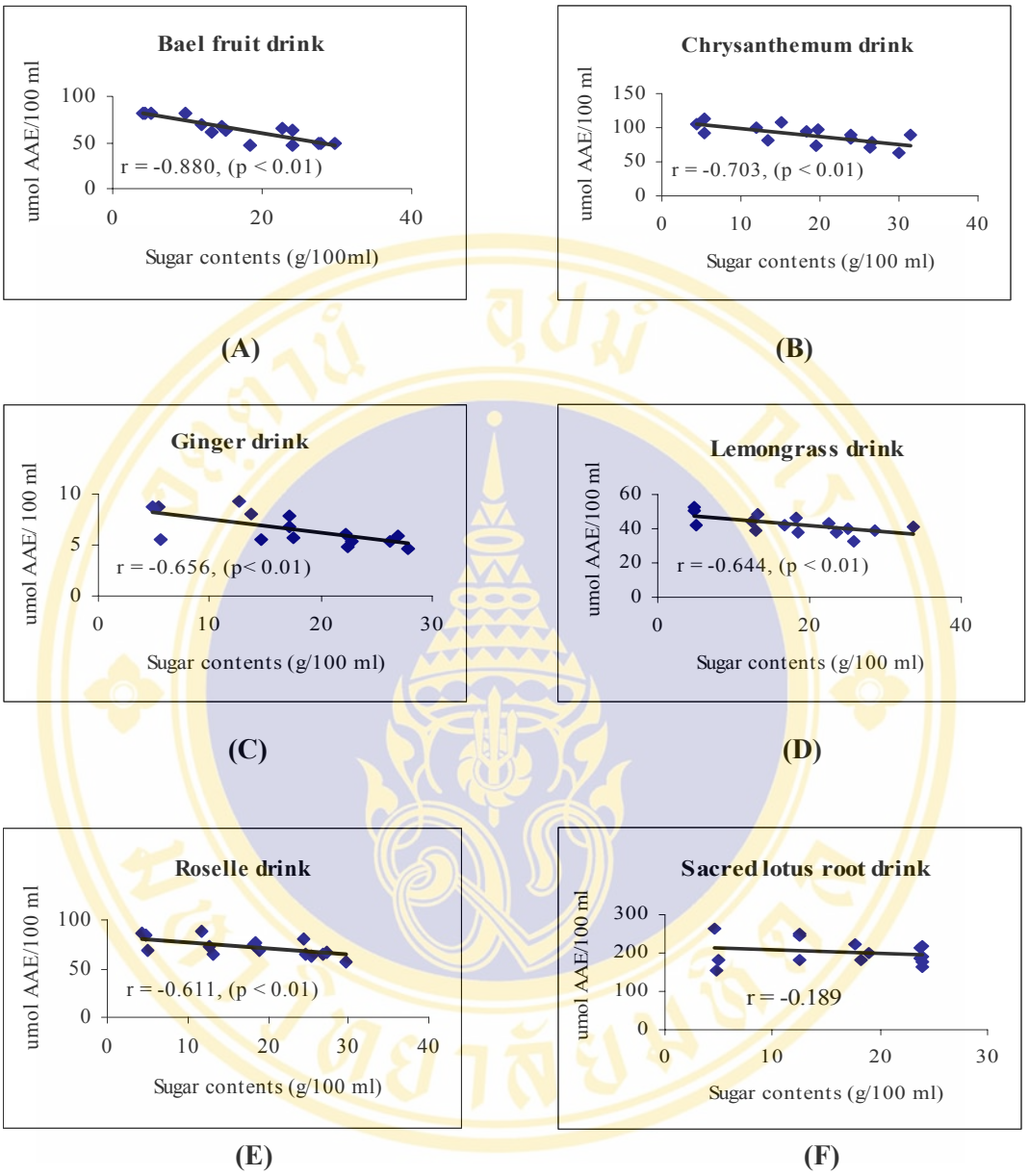


Figure 4-7. The correlation[1] between antioxidant capacities equivalent to ascorbic acid (AAE) of selected pasteurized beverages and sugar contents in DPPH assay: (A) Bael fruit drink, (B) Chrysanthemum drink, (C) Ginger drink, (D) Lemongrass drink, (E) Roselle drink, (F) Sacred lotus root drink. [1]Pearson's correlation coefficient (r)

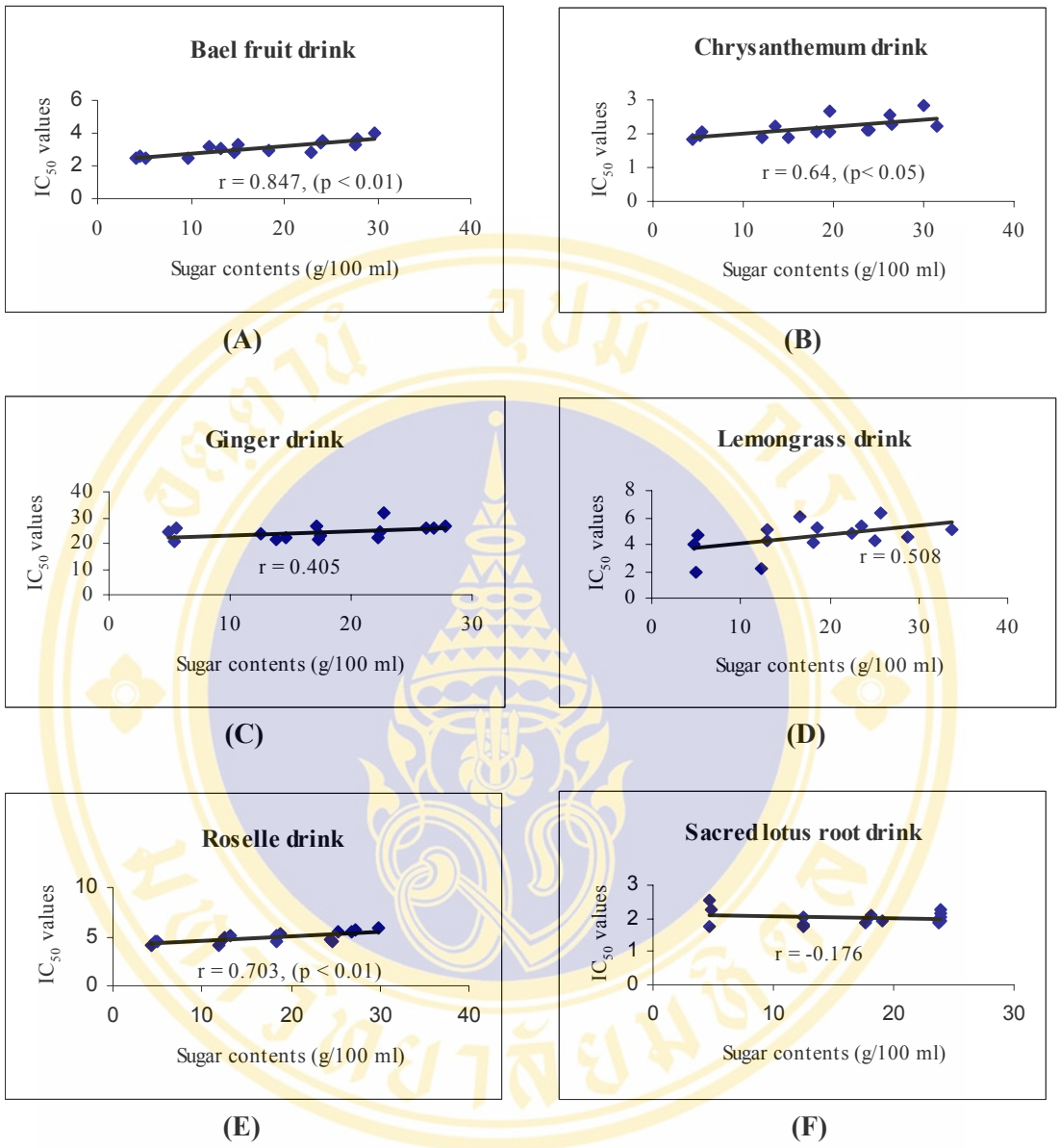


Figure 4-8. The correlation[1] between IC_{50} values of selected pasteurized beverages and sugar contents in DPPH assay: (A) Bael fruit drink, (B) Chrysanthemum drink, (C) Ginger drink, (D) Lemongrass drink, (E) Roselle drink, (F) Sacred lotus root drink. [1]Pearson's correlation coefficient (r)

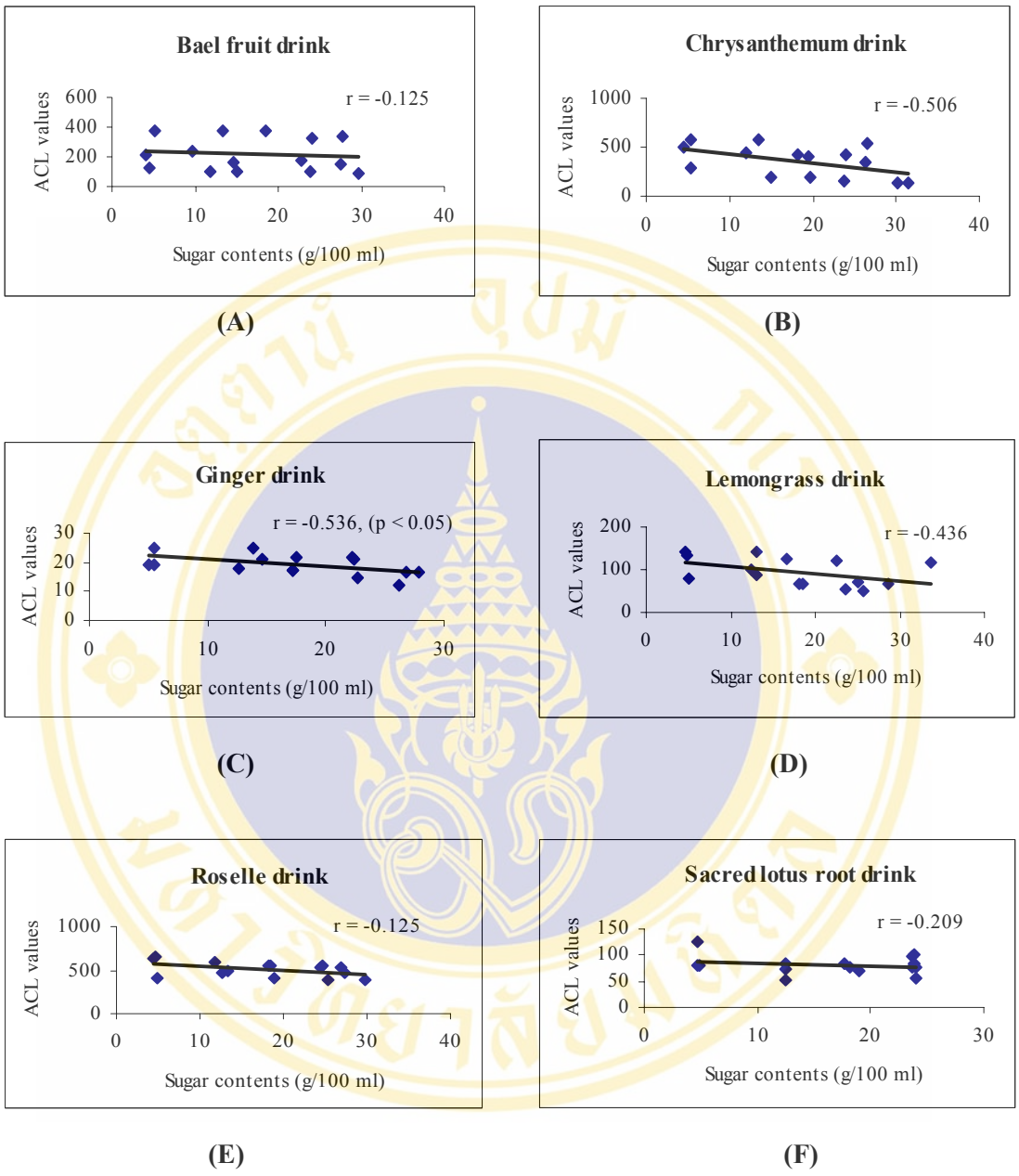


Figure 4-9. The correlation[1] between ACL values of selected pasteurized beverages and sugar contents in PCL assay: (A) Bael fruit drink, (B) Chrysanthemum drink, (C) Ginger drink, (D) Lemongrass drink, (E) Roselle drink, (F) Sacred lotus root drink. [1]Pearson's correlation coefficient (r)

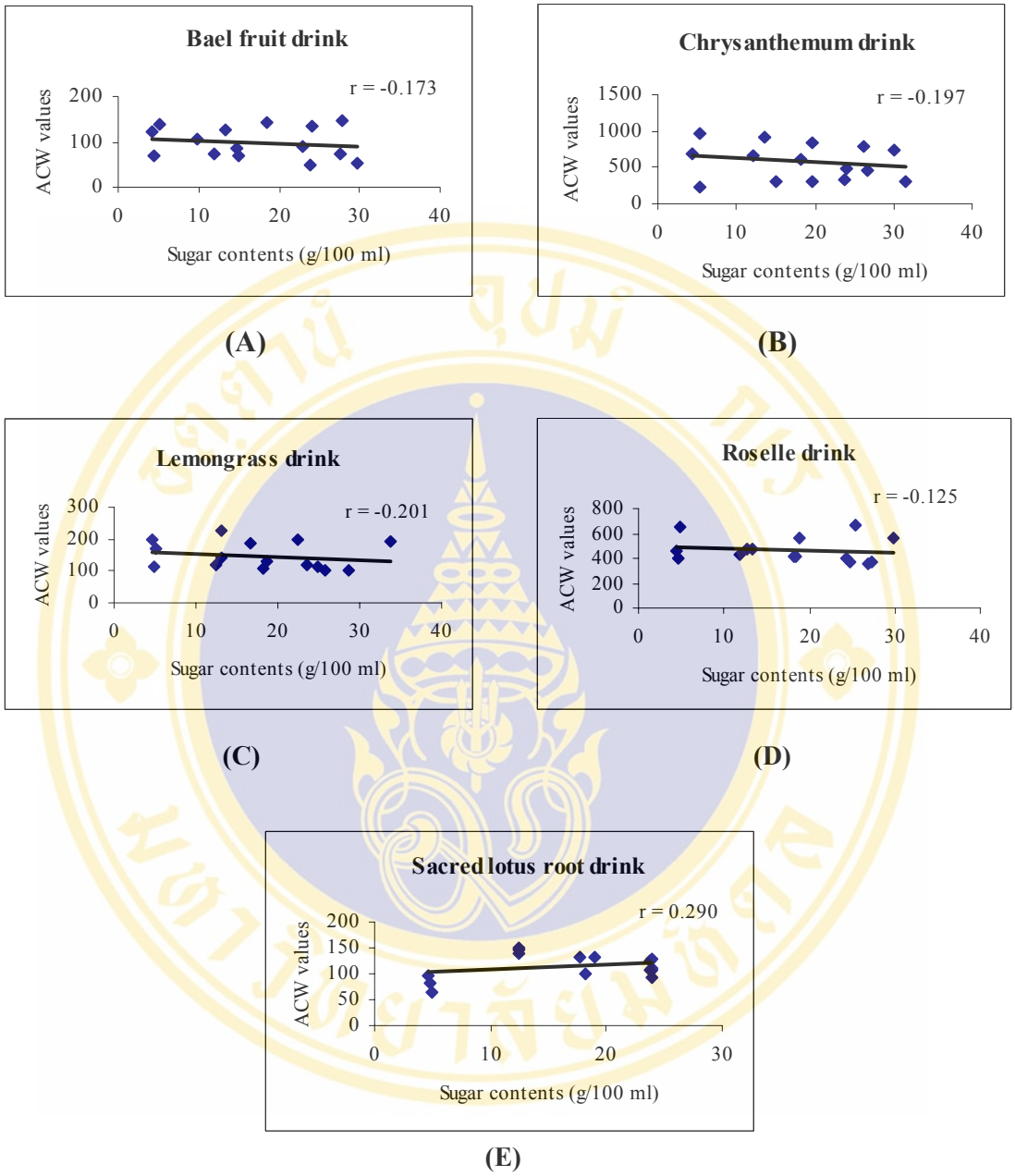


Figure 4-10. The correlation[1] between ACW values of selected pasteurized beverages and sugar contents in PCL assay: (A) Bael fruit drink, (B) Chrysanthemum drink, (C) Lemongrass drink, (D) Roselle drink, (E) Sacred lotus root drink, not detectable for Ginger drink. [1]Pearson's correlation coefficient (r)

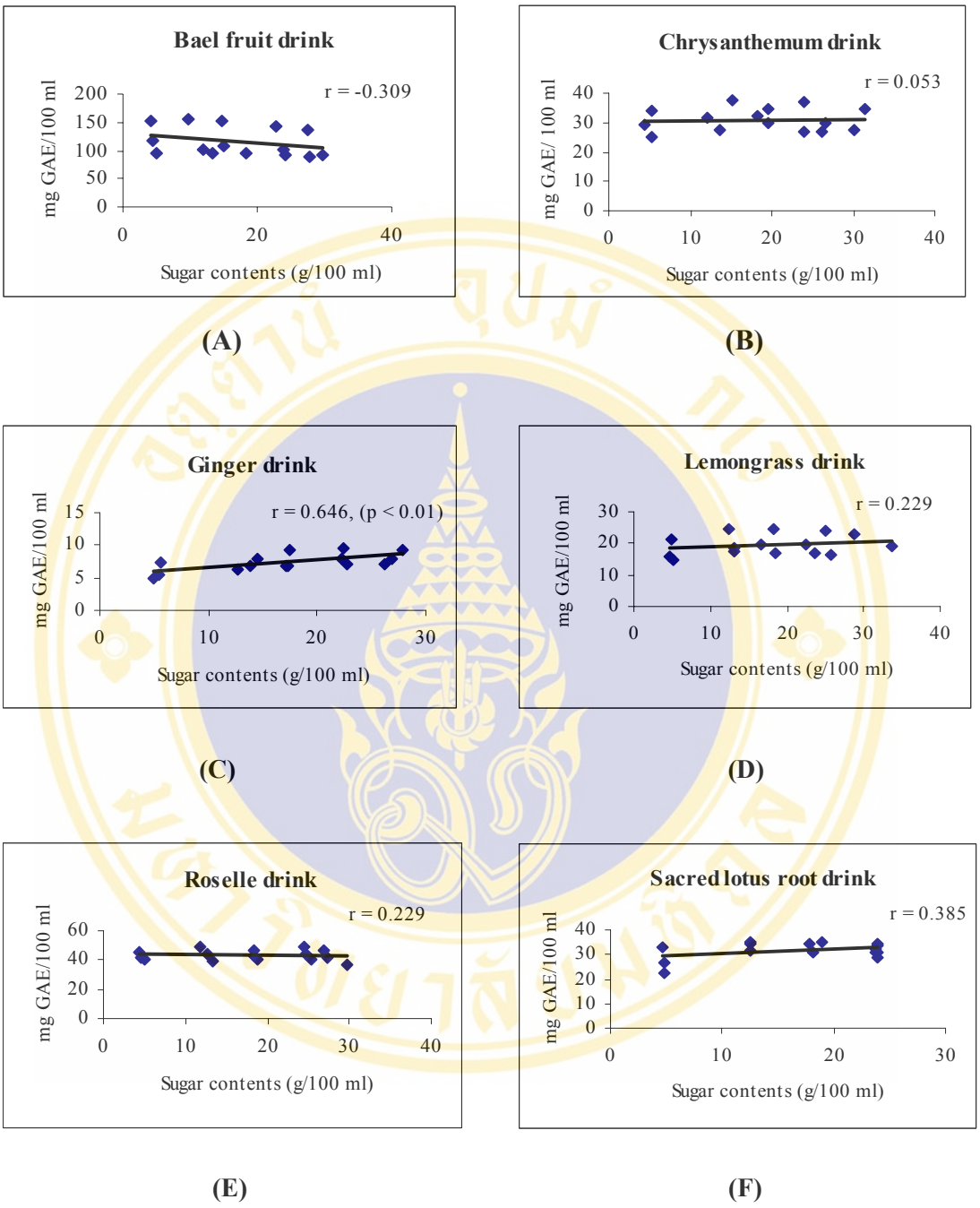


Figure 4-11. The correlation[1] between total phenolic compounds of selected pasteurized beverages and sugar contents: (A) Bael fruit drink, (B) Chrysanthemum drink, (C) Ginger drink, (D), Lemongrass drink, (E) Roselle drink, (F) Sacred lotus root drink. [1]Pearson's correlation coefficient (r)

4.3 Sensory evaluation of selected pasteurized beverages

The consumer evaluation consisted of 30 volunteers (n = 30) in each test of beverages. Panelists were general consumers with age range from 20 to 65 yr. They were investigator's family, neighbors, and community people, postgraduate students and staffs at Ramathibodi hospital. Although, the second sensory test was assessed by 23 panelists from early test and 7 new volunteers, there were no significant differences in age and gender of the panelists between two sensory tests. The initial beverage Brix was 6 °Brix for chrysanthemum drink and 7 °Brix for bael fruit drink, roselle drink, and ginger drink, according to the lowest beverage Brix of pasteurized juice produced by the pasteurized fruit juice plant of the Royal Chitralada Projects, which suggested from the most of panelists (by about 50% of all panelists) after the first sensory test that the sweetness of beverages should be increased. Therefore all of the beverages slightly increased Brix up to 8 °Brix for chrysanthemum drink and 9 °Brix for bael fruit drink, roselle drink, and ginger drink and sensory evaluation were repeated. There was no significant difference between the two sensory evaluations in each pasteurized beverage for age of the panelists group in the study. As shown in **Table 4-5**, the score between 6 and 7 from a nine – point hedonic scale indicate that the beverages were rated as "like slightly" to "like moderately". Therefore, all beverages were well accepted by the panelist after increase level of sweetness from 6 – 7 °Brix to 8 – 9 °Brix with significantly different score, especially in taste. Only ginger drink were significantly improved all of characteristic scores.

In addition, the total sugar contents and estimated calories in the samples were determined as shown in **Table 4-6.** The estimated calories of all beverages of the two different beverages Brix values were ranged between 70 – 97 kcal/serving.

Table 4-5. Sensory evaluation for consumer's acceptance in each characteristics of selected pasteurized beverages

Type of beverage	Beverage Brix	Score of consumer's preference[1]				
		Color	Odor	Taste	Texture	Overall
1. Chrysanthemum drink	6	6.6 ± 1.4	6.1 ± 1.5	6.5 ± 1.3	6.3 ± 1.7	7.0 ± 1.4
	8	7.2 ± 1.2	6.8 ± 1.3*	7.4 ± 0.9*	7.2 ± 0.9*	7.6 ± 1.0
2. Bael fruit drink	7	7.1 ± 0.9	6.5 ± 1.6	6.7 ± 1.6	6.6 ± 1.1	7.0 ± 1.2
	9	7.1 ± 1.3	7.0 ± 1.0	7.7 ± 0.8*	7.2 ± 0.9*	7.5 ± 0.7
3. Roselle drink	7	7.2 ± 1.7	5.6 ± 1.8	5.9 ± 1.7	6.1 ± 1.8	6.6 ± 1.5
	9	7.2 ± 1.1	6.5 ± 1.1*	6.9 ± 1.4*	6.5 ± 1.4	7.1 ± 1.0
4. Ginger drink	7	5.8 ± 1.8	5.8 ± 1.6	6.0 ± 1.8	5.5 ± 1.6	6.0 ± 1.4
	9	7.0 ± 1.2*	7.1 ± 1.5*	7.9 ± 0.9*	7.4 ± 1.1*	7.6 ± 0.8*

[1]All data are mean ± SD (n=30)
* Significantly different between two level of sweetness for sensory evaluation in each beverage at $p < 0.05$

Table 4-6. Total sugar contents and estimated calories of selected pasteurized beverages for sensory evaluation[1]

Type of beverage	Beverage Brix	Total sugar contents (g/serving)[2]	Estimated calories (Kcal/serving)[3]
1.Chrysanthemum drink	6	17.59 ± 1.32	70.34 ± 5.30
	8	22.45 ± 0.04	89.78 ± 0.16
2.Roselle drink	7	19.44 ± 0.27	77.77 ± 1.10
	9	23.89 ± 1.59	95.57 ± 6.35
3 Bael fruit drink	7	19.61 ± 0.52	78.42 ± 2.06
	9	24.23 ± 0.59	96.93 ± 2.38
4.Ginger drink	7	20.03 ± 0.38	80.11 ± 1.52
	9	23.93 ± 0.86	95.70 ± 3.43

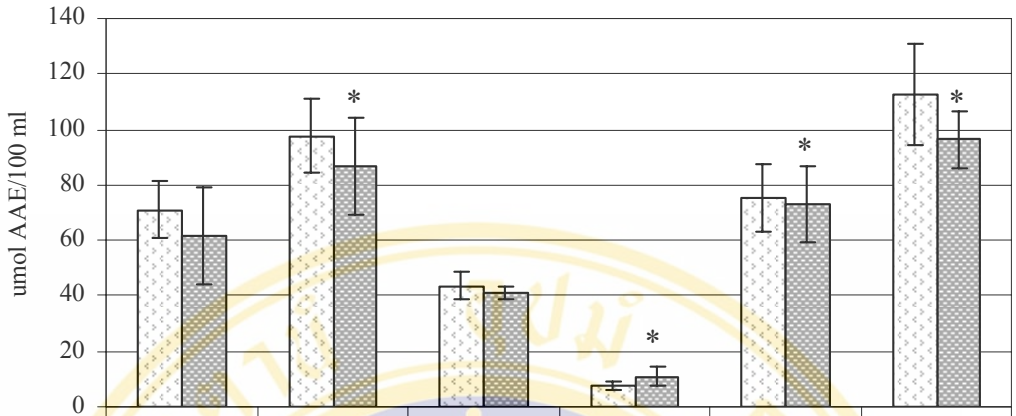
[1]All data are mean ± SD (n = 3)
[2] Analyzed by using Nelson's reducing sugar test
[3]Estimated total calories calculated from 1 gram glucose provides 4 kcal, 1 serving = 240 ml

4.4 Antioxidant capacity and total phenolic compounds during storage in selected pasteurized and sterilized beverages

4.4.1 Antioxidant capacity and total phenolic compounds during storage in selected pasteurized beverages

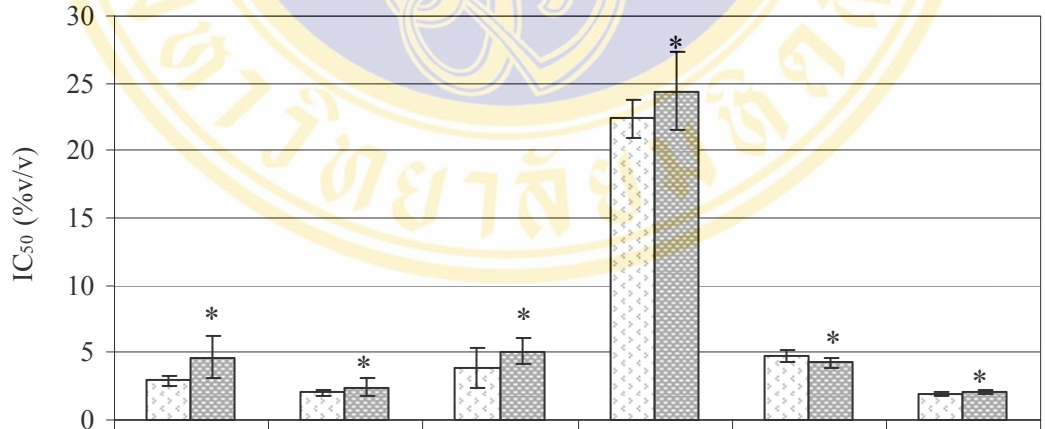
The DPPH antioxidant capacity changes in selected pasteurized beverages during storage at 5°C in refrigerator are shown in **Figures 4-12 and 4-13.** All of beverages showed significant decrease in the antioxidant capacity equivalent to ascorbic acid, except bael fruit drink and lemongrass drink. However, there were significant changes by increasing the IC_{50} values among the samples, but only roselle drink was found decreasing. According to the PCL, significant decrease in ACL values were found in bael fruit drink, lemongrass drink, ginger drink and roselle drink after 7 days of storage (**Figure 4-14**). In contrast, the ACW values of bael fruit drink and lemongrass drink were significantly increased, only that of sacred lotus root drink decreased significantly (**Figure 4-15**).

The significant decrease of total phenolic compounds after 7 days of storage among the samples were found in bael fruit drink, lemongrass drink, ginger drink, and roselle drink (**Figure 4-16**).



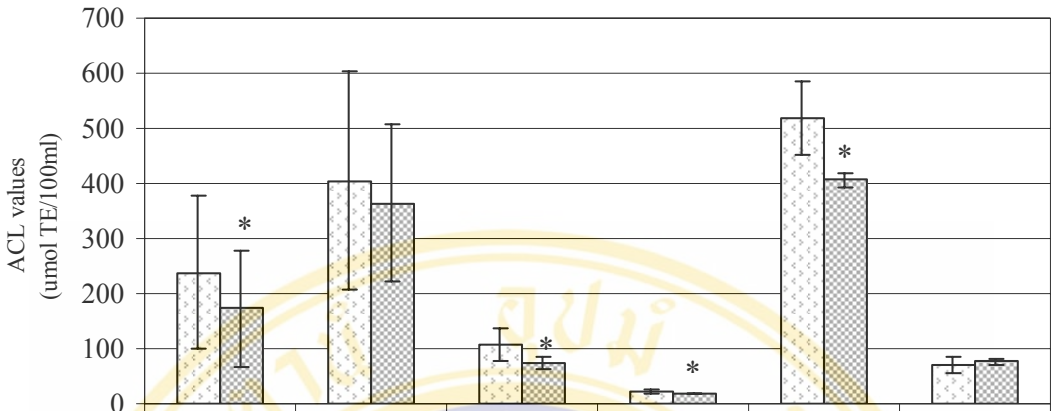
	Bael fruit drink	Chrysanthemum drink	Lemongrass drink	Ginger drink	Roselle drink	Sacred lotus root
0 day	71.11	97.70	43.73	7.59	75.16	112.53
7 days	61.30	86.40	41.02	11.02	73.32	96.56

Figure 4-12. Comparison of antioxidant capacity equivalent to ascorbic acid in selected pasteurized beverages for 0 and 7 days of storage by using DPPH assay. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars. * Significantly different at $p < 0.05$



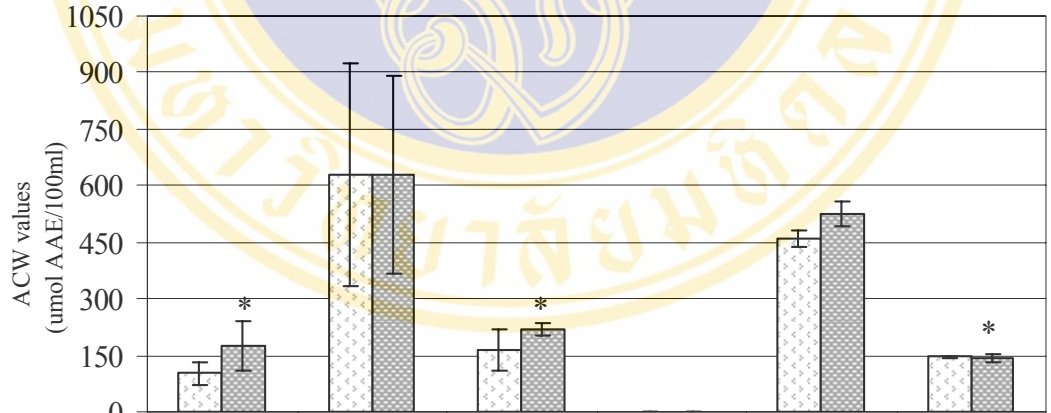
	Bael fruit drink	Chrysanthemum drink	Lemongrass drink	Ginger drink	Roselle drink	Sacred lotus root
0 day	2.90	2.01	3.86	22.37	4.71	1.88
7 days	4.66	2.45	5.11	24.42	4.30	2.04

Figure 4-13. Comparison of IC_{50} values in selected pasteurized beverages for 0 and 7 days of storage by using DPPH assay. 50% inhibitory concentrations: the concentration of antioxidant that causes 50% loss of the DPPH radical. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars.
* Significantly different at $p < 0.05$



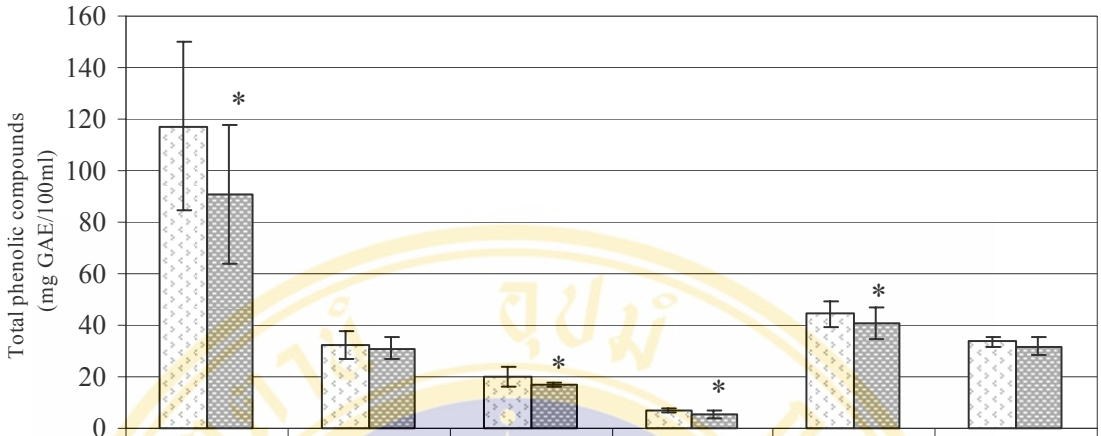
	Bael fruit drink	Chrysanthemum drink	Lemongrass drink	Ginger drink	Roselle drink	Sacred lotus root drink
0 day	237.45	404.06	108.58	21.36	518.81	69.68
7 days	173.59	363.84	74.48	18.15	405.58	76.03

Figure 4-14. Comparison of ACL values in selected pasteurized beverages for 0 and 7 days of storage by using PCL assay. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars. * Significantly different at $p < 0.05$



	Bael fruit drink	Chrysanthemum drink	Lemongrass drink	Ginger drink	Roselle drink	Sacred lotus root
0 day	101.43	629.23	161.90	0.00	459.43	145.01
7 days	174.74	628.79	219.65	0.00	525.30	140.01

Figure 4-15. Comparison of ACW values in selected pasteurized beverages for 0 and 7 days of storage by using PCL assay. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars. * Significantly different at $p < 0.05$



	Bael fruit drink	Chrysanthemum drink	Lemongrass drink	Ginger drink	Roselle drink	Sacred lotus root drink
0 day	117.15	32.29	20.28	7.01	44.28	33.68
7 days	90.73	31.03	16.99	5.48	40.58	31.73

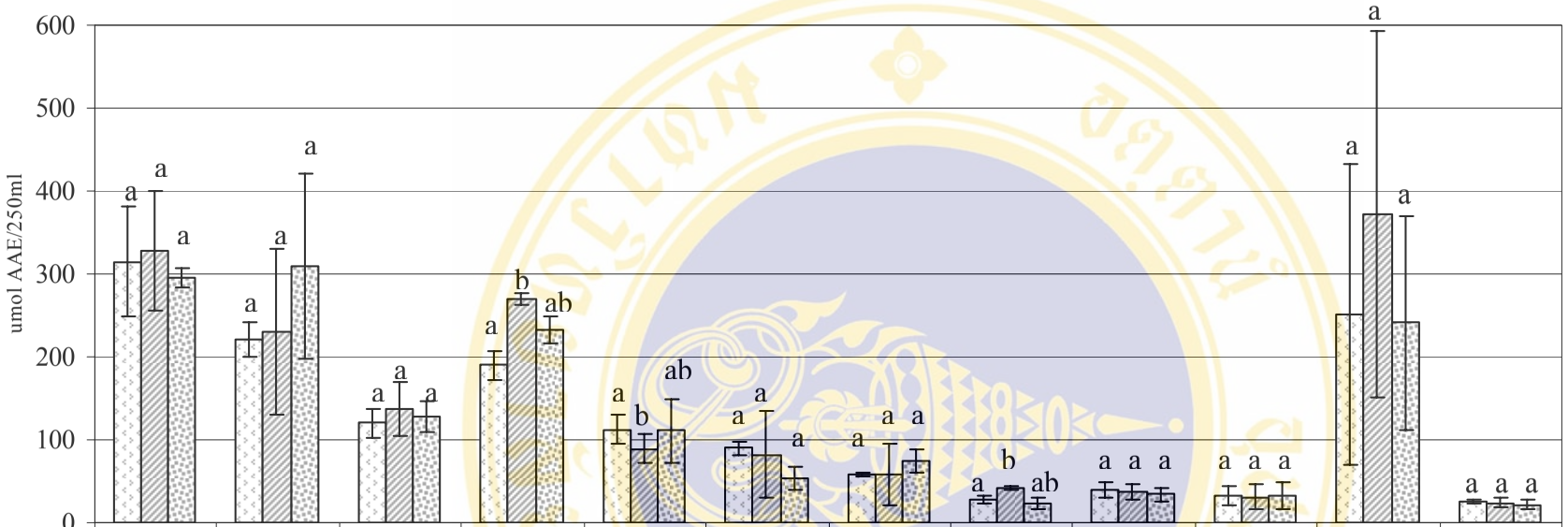
Figure 4-16. Total phenolic compounds of selected pasteurized beverages for 0 and 7 days of storage. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars. * Significantly different at $p < 0.05$

4.4.2 Antioxidant capacity and total phenolic compounds during storage in selected sterilized beverages

After storage at 0, 1, and 3 months at room temperature of sterilized beverages, there were the significant changes in the DPPH antioxidant capacity equivalent to ascorbic acid during storage of mango juice and tamarind juice by significant increase after 1 month of storage in mango juice, lemongrass drink, and pineapple juice (**Figure 4-17**). Only mango juice had significant decrease in the IC_{50} values after 3 months of storage (**Figure 4-12**). ACL values of orange juice was significantly decreased after 1 month of storage and ginger drink had significant differently decrease between 1 and after 3 months of storage as shown in **Figure 4-19**. For ACW values, pineapple with passion fruit juice was significantly increased between 1 and after 3 months of storage whereas that of tamarind juice was found significant increase between 0 and after 3 months of storage as shown in **Figure 4-20**.

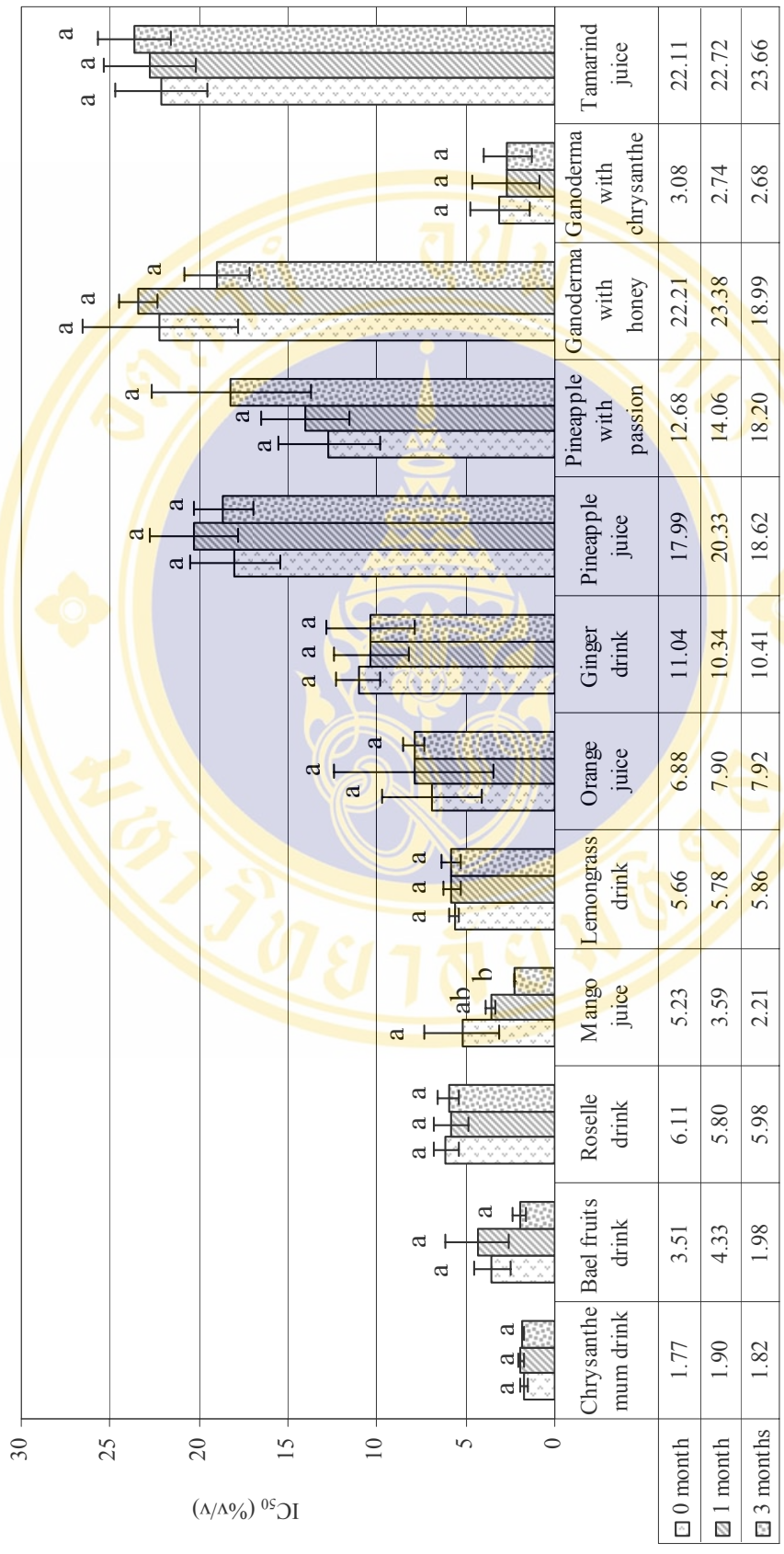
Total phenolic compounds were significantly increased in bael fruit drink between 1 and 3 months of storage. In contrast, significant decrease of those compounds between 1 and 3 months of storage was found in mango juice (**Figure 4-21**).

In addition, the samples were determined for total sugar contents and estimated calories as shown in **Table 4-7**. Ganoderma with honey drink had the lowest sweetness (8.1 ± 0.1 °Brix) and also showed the significantly lowest total sugar contents (19.0 ± 4.8 g D-glucose/serving) and estimated calories (75.9 ±19.3 kcal/serving), while tamarind juice had the highest sweetness (14.5 ± 0.5 °Brix) and showed the significantly highest values of total sugar contents (45.3 ± 3.1 g D-glucose/serving) and estimated calories (181.1 ± 12.3 kcal/serving).



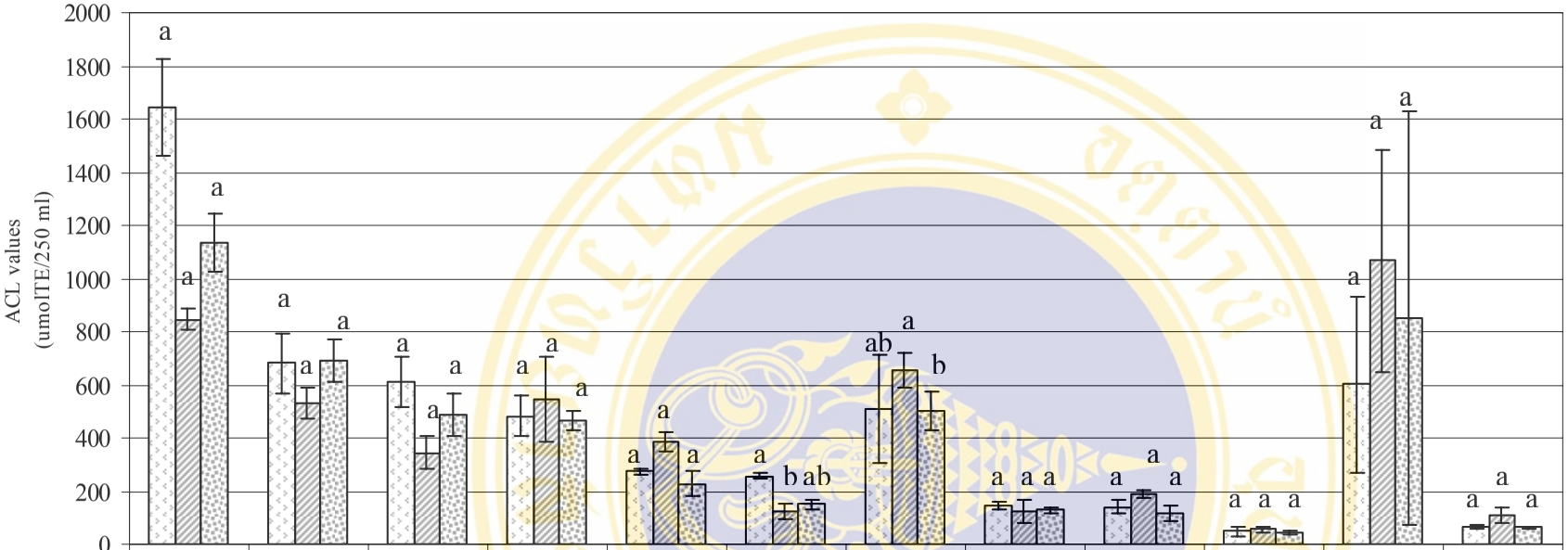
	Chrysanthemum drink	Bael fruits drink	Roselle drink	Mango juice	Lemongrass drink	Orange juice	Ginger drink	Pineapple juice	Pineapple with passion	Ganoderma with honey drink	Ganoderma with chrysanthe	Tamarind juice
0 month	315.08	220.89	120.53	190.00	112.39	89.75	58.06	27.63	39.04	33.18	251.16	25.87
1 month	327.86	230.47	136.52	269.93	89.42	82.01	57.95	42.17	37.37	31.19	371.88	24.07
3 months	295.38	309.63	127.97	232.32	110.52	53.54	73.47	23.83	33.87	31.62	241.57	21.64

Figure 4-17. Comparison of antioxidant capacity equivalent to ascorbic acid in selected canned beverages between 0, 1, and 3 months of storage, using DPPH assay. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars. Values with the same letters in each sample are not significantly different at $p < 0.05$



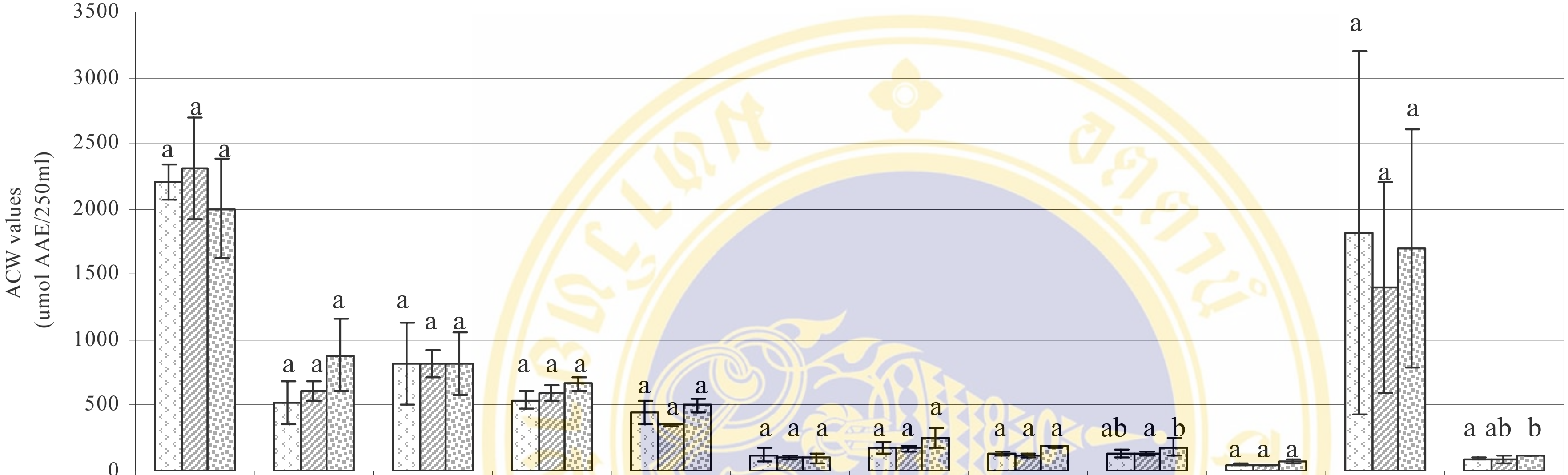
	Chrysanthemum drink	Bael fruits drink	Roselle drink	Mango juice	Lemongrass drink	Orange juice	Ginger drink	Pineapple juice	Pineapple with passion	Ganoderma with honey	Ganoderma with chrysanthe	Tamarind juice
□ 0 month	1.77	3.51	6.11	5.23	5.66	6.88	11.04	17.99	12.68	22.21	3.08	22.11
▨ 1 month	1.90	4.33	5.80	3.59	5.78	7.90	10.34	20.33	14.06	23.38	2.74	22.72
⊡ 3 months	1.82	1.98	5.98	2.21	5.86	7.92	10.41	18.62	18.20	18.99	2.68	23.66

Figure 4-18. Comparison of IC_{50} values in selected canned beverages between 0, 1 and 3 months of storage, using DPPH assay. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars. Values with the same letters in each sample are not significantly different at $p < 0.05$



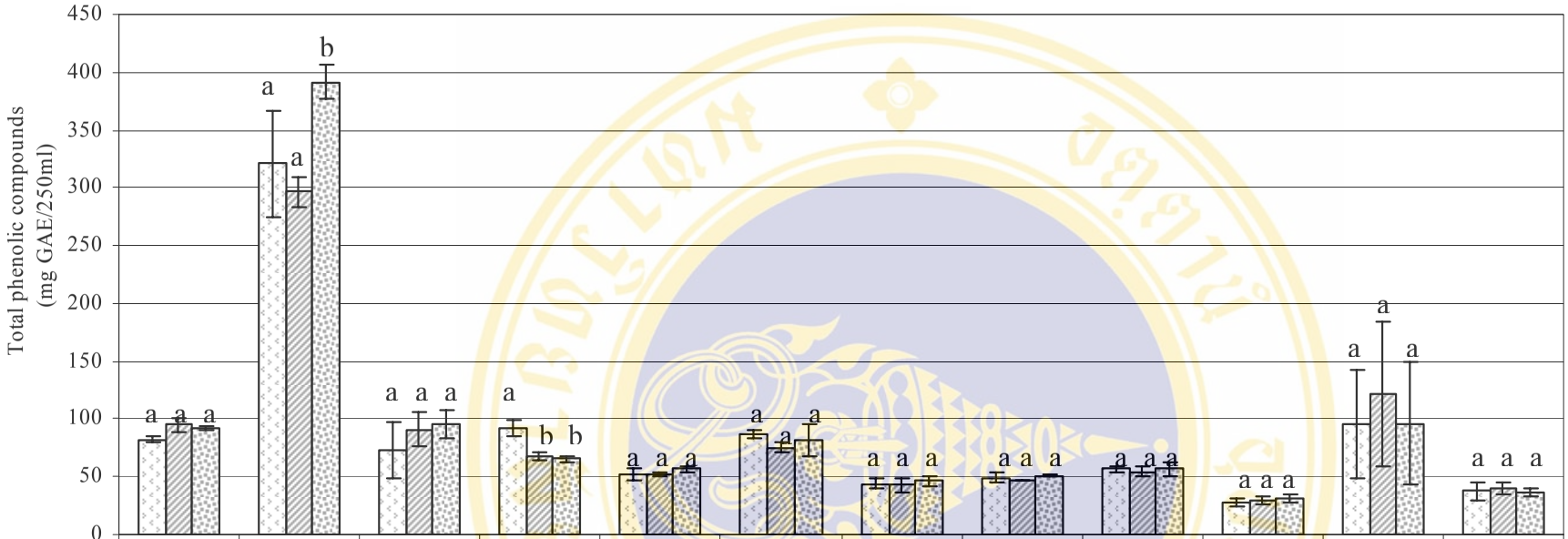
	Chrysanthemum drink	Bael fruits drink	Roselle drink	Mango juice	Lemongrass drink	Orange juice	Ginger drink	Pineapple juice	Pineapple with passion	Ganoderma with honey drink	Ganoderma with chrysanthe	Tamarind juice
0 month	1643.23	681.89	608.72	482.86	272.90	255.83	509.14	142.55	139.20	47.35	600.01	68.88
1 month	845.40	530.98	344.34	544.71	386.83	122.33	653.82	126.50	191.03	56.65	1066.56	108.50
3 months	1131.61	690.59	487.96	463.31	226.73	149.11	500.24	127.97	117.76	45.76	849.17	63.14

Figure 4-19. Comparison of ACL values in selected canned beverages between 0, 1 and 3 months of storage, using PCL assay. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars. Values with the same letters in each sample are not significantly different at $p < 0.05$



	Chrysanthemum drink	Bael fruits drink	Roselle drink	Mango juice	Lemongrass drink	Orange juice	Ginger drink	Pineapple juice	Pineapple with passion	Ganoderma with honey	Ganoderma with chrysanthe	Tamarind juice
0 month	2206.12	523.66	819.68	540.91	446.58	125.17	180.18	133.39	129.93	51.78	1815.56	90.35
1 month	2309.98	609.51	821.68	589.85	354.41	100.45	172.10	123.45	138.02	47.10	1401.06	89.80
3 months	1999.66	884.39	817.39	667.20	500.72	99.54	250.68	187.77	180.98	71.57	1701.37	122.69

Figure 4-20. Comparison of ACW values in selected canned beverages between 0, 1 and 3 months of storage, using PCL assay. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars. Values with the same letters in each sample are not significantly different at $p < 0.05$



	Chrysanthemum drink	Bael fruits drink	Roselle drink	Mango juice	Lemongrass drink	Orange juice	Ginger drink	Pineapple juice	Pineapple with passion	Ganoderma with honey	Ganoderma with chrysanthe	Tamarind juice
0 month	82.24	321.14	72.61	91.81	52.34	86.48	44.09	49.38	56.85	27.90	95.91	37.87
1 month	95.22	296.82	90.72	67.70	52.89	75.44	42.71	47.06	54.31	28.87	121.72	39.63
3 months	92.07	391.76	95.69	65.63	56.47	81.80	46.21	50.90	56.92	31.00	95.99	36.61

Figure 4-21. Comparison of total phenolic compounds in selected canned beverages between 0,1 and 3 months of storage. All analytical data are mean values of three independent samples (n=3) with standard deviations shown by vertical bars. Values with the same letters in each sample are not significantly different at $p < 0.05$

Table 4-7. Total sugar contents and estimated calories of canned beverages[1]

Type of beverage	Beverage Brix	Total sugar contents (g/serving)	Estimated calories[2] (kcal/serving)
1. Chrysanthemum drink	11.0 ± 0.0	33.1 ± 4.4	132.3 ± 17.8 [bc]
2. Bael fruits drink	11.5 ± 0.4	27.6 ± 3.1	110.5 ± 12.4 [bc]
3. Roselle drink	14.7 ± 0.7	37.0 ± 5.0	147.9 ± 19.9 [ab]
4. Mango juice	14.0 ± 0.0	35.0 ± 6.6	140.0 ± 26.2 [abc]
5. Lemongrass drink	11.7 ± 0.5	34.4 ± 4.6	137.7 ± 18.3 [abc]
6. Orange juice	14.2 ± 0.3	38.1 ± 5.8	152.4 ± 23.4 [ab]
7. Ginger drink	11.7 ± 0.5	29.2 ± 3.5	116.8 ± 13.9 [abc]
8. Pineapple juice	14.1 ± 0.1	39.0 ± 5.2	156.0 ± 20.9 [ab]
9. Pineapple with passion fruit juice	14.1 ± 0.1	41.8 ± 11.7	167.1 ± 46.9 [ab]
10. Ganoderma with honey drink	8.1 ± 0.1	19.0 ± 4.8	75.9 ± 19.3 [c]
11. Ganoderma with chrysanthemum drink	9.9 ± 0.1	25.3 ± 0.5	101.0 ± 1.9 [bc]
12. Tamarind juice	14.5 ± 0.5	45.3 ± 3.1	181.1 ± 12.3 [a]

[1] All analytical data are mean values of three independent samples (n=3) ± SD
[2] Estimated total calories calculated from 1 gram glucose provides 4 kcal, 1 can = 250 ml
Values with different letters (a – c) within column of each sample are significantly different at $p < 0.05$

CHAPTER V
DISCUSSION

5.1 Antioxidant capacity and total phenolic compounds of pasteurized and sterilized beverages

There is little evidence about antioxidant capacity and total phenolic compounds in fruit juices and herbal drinks of Thailand. Our study is the first study to investigate antioxidant properties of commonly consumed pasteurized and sterilized health beverages, produced by the pasteurized fruit juice plant and the fruit juice cannery under The Royal Chitralada Projects.

Because the different reactions and mechanisms of the measurement of antioxidant capacity, we use two methods as DPPH and PCL assay with different modes of actions in this study to evaluate the antioxidant capacity depending on the antioxidant potential expected and perhaps on the origin of substances in studied beverages (53,55). DPPH assay is simple, rapid and needs only a UV-vis spectrophotometer to perform while PCL assay is involved with superoxide radical, which is a deleterious by-product of oxygen metabolism, occurring in human body. By using PCL assay, the samples can be measured as lipophilic and hydrophilic antioxidants in nanomolar.

Different kinds of beverages were tested by DPPH and PCL methods. Although, the results showed significant correlation between the values of TE in DPPH assay and ACL in PCL assay ($r = 0.654$) as well as the values of AAE and ACW ($r = 0.661$) but PCL assay showed higher antioxidant capacity values as equivalent to trolox and ascorbic acid by about 2 – 6 and 1 – 9.7 times than DPPH assay, respectively. In addition, from all beverages, sacred lotus root drink showed the significantly highest amount of antioxidant capacity in both equivalent to trolox and ascorbic acid but not in PCL test. In contrast, chrysanthemum drink and roselle drink showed the significantly highest antioxidant capacity of both ACL and ACW values.

This is not unexpected in that two completely different radical sources are being evaluated. This may due to PCL assay has more sensitivity than DPPH assay, as described by Prior et al. (2005) and Huang et al. (2005). Since DPPH assay is not a competitive reaction because DPPH is both radical probe and oxidant. DPPH color can be lost via hydrogen atom transfer reaction (HAT) or single electron transfer reaction (SET) as well as unrelated reaction; steric accessibility of the DPPH radical site is a major determinant of the reaction, small antioxidants molecules that have better access to the radical site have higher apparent antioxidant capacity with this assay. Furthermore, many antioxidants that quickly react with peroxyl radicals, they may also react slowly or may even be inert to DPPH because DPPH is stable nitrogen radical that no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation (52,53,36). On the other hand PCL assay is combination of the simple and reliable photochemical generation of superoxide radical ($O_2^{\bullet -}$) with their very sensitive chemiluminometric detection. Compared to standard conditions, the oxidative reaction is accelerated by a factor of 1000. These results in a reduction in measuring times by a factor of 10 – 1000 compared to other methods (59-62).

As the results of total phenolic compounds in pasteurized and sterilized beverages, bael fruit drink had the significantly highest total phenolic compounds, but not highest in antioxidant capacity of both DPPH and PCL assays, while lemon juice had the lowest antioxidant capacity values of both DPPH and PCL assays and also contained the lowest total phenolic compounds. This can be explained on the basis of chemical characteristics of Folin-Ciocalteu reagent, using for the total phenols assay, a routine assay in studying phenolic antioxidants, which is nonspecific to phenolic compounds as it can be reduced by many nonphenolic compounds such as ascorbic acid, sugars, aromatic amines, sulfur dioxide, organic acid, Cu (I), and Fe (II), etc., although they are not effective radical scavenging antioxidants. Moreover, this assay have been unable to determine the total phenols of the lipophilic antioxidants because of Folin-Ciocalteu reagent is carried out only in water, an aqueous phase (52,53). However, there were significant correlation between the total phenolic compounds and the values of TE, AAE, and IC_{50} in DPPH assay ($r = 0.537$, 0.574, and -0.451, respectively), as well as significant correlation with ACL and ACW ($r = 0.459$ and 0.417).

From this study, sacred lotus and lemongrass drink showed the significant lowest estimated calories (95.2-90.7 kcal/serving) whereas pineapple and tamarind juice showed the highest estimated calories (149.7-151.8 kcal/serving). When compared to the sweetened beverage from common fruits, herbs, and tea collected from several cafeterias around Ramathibodi Hospital, all beverages in this study had lower estimated calories than those sweetened beverages by about 1.9 – 2.4 times (76 – 127 compared to 183 – 243 kcal/serving, respectively, 1 serving = 200 mL) (17). This finding shows that the beverage, produced from The Royal Chitralada Projects, which are well known and popular and also high total sales and productions, had lower calories than general consuming beverages. Therefore it is not necessary to fortify health beverages with high amount of sugar to enhance consumer acceptance.

5.2 The correlation between fortified sugar and antioxidant capacity and total phenolic compounds of selected pasteurized beverages

From the screening of antioxidant capacity and total phenolic compounds of 12 pasteurized and sterilized beverages, only 6 pasteurized beverages were selected to fortified sugar in ascending Brix as 0, 10, 15, 20, and 25 °Brix, respectively, according to the preliminary survey in the same sweetened beverages which found beverage Brix of those beverages range between 14 – 28.4 °Brix. To our knowledge, this is the first study to prove the relationship between fortification of sugar in pasteurized health beverages and the stability of antioxidant capacity and total phenolic compounds. Because there is little evidence to support this correlation and only two studies were shown. The first study from Chen et al. (2001) found that green tea catechins (GTC) added in commercial available soft drinks (Coca-Cola, 7-up, and Pepsi) degraded up to 45% in 7-up for 6 months and completely degraded in Pepsi and Coca-Cola only for 4 months (28). The last study from Tolcott et al. (2003) found that pasteurized yellow passion fruit juice fortified with 10% sucrose lowered antioxidant capacity values on average throughout processing and during storage in the first 14 days (28). For our study, results from both assays showed decreasing trends in antioxidant capacity with increasing trends of sugar contents in all selected pasteurized beverages. With DPPH assay, significantly inverse correlation between the values of antioxidant capacity and

sugar contents were observed in all beverages, except sacred lotus root drink. Unfortunately, with PCL assay, significant inverse relationship was found only in ginger drink between ACL values and the level of beverage Brix.

For the correlation between the increasing amount of sugar contents and total phenolic compounds, no significant changes were observed among the samples, except ginger drink had significant correlation between total phenolic compounds and sugar contents ($r = 0.646$). Likewise the previous study, Chen et al. (2001) reported that no effect of dissolved sucrose solution (0.15 g/mL) with GTC (classified as one of polyphenolic compounds) on stability of antioxidant capacity as compared to control samples (dissolved in distilled water) (29).

This study gives the primary data that evaluate the antioxidant capacity and total phenolic compounds of some health beverages with increasing amount of fortified sugar in the control condition. It seems likely that sugar might affect the stability of antioxidant capacity in beverages, including bael fruit drink, chrysanthemum drink, ginger drink, lemongrass drink and roselle drink with level of this inverse correlation (r) ranged from -0.6 to -0.9.

Although sugar may not show strong correlation with antioxidant capacity, but this findings could be useful for manufacturer to improve their products quality with high in nutritional quality and low calories by fortification of sugar in appropriate amount in commercial health beverages, and also disseminate to general consumers choosing fruit or herb beverages for improving their antioxidant status with low calories but high content of antioxidants. This information is very important because recently results from the study of Schulze et al. (2004) (18) have showed that higher consumption of sugar-sweetened beverages is associated with a greater magnitude of weight gain and an increased risk for development of type 2 diabetes in women, possibly by providing excessive calories and large amounts of rapidly absorbable sugars. Increased sugar-sweetened soft drink consumption and also fruit punch were the dependent risk factors. Look back to Thai population, Thai people had trend to eat more sweets with excessive amount of adding sugar from processed food and beverages, especially from beverages such as soft drink and fruit juices, in the meantime, the trend of prevalences of obesity and type 2 diabetes also have been increasing every year too. It should be noted that an increase in sugar-added beverages

leads to greater caloric consumption, therefore a decrease intake of these beverages or the reduced amount of adding sugar in beverages should be part of the solution and may help to secure the stability of bioactive components in them.

Nevertheless, significant linear correlation between estimated calories and the increasing level of beverage Brix was shown at high level, $R^2 = 0.969$ ($y = 3.8731x + 15.262$, $p < 0.05$). Therefore we can use beverage Brix as an indicator to estimate total calories in sweetened beverages. This may be helpful for small and medium manufacturers to consider about calories in their beverages when fortification of sugar in processing of beverages.

5.3 Finding out an appropriated amount of fortified sugar in selected pasteurized beverages

From data in part one, the products of The Royal Chitralada Projects showed estimated calories range between 91 – 152 kcal/serving, which contained sugar about 23 – 38 g/serving and also exceed about 1.6 times of the daily sugar intake recommended by Thai kids don't eat sweets campaign (not exceed 6 tsp or 24 g/d) (16). Only sacred lotus root drink and lemongrass drink were in recommended range. Therefore, 4 pasteurized beverages (chrysanthemum, roselle, bael fruit and ginger drink) were selected to find out an appropriate amount of fortified sugar with at minimum Brix and well acceptance in taste for general consumers.

Based on the lowest Brix of pasteurized beverages of The Royal Chitralada Projects which were more popular consumed by foreigners than Thais (data from the Research and Development units of The Royal Chitralada Projects), the studied beverages were tested for consumer acceptance with fortified sugar at 6 °Brix for chrysanthemum drink and 7 °Brix for roselle, ginger, and bael fruit drink and found that the rate of preference score from a nine – point hedonic scale as "like slightly" (score = 6) for taste, but there were suggestion from most of our panelist about the taste of all beverages which had less sweets and should be added more sugar. Therefore, the studied beverages were repeately tested for consumer acceptance with slightly increase fortified sugar up to 8 °Brix for Chrysanthemum drink and 9 °Brix

for roselle, ginger, and bael fruit drink and got better score as “like moderately” (Score = 7) for taste and also showed significantly improvement of the consumer acceptance in taste for all beverages between two tests. In theory, the degree of preference of nine-point hedonic scale with the strongly preferred is rated as 7 or “like moderately” (106). This is a good scientific evidence to introduce general manufacturers to reduce the sweetness of their beverage products because it is not necessary to fortify high amount of sugar to enhance consumer preference for increasing total sale of beverages. In addition, the decrease of added sugar in beverages may lead to the reduction of the capital investment of beverage production. On the other hand, this is also useful for the better health of general consumers.

Because caloric sweeteners have been liked to dental caries, increase energy intake, weight gain, and type 2 diabetes (18,81-84), and worldwide population have trend to increase daily energy intake. For Thai population, average sugar intake in the year 2001 (29.0 kg/mo. or 16 tsp/d) has increased about 2.3 times compared to the year 1985 (12.7 kg/y or 7 tsp/d) (14), and it is estimated that the largest source of added sugar comes from sweetened beverages such as soft drink and fruit drink. As a result of our preliminary survey of commonly consumed sweetened beverages from fruits, herbs and tea collected from several cafeterias around Ramathibodi Hospital have found high estimated calories range 220 – 292 kcal/serving (240 ml), contained sugar about 55 – 73 g/serving which 2-3 times higher than recommended by Thai kids don’t eat sweets campaign (24 g/d), and supply more than 10 % of the energy requirement for adult females and males [female and male, age ≥ 19 yr require energy intake 1,750 and 2,150 kcal/d, respectively (Dietary Reference Energy Intake for Thais 2003)] (17). Therefore, reduced intake of caloric beverages that provide no nutrient is needed. Nielson et al. (2004) reported that energy intake from sweetened beverages currently represents by 21 % of the total energy intake for Americans aged > 2 y according to US Dietary Guidelines for Americans and those calorically sweetened beverages has increased from 2.8 % to 7.0 % by about 3-fold between 1977 and 2001 (89). This hot issue lead to recently develop the Beverage Guidance System by Popkin et al. (2006), which ranks beverages in 6 levels, from the least preferred to the most preferred by the panel (Level 6 – beverages that should be consumed in limited quantities to Level 1 – beverages that should be consumed as the major beverage). The

goal of this guidance system is getting beverage intake from very-low-calorie beverage ≥ 80 %, which should be consist of water, unsweetened tea, and coffee and only about 20 % of low-fat milk, juice, alcohol, and calorically sweetened beverages. The recommendation of the panel for calorically sweetened beverages intake is about 0 – 8 fl oz/d or 0 – 237 ml/d (80).

With fortified sugar in minimum amount between 6 - 9 °Brix provides calories about 70 – 97 kcal/serving (240 ml) which is suitable amount intake following the new guideline and also low calorie contents. Therefore, food industry or small and medium enterprises (SME) of sweetened beverages should be consider to reduce calories content of their products from current levels, for example, sweetened beverages from several cafeterias around Ramathibodi Hospital and pasteurized beverages of The Royal Chitralada Projects could be reduced calories contents by 55 – 76 % and 36 – 54 % from current calories, respectively, as well as consumers should be concern their health by choosing and promoting less sweets and low calories content beverages.

5.4 Antioxidant capacity and total phenolic compounds during storage in selected pasteurized and sterilized beverages

There were many studies showed that the storage condition and storage duration can affect the stability of antioxidants in processed foods and beverages, especially pasteurized or sterilized beverages. They were interested in the stability of antioxidant capacity and total phenolic compounds during storage in their shelf life with suitable condition after processing. For products of The Royal Chitralada Projects, pasteurized beverages were recommended to keep in refrigerator at 4 – 5°C with shelf life at least 7 days of storage and sterilized beverages (canned juices) could be keep at room temperature with shelf life for 2 years but normally there were sold out in 3 months, therefore long term storage were observed for 1 and 3 months of storage.

For pasteurized beverages in this study, lower levels of antioxidant capacity equivalent to ascorbic acid (AAE) except ginger drink were found. With DPPH assay, the significant decreased values of AAE (2 – 14 %) after 7 days of storage were

observed in roselle, chrysanthemum, and sacred lotus root drink. With PCL assay, the significant decrease of ACL values were observed in bael fruit, lemongrass, ginger, and roselle drink (about 15 – 23 %), whereas bael fruit, and lemongrass drink had significant increase of ACW values by about 72 % and 36 %, respectively. Interestingly, chrysanthemum drink had a good retention of antioxidant capacity both ACL and ACW values in PCL assy. All of these pasteurized beverages had decreased content of total phenolic compounds with significant change in bael fruit, lemongrass, ginger, and roselle drink by about 8 – 23 %.

This finding showed that pasteurized beverages had changed in the stability of antioxidants during refrigerated storage of their shelf life. The changes of antioxidants stability in pasteurized juice during storage were investigated in several studies with the different results. As the study of Choi et al. (2002) found the completely degradation of ascorbic acid contents in pasteurized blood orange (*Citrus sinensis*) juice after 5 weeks of refrigerated storage at 4.5°C and total anthocyanin also had 25 % decrease with storage time (67). Likewise, Esteve et al. (2005) reported that pasteurized refrigerated Spanish orange juice lost its ascorbic acid more over 50 % during 3 weeks of storage at 4°C (68). Whereas, the study of Miguel et al. (2004) found no significant changes in anthocyanin contents during storage at 4°C for 72 hr or 3 days of pomegranate juice extraction, which a slight increase by about 0.3 % – 4 % in the first 5 hr of storage was observed in the amount of each anthocyanin (69) as well as Turker et al. (2004) found the highest anthocyanin retention at 4°C during storage in pasteurized fermented black carrot beverages (31). In contrast, Del Caro et al. (2004) reported that minimal processed citrus juices showed a decrease in flavonoid and ascorbic acid contents, whereas the trolox equivalent antioxidant capacity (TEAC values) of “red blush” grape juice significantly increased during storage at 4°C for 10 and 15 days**.** This may be due to the higher antioxidant potential of polyphenols in intermediate stages of oxidation (71). Johnson et al. (2002), recommended that ready-to-drink orange juice should be purchased 3 – 4 weeks before the expiration date and consumed within 1 week of opening because they found that ascorbic content was reduced after 4 weeks during storage at 4°C (64). And Patthamakanokporn O. (2004), also recommended that fruit juice should be consumed on the day of preparation or stored in a refrigerator at 5 °C not more than 3

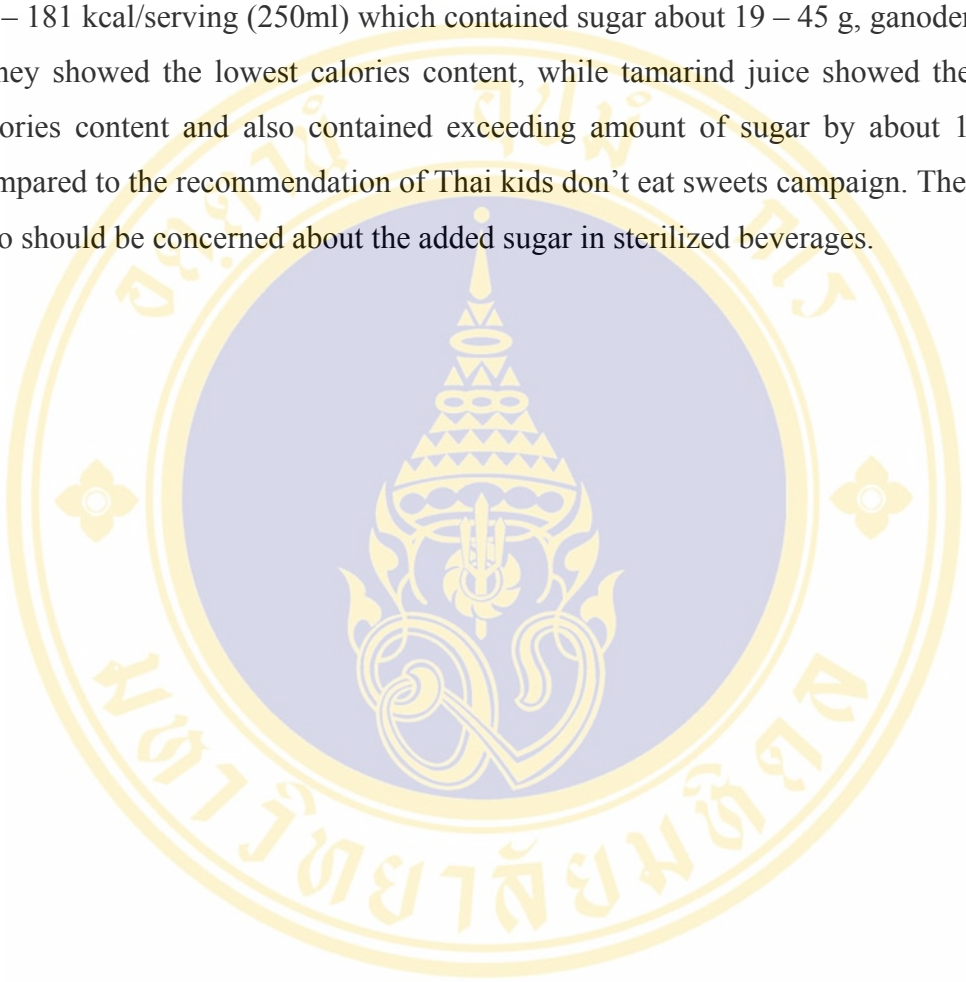
days because antioxidant activity of the juices were found not stable during storage at 5 °C (108). The lost or retention of bioactive components may affect on the decreasing or increasing of antioxidant capacity of pasteurized beverages during their shelf life storage, therefore consumers should be concern about storage time and storage condition of commercial beverages to preserve their nutritional quality and also to get the most nutritional values from them.

In this study, sterilized beverages (12 different kinds) showed a good retention of antioxidant capacity of both two assays and total phenolic compounds after 1 and 3 months during storage at room temperature. The significant increase of antioxidant capacity of AAE after 1 month of storage were found in 3 drinks as followed; mango juice, lemongrass drink, and pineapple juice by about 42 %, 20 %, and 53 %, respectively. In meantime, mango juice also found significant decrease in total phenolic compounds between 1 and 3 months of storage by about 26 % and 29 %, respectively. For PCL assay, the large decreasing of ACL values by about 52 % after 1 month of storage were found in orange juice and also found the decreasing of ACL values by about 23 % between 1 and 3 months of storage in ginger drink. While, ACW values of pineapple with passion fruit juice was significantly decreased by about 31 % of between 1 and 3 months of storage and that of tamarind juice was significantly increased by about 35 % between 0 and 3 months of storage. In addition, total phenolic compounds of only bael fruit drink was significantly increased of after 3 months of storage (increased from 0 and 1 month by about 22 % and 32 %, respectively); however there was no change in antioxidant capacity of both assays.

The good retention of antioxidant capacity and total phenolic compounds of most beverages may be due to the suitable containers of them; canned juices of the Royal Chitralada Projects were packed in metal cans (250 mL) with deaeration. Therefore, the canned beverages that are hermetically sealed could save the antioxidants or other ingredients and also preserve their physicochemical quality, nutritional and sensory characteristics. As a study of Lee et al. (2002) showed that red grapefruit juice concentrates contained in metal cans lost the pigments (carotenoids pigments; lycopene and β– carotene) slightly smaller than those with plastic containers after 12 months of storage at -23°C. Thus, this observation was explained that plastic bottles are permeable to oxygen, which can be one of the critical factors for pigment

stability during frozen storage, while metal containers are hermetically sealed (109). Therefore, the manufacturers should be concern about choosing suitable containers for beverages.

In addition, the calories content of sterilized beverages ranged between 76 – 181 kcal/serving (250ml) which contained sugar about 19 – 45 g, ganoderma with honey showed the lowest calories content, while tamarind juice showed the highest calories content and also contained exceeding amount of sugar by about 1.9 times compared to the recommendation of Thai kids don't eat sweets campaign. Therefore, it also should be concerned about the added sugar in sterilized beverages.



CHAPTER VI
CONCLUSION

The aim of this study was to determine antioxidant capacity and total phenolic compounds of some pasteurized and sterilized Thai health beverages. The study was conducted to find out an appropriate amount of fortified sugar in selected pasteurized health beverages with consumer acceptable in taste and they were also investigated the correlation between the varying amount of adding sugar and antioxidant capacity and total phenolic contents in pasteurized beverages. The storage effects of pasteurized and sterilized beverages on antioxidant capacity and total phenolic compounds were also evaluated as followed.

Antioxidant capacity and total phenolic compounds of selected pasteurized and sterilized Thai health beverages

The results showed that there were significant linear correlation between the total phenolic compounds and antioxidant capacity both DPPH and PCL assay, which sacred lotus root, chrysanthemum, and roselle drink had the significant highest amount of antioxidant capacity.

The relationship between fortified sugar and antioxidant capacity and total phenolic compounds of selected Thai health beverages

It was observed that increasing beverage Brix by adding sugar had decreasing trend in antioxidant capacity of pasteurized beverage by DPPH assay in most beverages, except sacred lotus root drink. There were no effects on total phenolic compounds.

An acceptance of consumers on selected Thai health beverages with minimum amount of fortified sugar in appropriated sweetness

It was found out that reduced adding sugar in selected pasteurized beverages to 8 – 9 °Brix showed well sensory acceptance, tested by 30 panelists.

The effect of storage on stability of antioxidant capacity and total phenolic compounds in selected pasteurized and sterilized Thai health beverages

Our data showed that after 7 days of storage, some pasteurized beverages showed the decreasing of antioxidant capacity. Most of sterilized beverages showed a good retention of antioxidant capacity and total phenolic compounds after 1 and 3 months of storage.

Even though adding sugars in the studied beverages may not show strong correlation with antioxidant capacities. However, general consumers should be perceived that not only antioxidants but also excess caloric intake will be provided by fruit and herb beverages with added sugars. Moreover, pasteurized beverages should be consumed within 7 days because our result showed the loss of antioxidant capacity and total phenolic compounds after 7 days of storage.

For further research, the effect of consumption of those sweetened health beverages on free radical formation and antioxidant status in Thai people should be performed.

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APPENDIX

APPENDIX A

สูตรการผลิตน้ำผลไม้พาสเจอร์ไรส์ที่ใช้ในการวิจัยโดยโครงการส่วนพระองค์ สวนจิตรลดา

ชนิดน้ำผลไม้	วัตถุดิบ (kg)	น้ำ (L)	pH	°Brix	เวลาในการต้ม (นาที)
1. น้ำกระเจี๊ยบ (Roselle drink)	1	50 ± 10	2.4 ± 1	7 ± 1 9 ± 1	15 – 20
2. น้ำเก็กฮวย (Chrysanthemum drink)	1	40 - 50	6 ± 1	6 ± 1 8 ± 1	30
3. น้ำขิง (Ginger drink)	1	8.2	7.5 ± 1	7 ± 1 9 ± 1	40
4. น้ำมะตูม (Bael fruit drink)	1	30	6 ± 1	7 ± 1 9 ± 1	40

ขั้นตอนการผลิตเครื่องดื่มพาสเจอร์ไรส์

1. การเตรียมวัตถุดิบ

- สำหรับวัตถุดิบที่เป็นของสด ได้แก่ ขิง จะต้องทำการล้างให้สะอาด และหั่นเป็นชิ้นเล็กๆ

- สำหรับวัตถุดิบที่เป็นของแห้ง เฉพาะ มะตูมจะต้องผ่านการอบที่อุณหภูมิ 80 – 100 องศาเซลเซียส เป็นเวลา 50 – 60 นาที เพื่อให้มีกลิ่นหอมของมะตูม

2. ทำการชั่งน้ำหนักของวัตถุดิบที่ต้องการใช้ โดยคำนวณตามสัดส่วนที่กำหนดเทียบกับปริมาณน้ำที่ต้องการผลิต แล้วใส่ลงในถุงผ้าขาวบาง

3. ต้มน้ำจนเดือดแล้วใส่วัตถุดิบลงไป ต้มตามเวลาที่กำหนด

4. เมื่อครบกำหนดเวลาที่ต้ม เอาถุงผ้าขาวบางออก และทำการเติมน้ำตาล โดยค่อยๆเพิ่มปริมาณน้ำตาลไปพร้อมๆกับการวัด Brix โดยใช้ refractometer จนกระทั่งได้ค่า Brix ตามที่ต้องการ และทำการวัดค่า pH ของเครื่องดื่ม

5. เทเครื่องดื่มใส่ขวดพลาสติกแล้วปิดฝาให้แน่น ก่อนจะใส่ลงในน้ำหล่อเย็นด้วยน้ำแข็งทันที และทำการเก็บไว้ในตู้เย็นก่อนทำการทดสอบชิม

APPENDIX B

แบบสอบถามการประเมินผลทางประสาทสัมผัส ผลิตภัณฑ์เครื่องดื่มเพื่อสุขภาพ

ผลิตภัณฑ์ .....

วันที่ทดสอบ ..... เวลา ..... เพศ ..... อายุ ..... ปี

วิธีทำ เมื่อท่านสังเกตโดยการมองและหลังจากชิมผลิตภัณฑ์แล้ว กรุณาขีดเครื่องหมาย √ ลงในช่องว่างที่ตรงกับความรู้สึกและความชอบของท่านมากที่สุด

กำหนดให้ 1 = ไม่ชอบมากที่สุด 4 = ไม่ชอบเล็กน้อย 7 = ชอบ
2 = ไม่ชอบมาก 5 = เฉยๆ 8 = ชอบมาก
3 = ไม่ชอบ 6 = ชอบเล็กน้อย 9 = ชอบมากที่สุด

ลักษณะผลิตภัณฑ์	คะแนนความชอบ								
	1	2	3	4	5	6	7	8	9
สี									
กลิ่น									
รสชาติ									
เนื้อสัมผัส									
ลักษณะโดยรวม									

ข้อเสนอแนะ

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ขอขอบคุณที่ให้ความร่วมมือ

APPENDIX C

Nelson's reducing sugar test

Reagent preparation

1. **Alkaric copper reagent**

Solution I – 25g of anhydrous sodium carbonate (Na_2Co_3) was dissolved in 250 ml of water and then 12g of potassium sodium tartrate ($C_4H_4KNaO_6 \cdot 4H_2O$), 40 ml of 10 % Copper sulfate ($CuSo_4 \cdot 5H_2O$), 16 g of sodium bicarbonate ($NahCO_3$) were added and mixed thoroughly

Solution II – 180 g of anhydrous sodium sulfate (Na_2SO_4) was dissolved in 500 ml of water

Finally, mixed the solution I and II and then diluted the mixture to a total volume of 1000 mL. After incubation at room temperature for 1 week, the solution was filtrated and store at 30 – 37 °C until used

2. **Arsenomolybdic reagent**

Solution III – 50 g of Ammonium molybdate [$(NH_4)_8MO_7O_{24} \cdot 4H_2O$] was dissolved in 900 mL of water and added 42 mL of concentrate sulfuric acid (H_2SO_4)

Solution IV – 6 g of disodium hydrogen arsenate ($Na_2HASO_4 \cdot 7H_2O$) was dissolved in 50 mL of water

Finally, the solution IV was added slowly in the solution III and then diluted the mixture to a total volume of 1000 mL and store at 30 – 37 °C until used

BIOGRAPHY

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