THE FIRST INTERNATIONAL CONFERENCE ON INNOVATION OF FUNCTIONAL FOODS IN ASIA

IFFA 2018

FUNCTIONAL FOODS: NEW TRENDS OF INNOVATION, RESEARCH AND MARKETS

JANUARY 22ND - 24TH , 2018

PROCEEDINGS

UNIVERSITY OF PHAYAO,
PHAYAO, THAILAND

Proceedings of international conference on innovation of functional foods in Asia (IFFA 2018)
Functional foods: New trends of innovation, research and markets

Conference theme	Trends in innovative research and markets of functional foods
Conference date	January 22nd -24th, 2018
Conference URL	http://www.iffa.up.ac.th/
Conference venue	UP main hall: PhayaNgumMuang Hall, University of Phayao

IFFA 2018 Proceedings

First published: January, 2018 (250 copies)

ISBN: **978-616-93040-1-2**

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Published by: Society for Free Radical Research-Thai
จัดทำโดย : สมาคมเพื่อการวิจัยอนุมูลอิสระไทย

Artwork design: Pisut Jirotekul
Published at: Health Innovation Publishing
189/365 M.9 Tambon Nongjom Sansai District, Chiangmai, 50210

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President, University of Phayao
Prof. Mondhon Sanguansermsri, PhD

Dear speakers and participants,

On behalf of the University of Phayao, it gives us great pleasure to welcome you to the First International Conference of "Innovation of Functional Foods in Asia (IFFA)" on Jan 22nd -24th, 2018, jointly hosted by University of Phayao (UP) and Society for Free Radical Research-Thai (SFRR-Thai). The focus of our conference theme will be "Trends in Research and Markets of Functional Foods". As recently reported by Grand View Research, during 2014 - 2024, Asia-Pacific region seems to be the leading one for the market size of functional foods. The functional food market revenue in Asia-Pacific region will rise up to 100,000 million USD.

A term "functional foods" is defined as foods and food components that provide a health benefit beyond basic nutrition. Having good food not only promotes optimal health but also reduces risk of many diseases. It is thus unsurprisingly that functional foods have gain more and more attention. Food and nutrition science have moved from identifying and correcting nutritional deficiencies to designing foods that promote good health. According to UP philosophy "Wisdom for Community Empowerment", the strong academic background and research quality enable our staffs to service local community. This conference gives us a great opportunities to facilitate interactive and in-depth discussion with international expertise. We sincerely hope that this conference will help all of us explore ways in the intersection of functional foods, dietary supplements, diseases and innovative technology. This understanding has the potential to revolutionize diet, nutrition and food products as well as to support local and natural production for global and functional food products through research and innovation.

In addition, virtually enveloped by mountains and valleys, Phayao is a peaceful province. Though it has only modest facilities and conveniences, Phayao and Kwan Phayao is an enchanting community with outstanding natural beauty and fascinating religious sites. Dating back more than 900 years, Phayao used to be an independent state before it became a part of the Lanna Thai kingdom in mid-14th century. Today, Phayao is a laid-back town surrounded by lush natural beauty. The Phayao lakeside strip of bars and restaurants is a perfect place to unwind and plan journeys into the countryside, within which many hill tribe communities retain much of their traditional lifestyles.

We wish you all an enjoyable and stimulating conference. Thank you for your participation and looking forward to seeing you in the IFFA2018 conference.

M. Sanguansermsri

Prof. Mondhon Sanguansermsri, PhD

President, University of Phayao

Introduction to IFFA 2018

Prof. Emeritus Dr. Maitree Suttajit

Introduction: This is the First International Conference on Innovation of Functional Foods in Asia (IFFA 2018) held in 3 days during Jan. 22-24, 2018 at Phaya Ngum Muang Hall, University of Phayao, Thailand. The IFFA conference is organized and hosted by the Schools of Medical Sciences and Pharmaceutical Sciences and Division of Research Administration and Educational Quality Assurance, University of Phayao, cohosted by the Society for Free Radical Research-Thai and Food Innopolis. Food Innopolis is a global food innovation hub of Thailand Science Park, a gateway to ASEAN.

Background: In the last decades, functional foods in different forms of health products become fast growing, well accepted by consumers and used in our daily life. Functional foods play a big role in the risk reduction, prevention and healing of several diseases especially non-communicable diseases (NCDs), health promotion in youths and aged persons and patients recovered from illness. Reportedly, the highest demand and markets of functional foods in Asia region are annually and rapidly increasing, however research, development and innovative products are still limited and have to be promoted. The significance and current situation of food product development and innovation stimulated us to organize the IFFA conference.

Objectives: The First International Conference on IFFA was initiated in 2016 and strongly supported by Prof. Dr. Mondhon Sanguansermsri, the UP President. The objectives of the IFFA conference are to bring together leading researchers in functional foods, nutritionists, dieticians, graduate students and research scholars to share and exchange their expertise, experiences and research data onto advanced aspects of functional foods. The Conference should be an exciting platform for them to present and discuss their most recent discoveries, innovations, trends, and others as well as practical problems and solutions in the areas of functional foods.

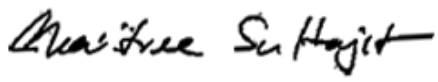
Program and Sessions: About 250 participants have early registered and 50 more on-site registrants will be expected. In the scientific program, there are 10 symposia by 37 invited distinguished speakers, 41 oral presentations and 82 poster sessions. More than 22 booths for functional foods and scientific exhibitors are joining the events. The IFFA participants are from 13 countries including Canada, New Zealand, Japan, Korea, China, India, Pakistan, Myanmar, Malaysia, Philippines, Singapore, Indonesia and Thailand. The symposia and sessions will introduce the cutting-edge research and the future perspectives relevant to the subjects covered as these topics: 1) Situation and trends in development of functional foods; 2) Role of Functional Foods in Health and Diseases; 3) Functional foods for diabetes; 4) Functional foods for brain memory and cognitive promotion; 5) Current situation and innovative trends of functional foods in Asia; 6) Probiotics, prebiotics and synbiotics; 7) Innovative approach for value-added functional foods; 8) Trends in consumption and

food preferences; 9) Update of traditional and functional foods /beverages in Asia and 10) Innovation in traditional food research and their health benefits. One IFFA innovative research award, six outstanding awards for oral and poster presentations will be given to the winners.

Lastly, IFFA is deeply grateful to all the invited speakers, participants, supporters and sponsors as well as the working committee for their precious time and effort that each of them expended on this conference,

Warmest welcome to the IFFA2018 conference. So, please enjoy the conference, have a pleasant stay in Phayao and bring your good memory back home.

Thank you very much.



Prof. Emeritus Dr. Maitree Suttajit
Chair of Organizing Committee,
President of SFRR-Thai

Dr. Wittaya Chaiwangyen
Secretariat

Message from Society for Free Radical Research-Thai (SFRR-Thai)

Assist. Prof. Chaiyavat Chaiyasut, Ph.D

Dear Friends and Colleagues,

Wish You Happy and Prosperous New Year-2018. Glad to meet you all at the beginning of New Year in a scientific gathering.

On behalf of the organizing committee, it gives us great pleasure to welcome you to the First International Conference of "Innovation of Functional Foods in Asia (IFFA)" on Jan. 22-24, 2018, jointly hosted by University of Phayao (UP), and Society for Free Radical Research-Thai (SFRR-Thai).

The society for free radical research (SFRR) was established in 1982, and now with over 2800 members worldwide, the Society consists of six autonomous regional groups: SFRR Asia, SFRR Europe, SFRR Australasia, the Oxygen Society (Pan America), SFRR Africa, and SFRR ASEAN. The Society for Free Radical Research-Asia established in 1995 as an Asian regional branch of the Society for Free Radical Research International. SFRR Asia currently consists of China, India, Korea, Malaysia, Taiwan, Thailand and Japan. In the present, Prof. Shinya Toyokuni is the President of SFRR Asia. The Society promotes attention in all aspects of research into the responses and properties of free radicals and their physical, chemical, biological, medical and industrial responsibility. Several successful scientific meetings were conducted on behalf of, SFRR-ASIA in various aspects of free radical research and its applications.

We sincerely hope that this conference will help all of us explore ways in which the intersection of genomics and molecular nutrition presents the opportunities to understand nutrient effects and individual variability in response to diet; this understanding has the potential to revolutionize diet, the nutrition and food products.

We wish you all an enjoyable and inspiring conference. Please let us have this opportunity to express our most profound appreciation and the warmest welcome to all of the participants, and invited speakers of IFFA-2018. Thank you very much.

Sincerely,

Assist. Prof. Chaiyavat Chaiyasut, Ph.D
Secretary, The Society for Free Radical Research-Thai

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Conference Program

January 22nd, 2018 (Monday) Phaya Ngum Muang Hall	
8:00-9:00	Registration
9:00-9:30	**Opening Ceremony:** - Introductory Address: Prof. Emeritus Dr. Maitree Suttajit, The President of SFRR-Thai - Inaugural Address: Prof. Dr. Mondhon Sanguansermsri, President of University of Phayao
9:30-10:10	**Keynote Lecture 1**: Prof. Emeritus Dr. Nuntavan Bunyapraphatsara "*Situation and trends in development of functional foods*"
10:10-10:30	Break
10:30-12.00	**Symposium 1: Role of Functional Foods in Health and Diseases** S1-1 Prof. Dr. Shinya Toyokuni "*Role of iron in carcinogenesis and tumor biology*" S1-2 Prof. Dr. Ki Baik Hahm "*FAT, fat and remedy for GI diseases*" S1-3 Dr. Frederick Warren "*Starch structure and its influence on digestion throughout the digestive tract*"
12:00-13:00	**Lunch and Special Seminar** Prof. Dr. Prachya Kongtawelert in "*Sesamin: From molecule to market*"
13:00-14:00	**Symposium 2: Innovation Ingredients of Functional Foods** S2-1 Prof. Dr. Veerapol Kukongviriyapan "*Rice bran hydrolysates from Thai Hom-Mali rice prevent metabolic syndrome and diabetes*" S2-2 Prof. Dr. Prachya Kongtawelert "*Sesamin: From research laboratories to functional foods*"
14:00-16:00	**Symposium 3: Functional Foods for Diabetes** S3-1 Prof. Dr. Umah Rani Kuppusamy "*Functional foods for prevention of diabesity"*

January 22nd, 2018 (Monday) Phaya Ngum Muang Hall (cont.)	
	S3-2 Assoc. Prof. Dr. Sirithon Siriamornpun **"***Trends in functional food products for diabetes*" S3-3 Prof. Dr. Shigeru Yamamoto **"***Control of high blood glucose and lipid concentrations by replacing white rice with pre-germinated brown rice*" S3-4 Prof. Dr. Angsumarn Chandrapatya "*Stevia products development in Thailand and potential benefits*"
16:00-18:00	**Oral Presentations (PN1, PN2 and PN3, Phaya Ngum Muang Hall ground floor)**
18:00-21:00	**Reception Banquets for Invited Speakers**

January 23rd, 2018 (Tuesday)		
8:00-8:30	Registration	
8:30-9:30	**Keynote Lecture 2**: Prof. Dr. Michael Gänzle "*Prebiotics-Superfood for the 21st century or only a marketing trend?"*	
9:30-12:30	**Symposium 4: Functional Foods for Brain Memory and Cognitive Promotion**	
Phaya Ngum Muang Hall	S4-1	Assoc. Prof. Dr. Naiphinich Kotchabhadi "*Functional foods for brain health*"
	S4-2	Assist. Prof. Dr. Natarajan Suganthy "*Nutraceuticals for healthy brain: Focus on dietary polyphenols against Alzheimer's disease*"
	S4-3	Prof. Emeritus Dr. Maitree Suttajit "*Phytochemicals rich of omega-3 fatty acids and rosmarinic acid in Perilla frutescens (Ngamon): health benefits to functions of brain and organs*"
	S4-4	Assist. Prof. Dr. Phakkharawat Sittiprapaporn "*Current trends in brain development studies of children supplemented with long-chain polyunsaturated fatty acids and omega fatty acids*"
	S4-5	Prof. Dr. Bungorn Sripanidkulchai "*Health benefit of aged garlic on cognitive and learning memory*"
	S4-6	Assoc. Prof. Dr. Worapol Aengwanich "*Functional foods against stress and anxiety*"
9:30-10:30	**Symposium 5: Current Situation and Innovative Trends of Functional Foods in Asia**	
PN1	S5-1	Assist. Prof. Dr. Akkharawit Kanjana-Opas "*Innovation trends of functional foods in Asia*"
	S5-2	Assist. Prof. Dr. Surapong Pinitglang "*Key trends in functional food and beverage ingredients for 2018*"

January 23rd, 2018 (Tuesday) (cont.)		
10:30-12:00	**Symposium 6: Probiotics, Prebiotics and Synbiotics**	
PN1	S6-1	Prof. Dr. Ravi Gooneratne "*Role of gut microbiome, probiotics and food safety and security*"
	S6-2	Prof. Dr. Sin-Hyeog Im "*Mining of probiotic strains for immune modulation*"
	S6-3	Assist. Prof. Dr. Chaiyavat Chaiyasut and Dr. Bhagavathi Sundaram Sivamaruthi "*Probiotic impacts on patient outcomes*"
12:30-13:30	**Lunch and Special Seminar**	
	Ms. Rajya Ramesh in "*Ingredient trends of functional foods 2018 Astaxanthin*"	
13:30-15:00	**Symposium 7: Innovation Approach for Value-Added Functional Foods**	
PN1	S7-1	Prof. Dr. Toshihiko Toida "*Anti-inflammatory effects of extracts form Phellinus mushroom*"
	S7-2	Assoc. Prof. Dr. Huang Dejian "*High throughput assay-guided approach for development of evidence based functional foods containing bioactive natural products isolated from tropical plants*"
	S7-3	Assoc. Prof. Dr. Surapol Natakankitkul "*Megatrend for nutraceuticals and cosmeceuticals in the future*"
13:30-15:00	**Symposium 8: Trends in Consumption and Food Preferences**	
PN2	S8-1	Prof. Dr. Shigeru Yamamoto "*Umami may contribute to the health of Japanese*"
	S8-2	Assoc. Prof. Dr. Venkatachalam Sivakumar "*Consumption pattern of functional food by Indian consumers*"

January 23rd, 2018 (Tuesday) (cont.)	
	S8-3 Ms. Shara Ng "*Functional plant-based foods and daily life: Long practice and experience in veganism*"
15:00-16:30	**Oral Presentations (PN1, PN2 and PN3, Phaya Ngum Muang Hall, ground floor)**
16:30-18:00	**Poster Presentations (Phaya Ngum Muang Hall, first floor)**
18:00-21:00	**Welcome Party**

January 24th, 2018 (Wednesday)		
8:00-8:30	Registration	
9:00-11:00	**Symposium 9: Update of Traditional and Functional Foods in Asia**	
PN1	S9-1	Assoc. Prof. Dr. Somdet Srichairatanakool "*Therapeutic approach of green tea in some diseases*"
	S9-2	Prof. Dr. Masami Suganuma "*Biophysical characteristics and anticancer immunity of green tea catechins and their effects of cancer prevention and treatment*"
	S9-3	Assoc. Prof. Dr. Tatsuro Watanabe "*Therapeutic impact of (-)-epigallocatechin gallate on human cancer stem cells and synergistic anticancer effects with the combination of green tea catechins and anticancer drugs*"
	S9-4	Prof. Dr. Sandip K. Bandyopadhyay "*L-Theanine: A magic molecule present in tea*"
9:00-11:00	**Symposium 10: Innovation in Traditional Food Research and Their Health Benefits**	
PN2	S10-1	Prof. Dr. Jaehong Han "*Korean ginseng, a representative Korean functional food*"
	S10-2	Dr. Susianto Tseng "*Role of tempe cereal as a source of vitamin B12 and its implementation for vegetarian diet*"
	S10-3	Dr. Kanti Bhooshan Pandey "*Polyphenols as anti-aging agents in food: Potential and performance*"
	S10-4	Assoc. Prof. Dr. Arunporn Itharat "*Research and development of nutraceuticals from Thai medicinal herbs*"
11:00-13:00	**Lunch, Award Announcement and Closing Ceremony**	
PN2	-Outstanding award -Oral presentation award -IFFA innovation award	
13:00-18:00	**Sightseeing Trip**	

Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Effect of high temperature short time pasteurization on availability of bioactive compounds and quality changes of watermelon juice during refrigeration storages

Nattawut Klong-Klaew[1], Tippawan Saeng-Arun[1] and Samart Sai-Ut[1]*

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Abstract

In the present study, the availability of bioactive compounds and quality in a watermelon juice processed by high temperature short time (HTST) pasteurizations and storage in the refrigerator were investigated. HTST at higher temperature (90 °C for 10 sec) significantly reduced total phenolic compounds, total carotenoid, ascorbic acid content and antioxidant activity. Pasteurized watermelon juice treated with heat of 80 °C for 40 sec maintained highest bio-accessibility of bioactive compounds, but was not effective in inactivating microorganism, when compare to other HTST-pasteurizations. The results showed that total carotenoid content in HTST-pasteurized watermelon juices ranged from 223 to 136 μg/dry sample after 4 weeks of storage at 4 °C. Bacteria, mold and yeast in watermelon juice were effectively inactivated by heat of 90 °C for 10 sec, during 4 weeks of storage at 4 °C. Consequently, higher-heat shorter time pasteurization (90 °C for 10 s) show good prospects for enhanced shelf life, but decrease the carotenoid and nutritional quality of watermelon juices.

Keywords: high temperature short time, pasteurizations, bioactive compounds, watermelon juices.

Introduction

Watermelon is one of the most popular fruit in Thailand due to a sweet, juicy, rich of nutritious and growing all year round. The most of the important bioactive compound in watermelon is carotenoids which act as free radical scavenger to protect cancers and cardiovascular diseases [1]. The production yield of the watermelon grown in Thailand is estimated to be around 166 kilotons per year [2]. Each growing zone has their own brand, which adds value to sales and increase production, and then watermelon for processing becomes more important. Recently, some researches have been found in focusing on development of pasteurized watermelon juice products [3][4].

Heat treatment is a general method to inactivate microorganisms and enzymes results in extends shelf life and maintains quality of fruit juices. Pasteurization process is commonly consumes heat to inactivate food born-pathogens in fruit juices which level of temperatures and times depend on the product type and the target organism. Without heating process, polyphenol oxidase enzymes can be cause of brown color formation [5]. However, conventional heat treatments for juices commonly changes in sensory and nutritive properties. Carotenoids, natural antioxidant colorant in watermelon, are highly sensitive to light, temperature, metals and oxygen during heat treatment. The most normal method of pasteurization is high temperature short time (HTST) pasteurization, which cause less damage to the nutrient constituent and sensory characteristics of products [6]. The HTST pasteurization encounters consumer need for improved flavor quality, less-processed products and makes the product closer to fresh juice. However, a final product with extend shelf life still requiring refrigeration storage.

Since the last decade, healthy drinking product become world wide's growing demand. Beverages have been developed the products acting as functional foods and foods rich in phytochemicals to support consumers' demands for highly nutritious foods. Pasteurized watermelon juice is not commercially available on the store in Thailand. The method of pasteurization can provide watermelon juice considered dietary sources of bioactive compounds, such as, carotenoids and phenolic compounds, which have shown to be good contributors to total antioxidant activity of food products. Thus, the objective of this study was to develop watermelon juice applying high-temperature/short-time on quality assurance of the watermelon juice during refrigerated storage. This study provides information support for commercialization of pasteurized watermelon juice.

Materials and Methods

Chemicals

Absolute ethanol was purchased from Merck (Darmstadt, Germany). 2,2′-diphenyl-picrylhydrazyl (DPPH•) and gallic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of watermelon juice

Watermelon was purchased from local market in Chonburi province and then cleaned with tap water and cut into pieces. Approximately 2 kg watermelon pieces were juiced with a blender and then filter through 4 layer of cheese cloth sheet. The watermelon juice was filled in to tube bottom (sized 20 ml) with cap.

HTST pasteurization

HTST treatment was carried out as previously described by water bath at temperature of 80 85 and 90 °C for 40, 20 and 10 sec, respectively. All samples were immediately cooled down to 4 °C in ice water after each treatment and stored in refrigerator, which were enclosed in aluminum foil to avoid oxidation by the interference of light with samples, at 4 °C until further analysis. The color of all the samples was measured by using a Hunter colour lab.

Refrigeration

After HTST treatment, samples were refrigerated and stored at 4 °C for a shelf-life study, with samples taken at 0, 1, 2, 3, and 4 weeks. On each analysis day, samples were taken at random and analyzed for quality changes.

Microbiological analysis

To monitoring microorganisms in watermelon juice, total plate count, total yeast and mold, coliform and *E. coli* counts were determined. Untreated and treated samples were serially diluted with sterile 0.85% NaCl solution, and 1.0 ml of each dilution was plated into duplicate plates of appropriate agar. Total plate count was determined using nutrient agar media. Coliforms and *E. coli* were determined on 3M petri-films. The nutrient agar media plate and petri-films were kept in the incubator at 35 °C for 24 hours. Total yeast and mold counts were determined by pour plate method in potato dextrose agar (PDA). The plates

containing media sample were incubated at 25 °C and yeast and molds were counted after two days. The results were expressed as log CFU/ml of watermelon juice sample.

Analysis of ascorbic acid

Determination of ascorbic acid content in watermelon was carried out using redox titration method. 10 ml of each of the watermelon juices was pipetted into a pre-washed conical flask and 5 ml of 10% potassium iodide with 1 ml of 0.3 M sulphuric acid were added into the flask. Then 10 ml of 0.01 M potassium iodate was also added into the flask. The excess iodine generated was titrated against 0.01 M sodium thiosulphate solution blank titration was carried out with 10 ml of distilled water. Calculate the concentration in mg of ascorbic acid in 100 g the solution obtained from juice

Determination of total phenols

Folin-Ciocalteau method was used for determination of total phenols. Watermelon juice of 0.4 mL was mixed with 2 mL of 10-fold diluted Folin-Ciocalteau reagent and set in the dark for 1 h at room temperature. This mixture was further mixed with 1.8 mL of sodium carbonate (7.5%) and reacted for 15 min at dark. And then it was immediately measured at 765 nm by a spectrophotometer. Results were expressed as mg of gallic acid equivalent (GAE) per 100 ml juice.

DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Brand-Williams, Cuvelier and Berset [7] with a slight modification. Samples (600 µl) were added to 600 µl of 0.60 mM 2, 2-diphenyl-1-picryl hydrazyl (DPPH) in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 520 nm using a UV-spectrophotometer (Biochrom/Libra S22, England). The sample blank at each concentration was prepared in the same manner except that ethanol was used instead of DPPH solution. The standard curve was logarithm between 2 and 25 µg/mL gallic acid. Results are expressed as gallic acid equivalents (GAE) mg/100g dry seed fruit. Additional dilution was needed if the DPPH value measured was over the linear range of the standard curve.

Sensory evaluation

The watermelon juice samples for sensory evaluation were placed and displayed on cleaned white porcelain plates. Thirsty trained panelists from department of Food Science and Technology, Burapah University were asked to evaluate the overall liking, odor, color and appearance attributes. The shrimp samples were examined by 9-point hedonic scales (9 = like extremely to 1 = dislike extremely).

Statistical analysis

All chemical analyses were performed in triplicate. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test. Analysis was performed by using a SPSS package (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL, USA).

Results and Discussion

Effects of HTST pasteurization and storage time on total carotenoid and ascorbic acid

Effects of HTST pasteurization on contents of bioactive compounds including, total carotenoid and ascorbic acid presented in Fig 1. Fig 1a presents the change in total carotenoid content of pasteurized watermelon juice during storage. Total carotenoid content of juices treated pasteurization of 80 ºC-40 s, 85 ºC-20 s and 90ºC-10 s were found as 481, 419 and 387 µg/g sample at 0 week ($P < 0.05$), respectively. The total carotenoid content of all pasteurized watermelon juices decreased substantially during 4 week storage. There were significant differences in the ascorbic acid content of pasteurized watermelon juices depending on storage times, ranging from 14.7 to 2.1 mg/100 g (4 weeks) for control and 7.6 to 0.94 mg/100 g for pasteurized watermelon juices at 90 °C. Total carotenoid content of water melon juice treated at 90 °C-10 s decreased considerably compared to the samples treated at lower temperature. This might be explained by the tendency of carotenoid to polymerize and to degrade easily at higher pasteurized temperatures. Carotenoids are unstable and susceptible to degradation, leading to a pale color during processing and storage. The red color deterioration in watermelon juices containing carotenoid occurs as a result of the degradation of carotenoid and loss of red pigments. Decreasing in lycopene and carotenoids content of fruit juices after increasing temperature or time of thermal processing depends on type of processing, temperature and time [8].

Ascorbic acid content in watermelon juice treated by HTST pasteurization during refrigerated storage are shown in Figure 1b, remarkable decrease of ascorbic acid content is observed, percentage of ascorbic acid content of pasteurization at 80 ºC-40 s, 85 ºC-20 s and 90ºC-10 s were 12.3, 9.7 and 7.6 mg/100 g sample, respectively. Results regarding the effect of pasteurized treatments on the contents of vitamin C of watermelon juice showed significant decrease in vitamin C in all the treated juice samples as compared to control. The decrease in vitamin C as a result of pasteurized of watermelon juice is retarded highly beneficial to the human health. Ascorbic acid contributes substantially towards prevention of many diseases including, protection against immune system deficiencies, cardiovascular disease, prenatal health problems, eye disease, and even skin wrinkling [9]. After 4 weeks, the ascorbic acid content of the all treatments was significantly decreased ($P < 0.05$). Heat and oxygen are the main responsible factors for its degradation.

Effects of HTST pasteurization and storage time on phenol compounds and antioxidant activity

In order to study the effect of the HTST pasteurization and optimal storage time, watermelon juices were treated with different pasteurized temperatures and then the phenolic content and antioxidant activity in samples were determined. The value of phenolic content of control was higher than that pasteurized at 80 ºC-40 s, 85 ºC-20 s and 90ºC-10 s. In addition, the effect of pasteurized at the temperature of 80 ºC-40 s was better than that at 85 ºC-20 s and 90ºC-10 s for the keeping high in phenolic content. When storage the juices for 4 weeks, the phenolic content also decreased during storage time increased. Increase in temperature during pasteurization might be attributed to the decreased amount of phenolic compound as a result of phenol compound degradation. Phenol compounds in watermelon are important biochemical substances juice. Spanos and Wrolstad [10] reported that total phenol concentration in pasteurized apple juice was reduced after processed thermally at 80 °C for 15 min. Phenols are also used as indicators of physiological state and potential damage in quality of fruit products [11]. The study of Gardner et al. [12] reported that significant losses in phenolics in fruit juice due to thermal processing.

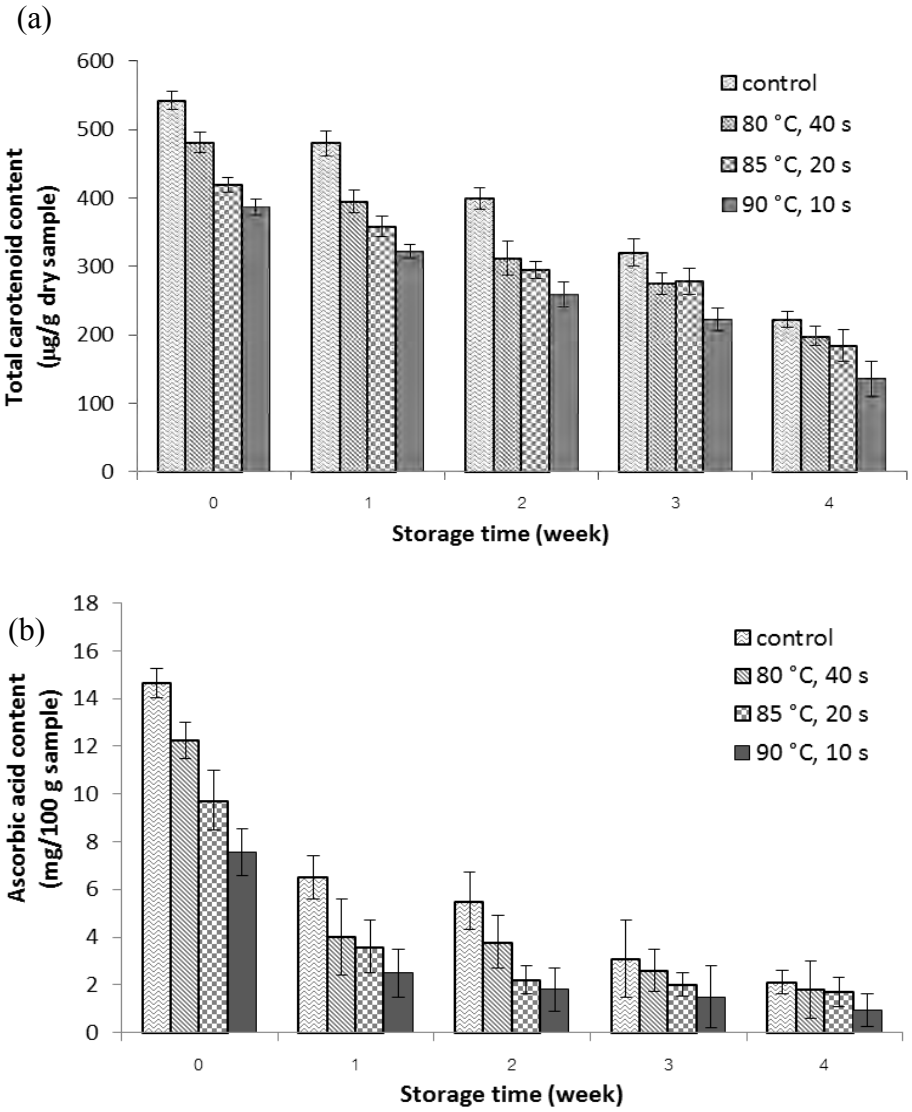

Figure 1 Changes in total carotenoid (a) and ascorbic acid content (b) in watermelon juice treated by HTST pasteurization during 4 weeks of storage at 4 °C.

The effect of HTST pasteurization on DPPH free radical scavenging activity of watermelon juice is shown in Fig 2b. There was significant decrease exists in both total phenolic content and DPPH free radical scavenging activity in all HTST treated juice samples during storage for 4 weeks. Bioactive compound such as phenolic compounds and vitamin C in watermelon are the major components responsible for DPPH free radical scavenging activity. These compounds have potential to scavenge the free radicals that cause damage to cell and also reduce the microorganism from contaminant. Efficiency of the antioxidant activity in fruit juice is related to presence of high concentration of total polyphenol content.

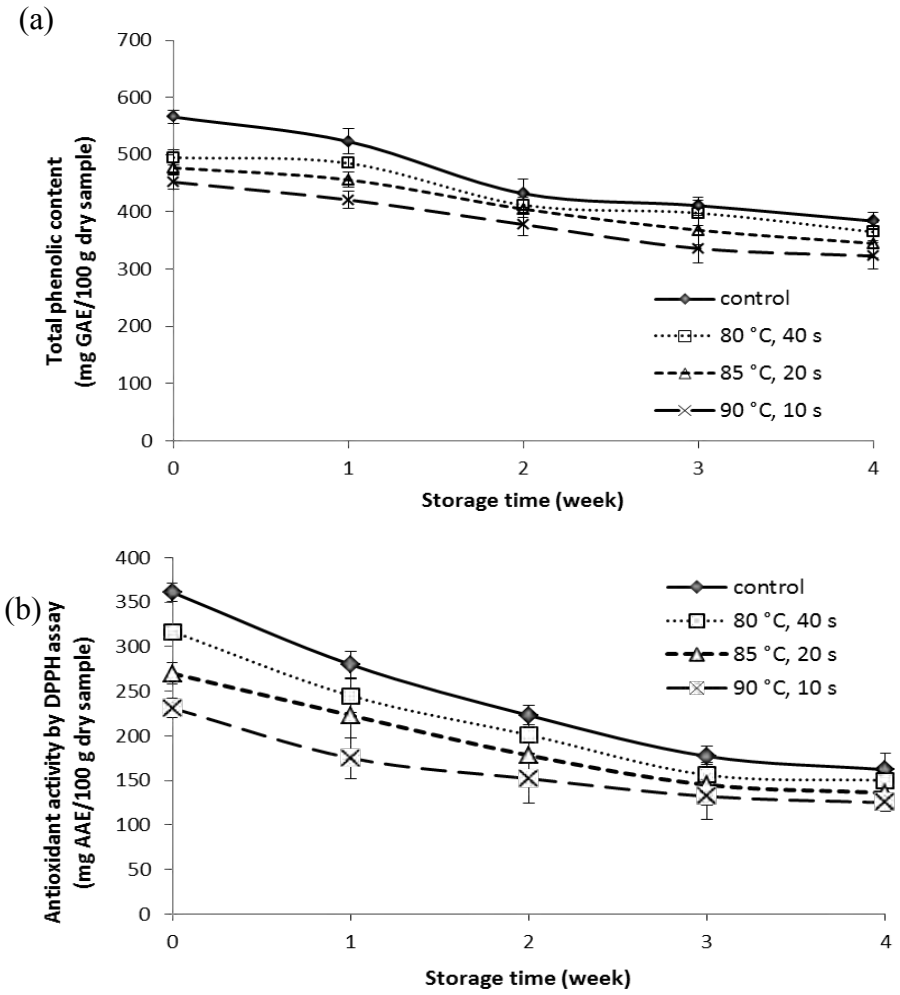

Figure 2 Changes in total phenolic content (a) and antioxidant activity (b) in watermelon juice treated by HTST pasteurization during 4 weeks of storage at 4 °C.

Effects of HTST pasteurization and storage time on color

In fruit juice quality and sensory acceptability, a primary factor considered by the consumer is color. There were significantly ($P < 0.05$) different in color of the watermelon juice after the pasteurized-treatments, whereas an increase in L^* value was found after treated by higher temperature (Table 1). As a consequence of storage time, color parameters of all samples showed decreasing in a^* and b^* value whereas L^* value was increased. Akgün and Ünlütürk [13] reported that thermal pasteurization at 70 °C-100 s and 80 °C-15 s affected L^* value of apple juice owing to the partial precipitation of suspended particles. Lower in $a*$ and b^* values correspond with higher pasteurized temperature and storage time of juices. Total carotenoid concentration of watermelon juice highly correlated with a$*$ value. Decreasing in

a∗ and b* values can be due to the degradative process of lycopene and carotenoids which is the main source of watermelon juice. Carotenoids are unstable and susceptible to degradation leading to a brownish color during storage. These were in agreement with the previous studies on negative effect of higher temperatures and longer storage time on the color of fruit juice [14][15]. Color quality changes compactly affect consumer acceptability as well as lead to a loss of marketability of fruit juice products [15]. Thus, color of watermelon juice changes during thermal processing and storage time.

Table 1 Color parameter changes in watermelon juice during 4 weeks of storage at 4 °C.

Parameter	Treatment	Storage time (week)				
		0	1	2	3	4
*L**	Control	23.65 ± 0.01	24.79 ± 0.04	25.56 ± 0.02	25.75 ± 0.01	26.26 ± 0.02
	80 °C-40 s	24.06 ± 0.02	25.16 ± 0.02	27.53 ± 0.03	27.85 ± 0.03	28.81 ± 0.02
	85 °C-20 s	24.52 ± 0.02	25.63 ± 0.03	27.94 ± 0.01	28.47 ± 0.06	29.43 ± 0.03
	90 °C-10 s	24.96 ± 0.01	26.25 ± 0.03	28.67 ± 0.01	28.98 ± 0.02	30.42 ± 0.03
*a**	Control	28.23 ± 0.02	27.26 ± 0.02	26.84 ± 0.10	25.28 ± 0.02	23.92 ± 0.04
	80 °C-40 s	25.82 ± 0.04	24.95 ± 0.01	24.54 ± 0.03	24.33 ± 0.04	22.75 ± 0.60
	85 °C-20 s	25.34 ± 0.02	24.74 ± 0.02	24.43 ± 0.03	24.17 ± 0.03	21.97 ± 0.02
	90 °C-10 s	25.13 ± 0.02	24.63 ± 0.02	24.28 ± 0.02	23.87 ± 0.03	21.44 ± 0.03
*b**	Control	20.73 ± 0.04	20.08 ± 0.05	19.73 ± 0.04	19.17 ± 0.02	18.32 ± 0.07
	80 °C-40 s	17.86 ± 0.02	17.66 ± 0.03	17.26 ± 0.02	16.98 ± 0.02	16.65 ± 0.03
	85 °C-20 s	17.56 ± 0.03	17.15 ± 0.02	16.85 ± 0.02	16.55 ± 0.04	16.43 ± 0.03
	90 °C-10 s	17.25 ± 0.02	16.83 ± 0.02	16.55 ± 0.03	16.35 ± 0.02	16.11 ± 0.02

Effects of HTST pasteurization and storage time on sensory properties

The sensory evaluation determined by thirsty trained panelists was performed with a control sample and HTST pasteurized sample. The sensorial analysis scores attributes are shown in Table 2. The HTST pasteurized-treated juices had lower in sensory score in all attributes compare with the control. However, all scores were within the acceptance range by consumer and with no significance difference among HTST pasteurized-treated juices ($P >$

0.05). In the HTST pasteurization, watermelon juice treated at 80 ºC-40 s exhibited better appearance and color, which could be distinguished in appearance attribution evaluated by the panelists, indicating that lower temperature was better at maintaining the sensory quality of watermelon juice during thermal processing. However, odor, and overall liking attribution of the HTST pasteurized samples (80 ºC-40 s, 85 ºC-20 s and 90ºC-10 s) were not significant different. Too high pasteurized temperature had a negative effect on the odor and taste of the watermelon juices. In addition, lower in color score might be due to pasteurized temperature on the carotenoids degradation, the mainly source of pigment of watermelon juice, which detected by consumers.

Table 2 Sensorial analysis of watermelon juice treated with HTST pasteurization compare with untreated juice control.

Pasteurization	Appearance	Color	Odor	Overall liking
Control	7.13 ± 1.14	7.03 ± 0.96	7.23 ± 1.07	7.23 ± 10.1
80 °C-40 s	6.33 ± 1.12	6.13 ± 0.90	5.53 ± 1.25	5.87 ± 1.14
85 °C-20 s	5.90 ± 1.35	5.90 ± 0.88	5.13 ± 1.48	5.57 ± 1.19
90 °C-10 s	5.83 ± 1.21	5.47 ± 0.97	5.07 ± 1.39	5.67 ± 1.15

Effects of HTST pasteurization and storage time on microorganism

The effects of HTST pasteurized treatments on coliform, *E. coli*, total plate counts and mold and yeast counts in watermelon juice are shown in Fig 3 and Table 3. There were no counts detected for coliform and *E. coli* in HTST pasteurized juice, whereas the control juice was found the bacterium counts at storage time of 0 and 2 weeks, respectively (Table 3). According to the study of Akgün and Ünlütürk [13], the HTST pasteurized treatment conditions (70 °C-100 s, 70 °C-120 s, and 80 °C-15 s) were found to be sufficient to obtain 5 $\log^{10}$ reduction of *E. coli* K12 in apple juice. Decrease of total plate count (TPC) of bacteria was notably accelerated by higher pasteurized temperature at day 0 with the value of 0.53, 0.35 and 0.19 log cfu/ml for 80 ºC-40 s, 85 ºC-20 s and 90ºC-10 s, respectively (Figure 3 & 4). The total plate counts varied depending on the pasteurized temperature and storage time. HTST pasteurized juices had a shelf-life of at least 4 weeks, whereas the control and 80 ºC pasteurized-treated juice had found a mold and yeast count when stored at 4 °C for 0 and 2

weeks, respectively. Thermal pasteurization at higher temperature was the most effective treatment to inactivate mold and yeast. As a result, there were not found mold and yeast counts after storage for 3 weeks. These results also indicated that mold and yeast were more responsive to HTST pasteurized process compared to bacteria, which was mainly because of their cell wall types [16]. During refrigerated fruit juices, *Saccharomyces cerevisiae* are usually the most common causes of spoilage [17]. Owning high pH and sugar contents are the most susceptible to yeast spoilage in fruit juice [18]. The study of Zhao et al. [19] reported that mold and yeast count in pear juice were prolong shelf life after HTST (110 °C-8.6 s) treatments up to 50 days when storage at 4 °C. Thus, HTST treatments in fruit juice reducing the counts of bacteria, mold and yeast to extend quality and safety in food products.

Table 3 Population of total coliform bacteria in watermelon juice treated with HTST pasteurization during 0 and 2 weeks of storage at 4 °C.

Coliform bacteria	Treatment	Storage time (week)	
		0	2
Coliform	Control	2.60 ± 2.08 cfu/ml	2.81 ± 1.15 cfu/ml
	80 °C-40 s	ND	ND
	85 °C-20 s	ND	ND
	90 °C-10 s	ND	ND
E. coli	Control	ND	0.52 ± 0.57 cfu/ml
	80 °C-40 s	ND	ND
	85 °C-20 s	ND	ND
	90 °C-10 s	ND	ND

ND: Not detected.

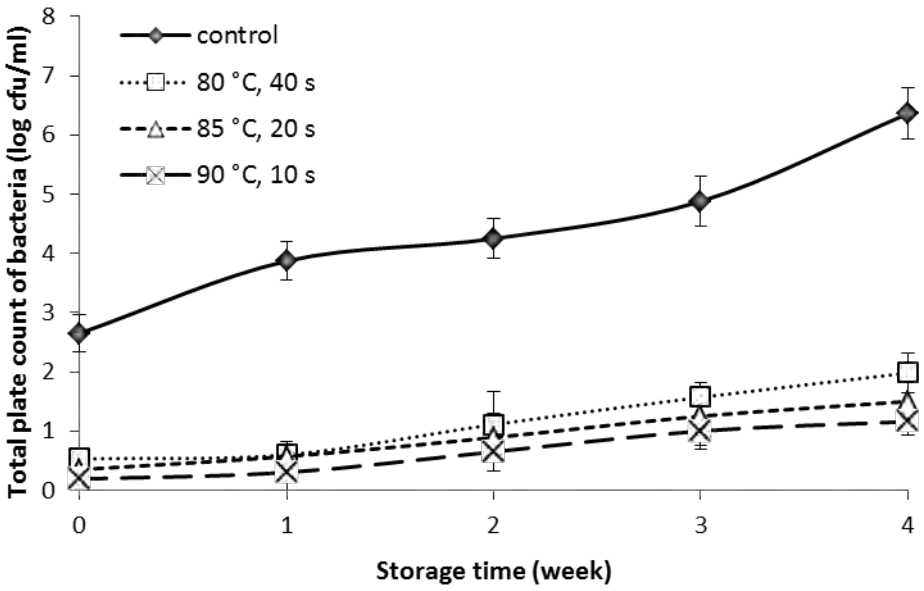

Figure 3 Evolution of total plate count of bacteria in watermelon juice treated by HTST pasteurization during 4 weeks of storage at 4 °C.

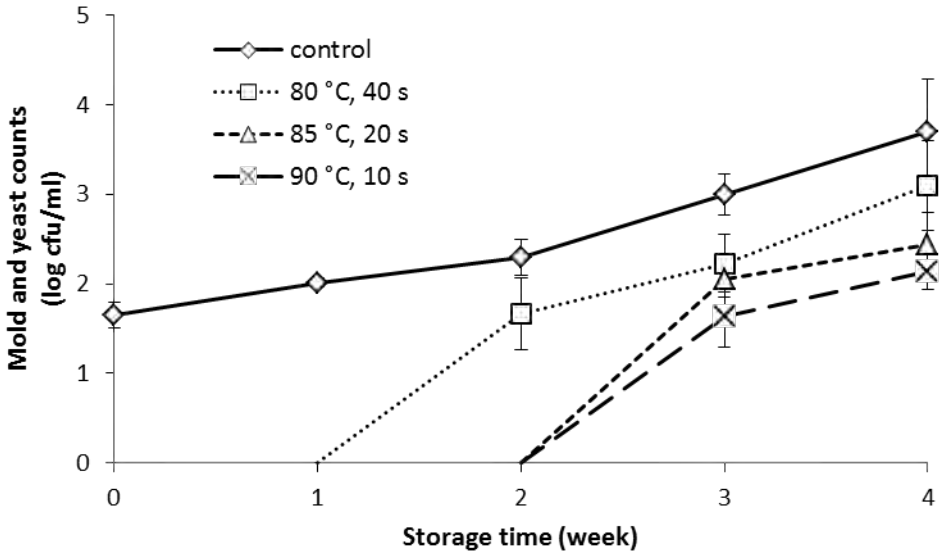

Figure 4 Evolution of mold and yeast counts in watermelon juice treated by HTST pasteurization during 4 weeks of storage at 4 °C.

Conclusions

Pasteurized treatments have a strong effect on bioactive compounds including, total phenolic compounds, total carotenoid, ascorbic acid content and antioxidant activity. Pasteurization treatment of 85 °C-20 s and 90 °C-10 s storage at 4 °C could help to ensure the microbiological safety of pasteurized watermelon juices, but impact on its and negative effects on color quality and sensory properties. Therefore, HTST pasteurization could be an efficient processing for fruit juice safety, and should be pronounced at shorter time for preserve the health benefits compounds in the fruit juices.

Acknowledgements

The authors would like to thank Department of Food Science, Faculty of Science, Burapha University for financial support.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
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The effect of proteases and degree of hydrolysis on production of gelatin hydrolysate with antimicrobial activities

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Abstract

This research aimed to study the effect of various enzymes and degree of hydrolysis (DH) on antimicrobial activity of gelatin hydrolysate against *Escherichia coli* and *Staphylococcus aureus*. Gelatin hydrolysates were produced by various proteases (trypsin, neutrase, bromelain and papain) with different DHs (10-30% and 31- 43% for flavourzyme). The result showed that increase the DH of gelatin hydrolysate hydrolyzed by trypsin and flavourzyme increased antimicrobial activity against *E. coli* and *S. aureus*. Gelatin hydrolysate prepared by trypsin at DH of 30 (GT30) exhibited the highest antimicrobial activity. The MIC, MBC and IC_{50} values of gelatin hydrolysate prepared by flavourzyme at 43% DH (GF43) were 80 mg/mL, 80 mg/mL, and 43.57 mg/ml, respectively. The gelatin hydrolysate prepared by selected enzymes (trypsin and flavourzyme) were applied in orange juice prepared by pasteurization (80 ºC for 15 seconds) and then kept at 4ºC for 2 weeks. During storage, orange juice added GT30 and GF43 showed no different in color, while pH valve was different ($P \leq 0.05$) when compared with control.

Keywords: gelatin hydrolysate, antimicrobial activity, protease, degree of hydrolysis

Introduction

The bioactive peptides derived from protein-based foods with low molecular weight are easily absorbed in the digestion system. These peptides have gained more attention for they are safer and healthier than synthetic drugs [1]. Bioactive peptides basically consist of 3-20 amino acids, and their bioactivity base on sequence and amino acid compositions [2]. A growing interest in antimicrobial peptide from natural sources, especially dietary proteins, has been interested in consumers and food industries. Gelatin hydrolysate has been known as the potential source of biologically active peptides with many useful properties such as antioxidative activity [3], angiotensin I converting enzyme inhibition [4], chemotactic activity [5], and antimicrobial activity [6], etc. In addition, a discovery natural antimicrobial compound from natural sources is needed. Recently, bioactive peptides with antibacterial activity have received a gain interested in the food industry, due to their low toxicity and biological properties to inhibit the food borne pathogens (Hong et al., 2001).

Several antimicrobial bioactive peptides from marine sources, including oyster [7], shrimp [8], green sea urchin [9] and fish [10] have been produced by enzymatic hydrolysis. A novel antimicrobial peptide from oyster can exhibit against *E. coli*, *B. subtilis*, and *P. aeruginosa* [7] (Liu et al., 2008). Production of hydrolysate from oyster hydrolyzed by alcalase and bromelin generated a peptide with a low molecular weight that exhibited inhibitory activity against the herpes virus [11]. Bioactive peptides from American lobster exhibited antibacterial activity against some Gram-negative bacteria and both protozoastatic and protozoacidal activities [12]. Fish gelatin hydrolysate can be used as a functional food [10]. The antibacterial activities of peptide–zinc complexes prepared from silver carp protein hydrolysates treated by flavourzyme exhibited the highest antibacterial activities against both *S. aureus* and *E. coli* [13]. Commonly, gelatin hydrolysate has been prepared by various proteases such as alcalase, properase E, Neutrase, flavozymes, and protamex [4][14][15][16]. However, the peptides generated via its cleavage of gelatin might not have the maximized bioactivity. As a consequence, the protease with high capacity of hydrolyzing peptides and yielding the active peptides with desirable bioactivity is still needed. Thus, this study aimed to comparatively determine antimicrobial activities of fish gelatin hydrolysates produced with the aid of various proteases with different DH.

Materials and Methods

Chemicals

2,4,6-Trinitrobenzenesulphonic acid (TNBS) was purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). Neutrase and flavourzyme were obtained from Novoenzyme (Denmark). Bromelain and trypsin were purchased from Sigma-Aldrich (USA). All chemicals were of analytical grade.

Crude papain extraction

Preparation of crude extract was prepared from papaya (*C. papaya*) latex. Fresh papaya latex was collected from Chonburi, Thailand. The exuded latex was collected using a receiving container. The latex was then transferred to a beaker and stored below 10 °C and used within 3 h. To prepare the crude extract, the latex was mixed with cold distilled water (⩽4 °C) with a latex to water ratio of 1:1 (w/v). The mixture was gently stirred at 4 °C for 1 h. Then, the mixture was centrifuged at 9000g at 4 °C for 20 min using a refrigerated centrifuge. The supernatant was filtered using a Whatman No. 1 filter paper, followed by freeze-drying. The crude extract powders from papaya latex referred to as papain.

Production of gelatin hydrolysate with different degrees of hydrolysis (DH)

Unicorn leatherjacket skin gelatin extracted following the method of Kaewruang et al. [17] (4 g) was dissolved in distilled water. The pH of mixture was adjusted with 0.5 M NaOH or 0.5 M HCl. The volume of solution was made up to 50 ml by distilled water previously adjusted to pH optimum (as stated in Table 1) to obtain a protein concentration of 2% as determined by the Lowry method [18]. The hydrolysis reaction was started by the addition of various enzymes (trypsin, neutrase, bromelain, and papain), which were calculated from the plot between log (enzyme concentration) and DH to obtain DH of 10%, 20% and 30% (DH of 31, 35 and 43% for flavourzyme) [19]. Hydrolysis was performed for 1 h in a water bath shaker at the optimum temperature of each enzyme. After 1 h of hydrolysis at optimum temperature, the enzyme was inactivated by heating at 90°C for 15 min in a temperature controlled water bath. The mixture was then centrifuged at 5000 × *g* at room temperature for 10 min. The supernatant was freeze-dried using a freeze-dryer. Hydrolysates prepared by various enzymes with different DHs were subjected to analysis.

Table 1 Optimum conditions for enzymatic hydrolysis of gelatin hydrolysate by different proteolytic enzymes**.**

Enzymes	pH	Temperature (ºC)
Trypsin	7.8	37
Neutrase	7.0	50
Bromelain	7.0	50
Papain	7.5	40
Flavourzyme	7.0	50

α-amino acids content determination

The α-amino acids content in the mixture was determined according to the method of Benjakul and Morrissey [19]. Gelatin hydrolysate sample (125 µl), 0.20 M phosphate buffer, pH 8.2 (2.0 ml) and 0.01% TNBS solution (1.0 ml) were added into a test tube. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50 °C for 15 min in the dark. The reaction was stopped by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature before measured absorbance at 420 nm. α-amino acids content was expressed in terms of L-leucine.

Cultivation and bacterial inoculum perpetration

Antibacterial activity of peptides was evaluated against both Gram negative (*Escherichia coli*) and Gram positive (*Staphylococcus aureus*). Selected bacteria were cultivated aerobically at 37 °C overnight for 18 h in sterile nutrient broth. The prepared cultures were re-cultivated for acquiring maximum growth under the same conditions, by transferring 0.5 ml of the culture into nutrient broth. Bacterial inocula were prepared for antibacterial assay from the mid-logarithmic phase of their growth culture. The optical density cultures were measured at 600 nm and adjusted to around 0.5 by addition of the TSB (OD600 = 0.5) which contains approximately 10^3 colony-forming units/mL

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC value was defined as the peptide concentration at which bacterial growth was inhibited after 24 h of incubation at 37 °C. *E. coli* and *S. aureus* were used for the determination First, each bacterium was grown separately in nutrient broth at 37 °C for 18 h. Approximately 1 ml of culture was transferred to 9 ml of broth medium and incubated at 37 °C for another 15 h, cell concentration was then adjusted to obtain a final concentration of 10^3 cfu/ml using nutrient broth. To a 96-well microplate, 10 µl of bacterial suspension to yield a final concentration of 10^3 cfu/ml in each well and 90 µl of nutrient broth medium with gelatin hydrolysate prepared by dilutions of 10-80 mg/ml were poured into the microplate. The microplate was incubated at 37 °C for 24 h. Later, the absorbance at 600 nm for each well was read using micro plat reader, and the results were compared to the control sample. The minimum bactericidal concentration (MBC) is determined as the highest dilution at which no visible growth occurred in medium. Each experiment was repeated twice

Determination of antibacterial activity and IC_{50}

To conduct the experiment, *E. coli* and *S. aureus* were used. After incubating in a nutrient broth at 37 °C, the bacterium suspension was prepared, and then diluted with saline until the final concentration reached 10^3 cfu/ml. Antibacterial activity of gelatin hydrolysate was determined by the percentage of inhibition. The percentage of inhibition was calculated as $[(OD_{control} - OD_{sample})/OD_{control})] \times 100$. Experiments were carried out in triplicate. The IC_{50} value is calculated using the linear relation between the inhibitory probability and concentration logarithm

Application of gelatin hydrolysates as antimicrobial agents in fruit juices

The antimicrobial activity of selected gelatin hydrolysate was tested in orange juices. The fruit juices were supplemented with 100 ppm GT30 and GF43, respectively. The fruit juices were pasteurized at 90ºC for 30 sec. After that, the juices were kept at 4 °C for 2 weeks. After 0, 1, and 2 weeks, samples were withdrawn, diluted (in ten-fold increments) and viable cell numbers were determined by plate counting. The color of all the samples was measured by using a Hunter colour lab. pH was determined using a digital pH meter.

Statistical analysis

All experiments were carried out in triplicate using three different lots of samples. Data were subjected to the analysis of variance (ANOVA) and mean comparisons were performed using Duncan's multiple range test (Steel and Torrie, 1980). For pair comparison, *t*-test was used. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

Results and discussion

Effect of DH on antibacterial activity of gelatin hydrolysates

To investigate the effect of DHs on gelatin hydrolysates with antimicrobial activity, unicorn leatherjacket skin gelatins were hydrolyze with bromelain, flavourzyme, neutrase, papain and trypsin for 1 h and assayed for antibacterial activities. The antibacterial abilities of different hydrolysates against *E. coli* and *S. aureus* were studied. During enzymatic hydrolysis, cleavage of peptide bonds releases the α-amino groups, which are reacted with TNBS in the presence of a yellow complex compound detectable at an absorbance of 420 nm. Figure 1-4 shows that the patterns of gelatin hydrolysate by different proteases were found to be differences DHs. DH is the most widely used indicator for comparing different protein hydrolysates. The gelatin hydrolysates produced without hydrolyzed by enzymes did not show any antibacterial activities, while only hydrolysates produced with higher DH exhibited antibacterial activities. In addition, the relationship between DH and antibacterial activity showed that an increasing DH value exhibited low in bacteria growth. The growth of *E. coli* and *S. aureus* was reduced by different proteases by DH 20, 30 and 40%, respectively (Figure 2), whereas hydrolysate hydrolyzed by flavourzyme showed antibacterial activity of 43% against *S. aureus* ($P < 0.05$) (Figure 2b).

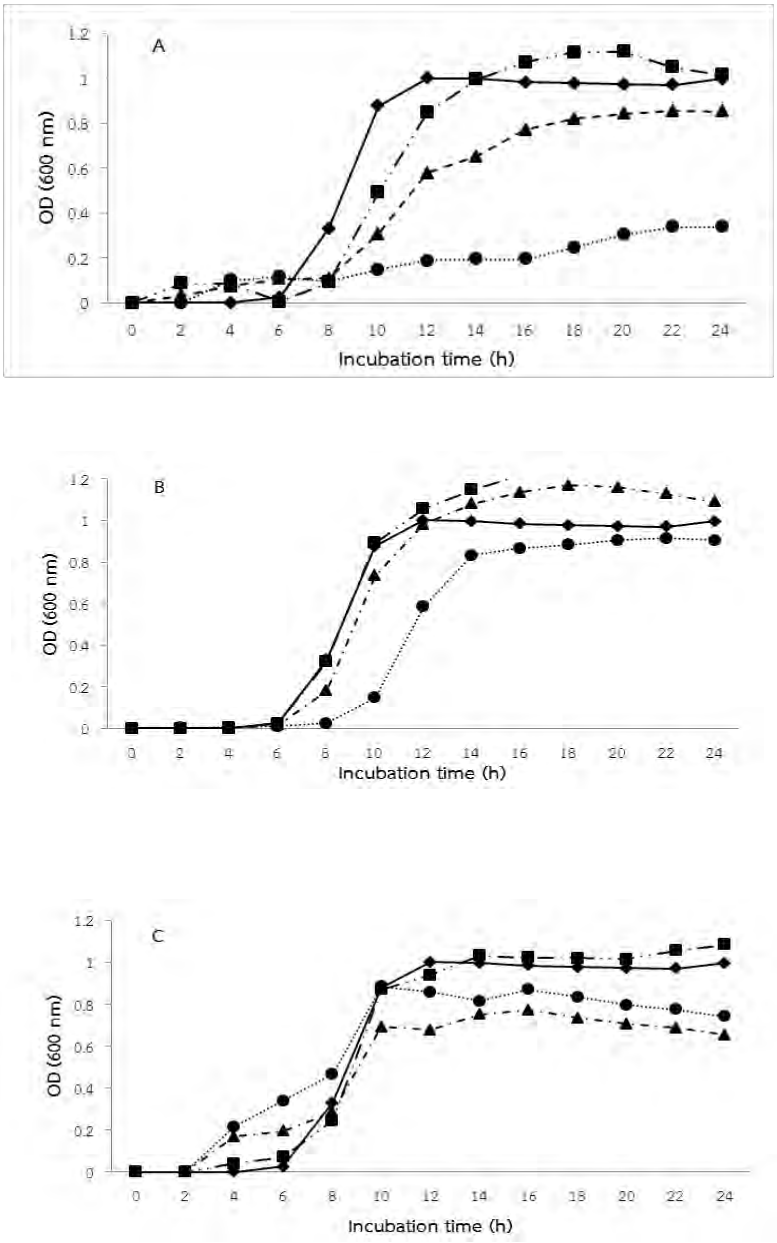

Figure 1 *E. coli* inhibition of gelatin hydrolysates by trypsin (A), neutrase (B), and bromelain (C) with different DH (◆: control; ■: DH 10; ▲: DH 20; ●: DH 30)

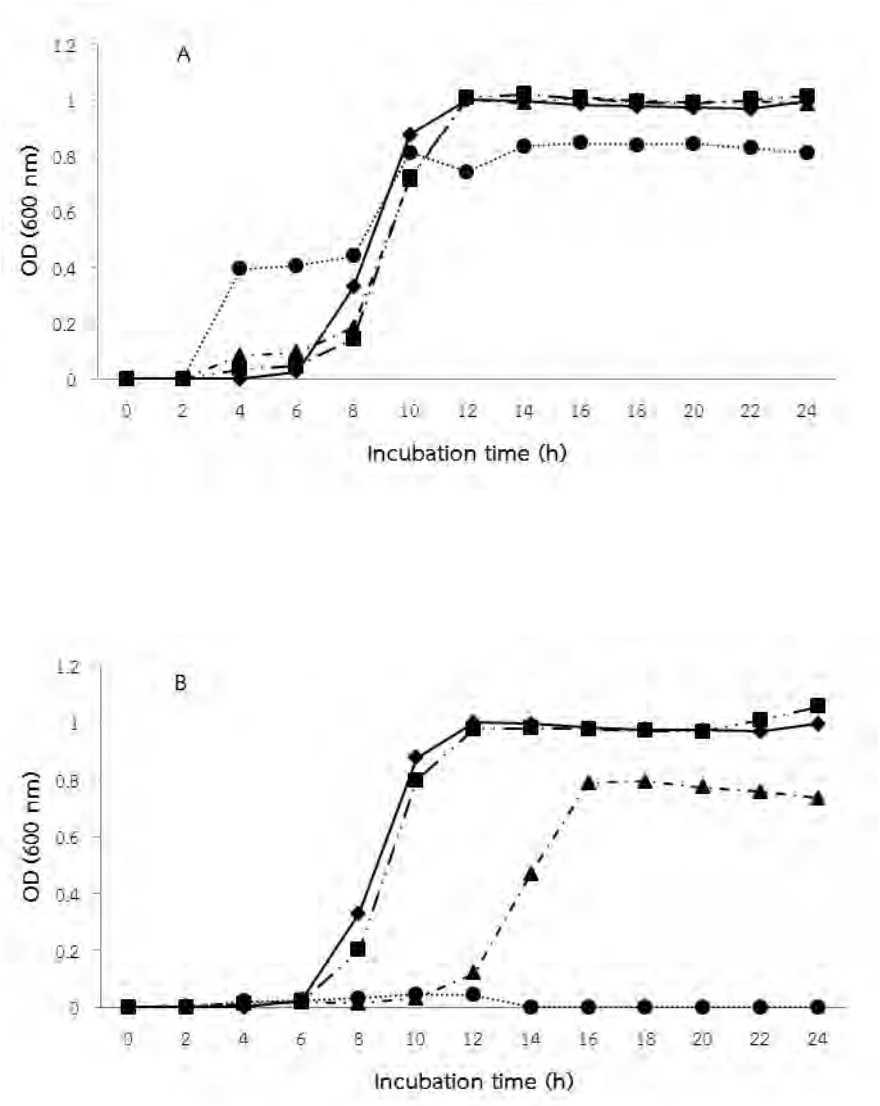

Figure 2 *E. coli* inhibition of gelatin hydrolysates by papain (A) and flavourzyme (B) with different DH (◆: control; ■: DH 10; ▲: DH 20; ●: DH 30)

The results showed that hydrolysis time had a significant effect on the growth inhibition percentage of each bacterium ($P < 0.05$). Song et al. [20] reported that halffin anchovy hydrolysates had antibacterial activities against *E. coli*. Moreover, Salampessy et al. [21] noticed the leatherjacket prepared by bromelain hydrolysis had antibacterial activity against *S. aureus* and *B. cereus*. On the other hand, this finding showed that the gelatin hydrolysates were more active in inhibiting the growth of Gram negative bacteria (*E. coli*) than that of Gram positive bacterium (*S. aureus*). Thus, the selections of suitable protease and time of hydrolysis are crucial due to the enzyme specificity and activity, producing peptides,

which vary in molecular size, amino acid sequences and consequent differences in antibacterial activity. Antibacterial activity of gelatin hydrolysates affected by the type of the protease used due to amino acid sequence of hydroltsates which different in amino acid sequence, secondary structure, length, molecular weight and charge, and DH Commonly, bromelain is used to enhance the hydrolysis especially, fermentation processes for preparation of soy and fish sauces from different protein sources which release antibacterial peptides [20]. Thus, antimicrobial peptides derived from the gelatin hysrolysate have the potential to be used as natural preservatives in food products.

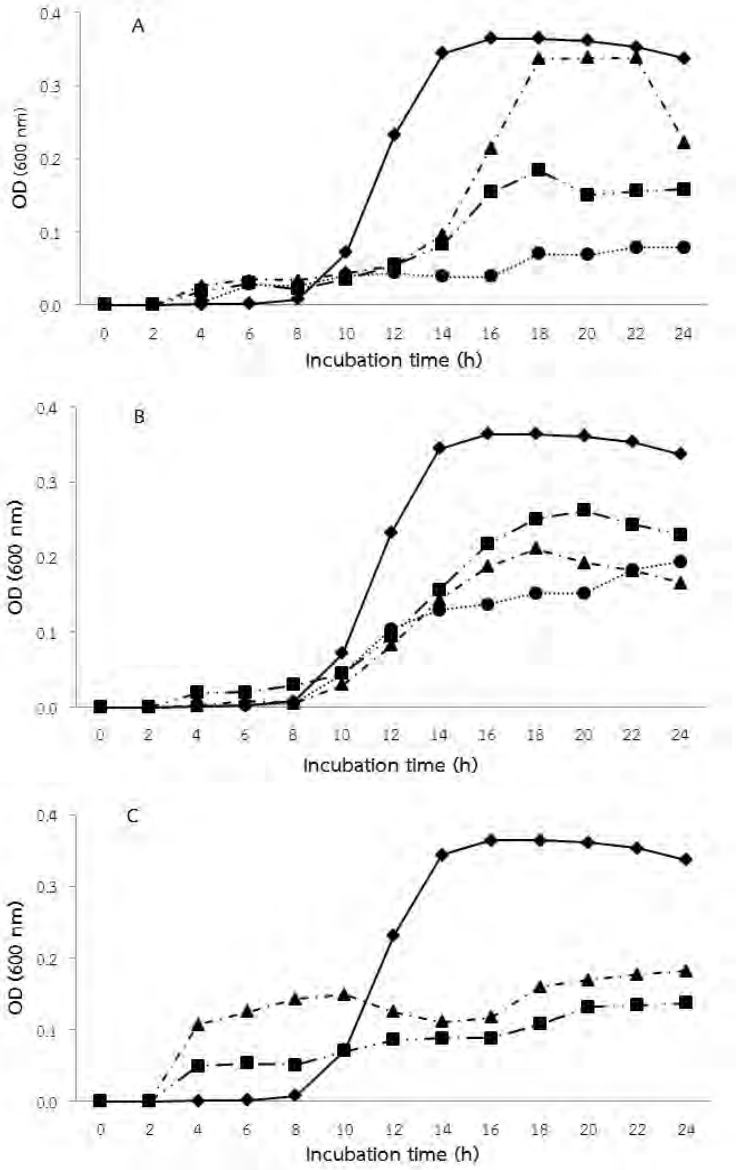

Figure 3 *S. aureus* inhibition of gelatin hydrolysates by trypsin (A), neutrase (B), and bromelain (C) with different DH (◆: control; ■: DH 10; ▲: DH 20; ●: DH 30)

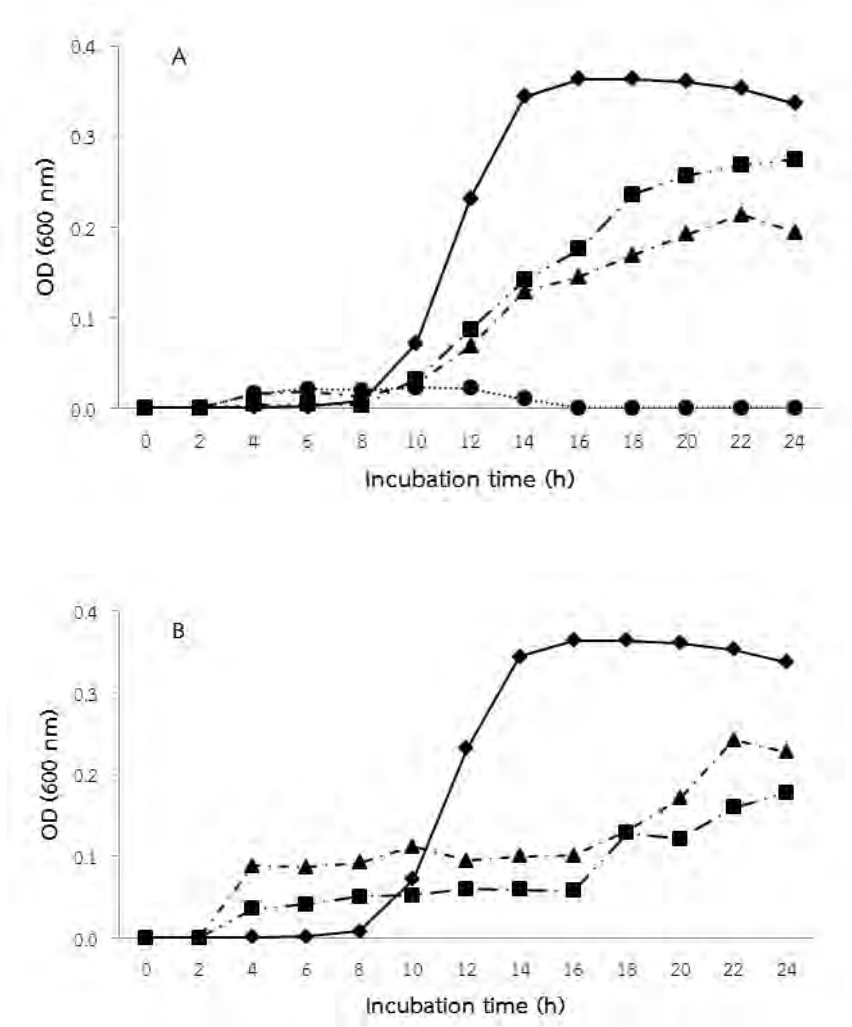

Figure 4 *S. aureus* inhibition of gelatin hydrolysates by papain (A) and flavourzyme (B) with different DH (◆: control; ■: DH 10; ▲: DH 20; ●: DH 30 for papain and ■: DH 31; ▲: DH 35; ●: DH 43 for flavourzyme)

Antibacterial activity of gelatin hydrolysates produced by different proteases against *E. coli* and *S. aureus*

Antibacterial activity of gelatin hydrolysates produced by different proteases against *E. coli* and *S. aureus* are showed in Figure 1. Among gelatin hydrolysates hydrolysed different proteases, gelatin produced by trypsin and flavourzyme showed the highest activity in bacteria inhibition. The α-amino acids content increased with increasing amount of enzyme ($P < 0.05$).

The result indicated that gelatin served as the better substrate for hysrolyse by trypsin and flavozyme than papain, bromelain and nutrease. It was noticed that there were no differences in antimicrobial activity of hydrolystes hydrolysed by papain with DH of 10 and 20% ($P > 0.05$). Gelatin hydrolysate produce by flavourzyme with DH of 20-40% exhibited higher activity than did trypsin ($P < 0.05$). Active antimicrobial peptides were more produced when DH increased. Increases in bioactive of hydrolysate with increasing DH have been reported in gelatin hydrolysate from bigeye snapper prepared using alcalase, neutrase and pyloric caeca extract [22], and gelatin hydrolysate from blacktip shark skin prepared using papaya latex enzyme [3]. Generally, the rate of enzymic cleavage of peptide bond controls the overall rate of hydrolysis [19]. DH directly influenced the peptide chain length and the exposure of terminal amino groups of products obtained [22][3]. Cleavage of peptides led to an enhanced metal ion binding capacity, plausibly due to the increased concentration of amino groups such as aromatic residues, Hyp, Pro, Ala or Gly of the N-terminus end of peptides [23][7][3]. Thus, gelatin hydrolysate with higher DH could act as a antibacterial agent, which was able to prevent spoilage in food system. Polar portions of peptides might interact with the liposomes of phospholipids, where they function effectively as antimicrobial agent which the activity of peptides or proteins is dependent on molecular size and properties such as hydrophobicity and electron transferring ability of the amino acid residues in the sequence [24]. Basically, antibacterial peptides inactivate bacteria through direct interaction with the target cell membrane cause disintegration of the lipid bilayer structure [25]. Therefore, peptides in unicorn leatherjacket fish skin gelatin hydrolysates produced either by protease from flavourzyme or trypsin had the antimicrobial properties in food system. Thus, GT30 and GF43 were used for further studies due to the highest in antimicrobial activity.

Table 2 Antimicrobial activity of GT30 and GF43 against *E. coli* and *S. aureus*.

Sample	Bacteria	Concentration (mg/ml)	Antimicrobial activity (%)
GT30	*E. coli*	10	13.42
		20	22.45
		40	22.66
		80	23.10
	S. aureus	10	No observed
		20	13.79
		40	No observed
		80	26.42
GF43	*E. coli*	10	8.30
		20	17.68
		40	55.40
		80	89.77
	S. aureus	10	14.69
		20	20.04
		40	37.76
		80	79.19

MIC, MBC and IC_{50} of GT30 and GF43 against *E. coli* and *S. aureus*

The results of MIC, MBC and IC_{50} showed that the GF43 has an antibacterial effect against *E. coli* and *S. aureus* bacterial species. As shown in Table 3, antimicrobial activity of GT30 showed no antibiotic activity in the concentration of 80 mg/mL. The MIC, MBC and IC_{50} values of GF43 were 80, 80 and 43.57 mg/ml against *E. coli* and *S. aureus*, respectively. Similarly, Gottardi et al., [26] reported that enhanced antimicrobial activity against *E. coli* was

observed in the glycated alcalase-derived hydrolysates and in the glycated flavourzyme-derived hydrolysates with both MIC and MBC of 40 mg/ml. In addition, the study of Taniguchi et al., [27] showed that IC50 values of peptic hydrolysates of rice bran protein were 75.6-78.5 μM against *P. gingivalis*. Antimicrobial activities of peptides are significantly

affected by positive charge and amphipathicity of their amino acid residues, reflecting binding to negatively charged surfaces and microbial membrane permeability [28]. The study of Mine et al. [29] reported that isolated antimicrobial peptides from hen egg white lysozyme hydrolysate using peptic and tryptic digestion showed the most active peptides against *E. coli* K-12 and *Staphylococcus aureus* 23-394. Peptide bioactivities are depended on numerous factors, including size, net positive charge, charge distribution, protein structure, and amphipathicity [27].

Table 3 MIC, MBC and IC_{50} of GT30 and GF43 against *E. coli* and *S. aureus*.

Sample	Bacteria	MIC (mg/mL)	MBC (mg/mL)	IC_{50} (mg/mL)
GT30	*E. coli*	-	-	-
	S. aureus	-	-	-
GF43	*E. coli*	80	80	43.57
	S. aureus	-	-	-

–: no activity was observed in the range of concentrations used.

Application of GT30 and GF43 in food system

The application of GT30 and GF43 as preservatives was added in orange juices. The gelatin hydrolysates as preservatives in fruit juices can be limited by their properties such as color and pH. The interaction of GT30 and GF43 with the juices components could be changed the juice pH and appearance of juice. Therefore, the physic-chemistry property is an essential characteristic for the gelatin hydrolysates to be used in the conservation of fruit juices. After 2 weeks, fruit juices supplemented with both gelatin hydrolysates showed no bacterial growth observed by total plate counts (data not show). When fruit juices were stored at 4 °C with GT30 and GF43, no significant differences in colour (L^* and b^*) were observed after 2 weeks of storage (Figure 5). Our results showed that GT30 and GF43 increased in a^* value of fruit juices when compare with control. However, value of the juices added with GT30 and GF43 were no difference during storage for 2 weeks. The differences in the colour of the juice added gelatin hydrolysates might be due to pigments and protein interaction.

When fruit juices were stored at 4 °C with or without GT30 and GF43, significant increase in pH were observed after 2 weeks of storage. For GF43, the pH reduction of juice

after added antimicrobial agents was found when compared with control samples and the juice added GT30 (Table 3). However, juices added with GF43 showed decrease in pH after storage for 2 weeks. In overall, these results indicated that GT30 and GF43 could remain stable in fruit juices to ensure the microbiological safety and appearance acceptability of the product. Thus, the application of GT30 and GF43 as preservative agent resulted in a new alternative for inactivate microorganism and suitable for fruit juice.

Table 4 pH value of orange juice treated with GT30 and GF43 during storage for 2 weeks.

Treatment orange juice	Storage time (week)		
	0	1	2
Control	4.13±0.01bA	4.18±0.01bB	4.35±0.14bC
GT30	4.17±0.04bA	4.23±0.01cB	4.30±0.01bC
GF43	4.00±0.07aB	4.03±0.01aB	3.58±0.15aA

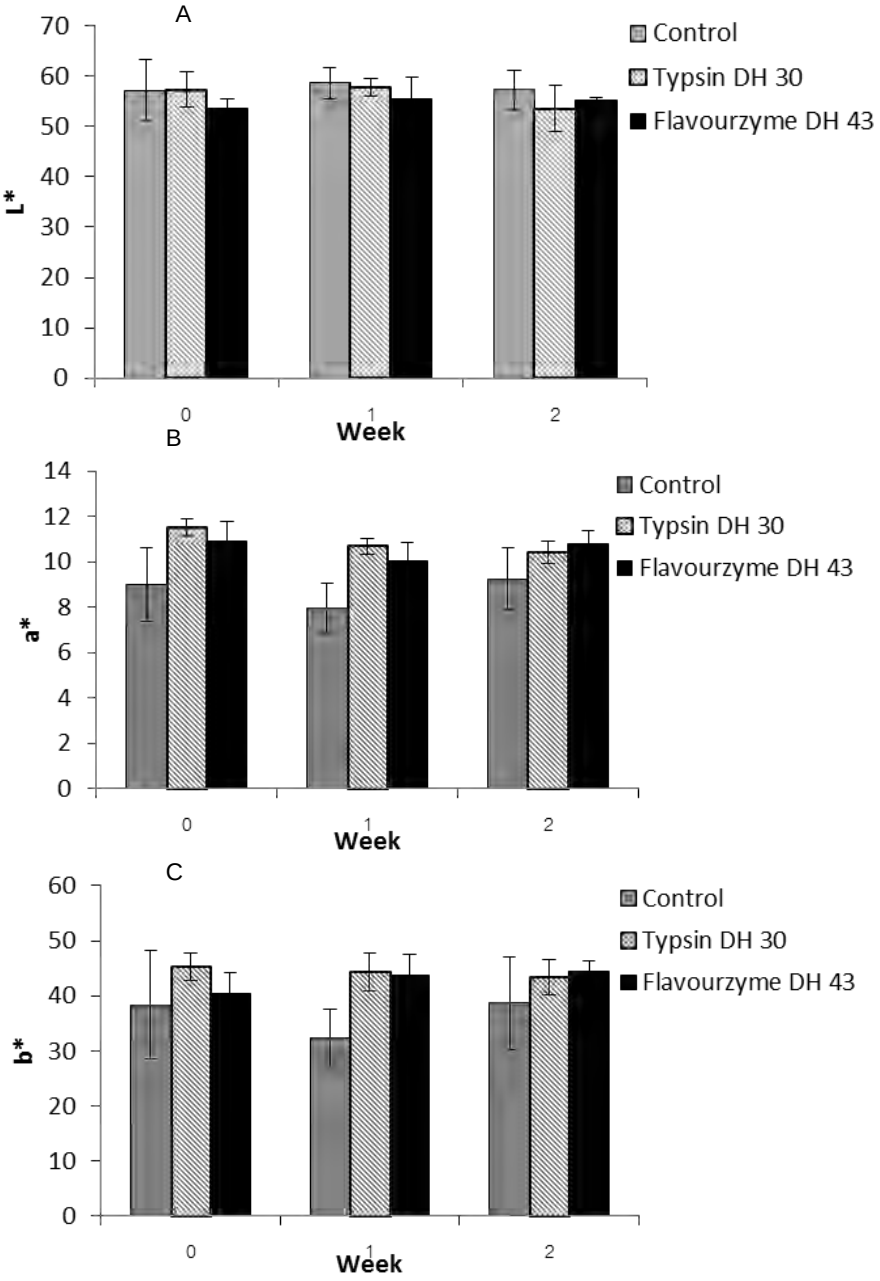

Figure 5 Color changes (*L**, *a** and *b**) of orange juice added gelatin hydrolysate prepared by trypsin with different DH during storage for 2 weeks.

Conclusion

Production of gelatin hydrolysates with antimicrobial activity could be achieved with the aid of trypsin and flavourzyme. The hydrolysate with high DH showed higher antimicrobial activity than that with low DH. Therefore, gelatin hydrolysate prepared using

protease from trypsin and flavourzyme might serve as a potential source of natural antimicrobial, which can prevent food bone pathogen.

Acknowledgements

The authors would like to thank Department of Food Science and Faculty of Science, Burapha University for financial support.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
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Effects of combined iron chelator with antioxidant on cardiac autonomic balance and cardiomyocytes calcium regulation in iron-overloaded mice

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Abstract:

Iron overload cardiomyopathy (IOC) is a major cause of morbidity and mortality in patients with secondary iron-overloaded and genetic hemochromatosis. A previous study has revealed that intracellular calcium ($[Ca^{2+}]i$) dysregulation played an important role in the pathophysiology of IOC. Iron overload could impair cardiac $[Ca^{2+}]i$ transient amplitude causing diminished contractility and leading to heart failure. Although either iron chelators deferiprone (DFP) or antioxidants N-acetyl cysteine (NAC) could provide cardioprotection, a combination of DFP plus NAC on cardiac autonomic balance and cardiac $[Ca^{2+}]i$ transient amplitude in chronic iron-overloaded mice has never been investigated. Adult C57/BL6 male mice were fed with either a normal diet or high iron diet (FE) for 4 months. At 3rd month, FE mice were divided into 4 groups and treated with either a vehicle, DFP (75 mg/kg/day), NAC (100 mg/kg/day), or combined DFP plus NAC. At 4th month, the levels of plasma NTBI and

cardiac iron concentration were increased in iron-overloaded mice, leading to cardiac iron overload, impaired cardiac autonomic balance and decreased cardiac $[Ca^{2+}]i$ transient amplitude. All pharmacological interventions decreased plasma NTBI and cardiac iron concentration (CIC), resulting in improvement of cardiac autonomic balance. Although DFP or NAC alone showed similar efficacy in reducing plasma NTBI and CIC, only combined DFP plus NAC could reduce CIC greater than individual treatment and resulting in restoration of the cardiac $[Ca^{2+}]i$ transient amplitude in iron-overloaded mice. Therefore, the combined DFP plus NAC was more effective than any monotherapy in reducing CIC and restoration of cardiac $[Ca^{2+}]i$ transient amplitude, which might be due to improvement of cardiac contractility in iron-overloaded mice.

Keyword: iron overload; iron chelator; antioxidant; cardiac autonomic balance; calcium regulation

Introduction

Iron overload condition is often seen in transfusion dependent thalassemia (TDT) patients because of the repeated blood transfusions, and also in hereditary hemochromatosis patients due to increased dietary iron absorption into the duodenal enterocytes (1). Regular blood transfusion is very important for patient with anemia for the improvement of the quality of life (2, 3). However, blood transfusions therapy and excessive iron absorption leading to plasma transferrin saturated and non-transferrin-bound iron (NTBI)can be found in plasma which increase accumulation in various organs and resulting in increased reactive oxygen species(4-8). Emphasis in heart, excess iron deposition in the heart increases the relative risk of cardiac dysfunction known as iron overload cardiomyopathy (IOC)(7). Moreover, IOC is a major cause of morbidity and mortality in patients with secondary iron-overloaded and genetic hemochromatosis (3, 9).

Oxidative stress-mediated iron toxicity can cause cardiac damage due to alteration of cardiac autonomic balance and several regulators of cardiac excitation-contraction coupling which can directly affect the myocyte electrical and contractile properties (5, 10). In addition, IOC probably impaired cardiac intracellular Ca^{2+} ($[Ca^{2+}]i$) transient amplitude resulting in diminished contractility and leading to heart failure as observed in iron overload cardiomyopathy (10, 11).

Iron chelation therapy is a gold standard treatment for a clinical problem with iron overload condition (1). Deferiprone (DFP) is one of the three iron chelators available for clinical use, to remove excess iron for the prevention of iron accumulation (1, 12). Previous studies reported that DFP monotherapy was superior to deferoxamine (DFO) and deferasirox (DFX) for the removal of cardiac iron and resulting in improvement of cardiac function (12-16). Recently, antioxidant compound N-acetyl cysteine (NAC) has been shown to effectively decrease systemic and cardiac iron overload as well as lowering of plasma and cardiac oxidative stress levels, leading to improved cardiac autonomic balance and left ventricular function in iron-overloaded rats (17). Interestingly, a combination of iron chelator DFP plus antioxidant NAC therapy has been shown to significantly decrease iron accumulation, oxidative stress and restore heart and brain functions in iron overloaded rats (11, 17-19).

However, the effects of either of iron overload condition and pharmacological intervention with iron chelator (deferiprone:DFP), antioxidant (N-acetyl cysteine:NAC) or combined DFP plus NAC on cardiac autonomic balance and cardiomyocytes intracellular Ca^{2+} transient amplitude in iron-overloaded mice has never been investigated.

Materials and Methods

Animals Model and Research Designs

Adult C57/BL6 male mice aged 3-6 months old (20-25 g.) were used (11). The study followed appropriate Chiang Mai University Standard Operating Procedures for animal identification. An animal holding room was used to house all mice in a controlled environment of 20-22°C, humidity (50 ± 10 %) and controlled lighting (12-hour day/night cycle). The mice were provided access to drinking water ad libitum throughout the entire experiment. Iron overload condition was induced by feeding mice with iron (FE) diet (0.2% ferrocene w/w), whereas mice in the control group(n=4) were fed with a normal diet (ND)for 3 months(20). Then, mice in the control group were treated with vehicle for 1 month. The mice in FE group were randomly divided into 4 subgroups (n=4/group) and treated with either a vehicle, DFP (75 mg/kg/day), NAC (100 mg/kg/day), or combined DFP (75 mg/kg/day) plus NAC (100 mg/kg/day) for 1 month with continuous Fe diet feeding.

Measurement of heart rate variability (HRV)

Heart rate variability (HRV) is a measurement of variation in the heart rate. Under physiological condition, heart rate is controlled by cardiac autonomic nervous activity involving the sympathetic and parasympathetic nervous system and can evaluate RR interval variability by spectral analysis. All mice underwent measurement of HRV at 4th-month. The lead II electrocardiogram (ECG) was recorded using needle electrodes and recorded continuously using Power Lab with chart 5.0. During the recording of the ECG, mice were placed in a familiar environment with unnecessary noise, put to calm, and prohibited from movement. The ECG data was analyzed using MATLAB program (21). The power spectra of RR interval variability were obtained using fast Fourier transform algorithm, and the high-frequency (HF: 0.6–3 Hz) and the low frequency (LF: 0.2–0.6 Hz) components were determined. The power below 0.2 Hz was considered as a very low frequency (VLF). Each spectral component was calculated by determining the area under the respective part of the power spectral density function and was presented in absolute unit (ms^2). To minimize the effect of changes in total power on the LF and HF components, LF and HF were expressed as normalized units (LFnu and HFnu) by dividing the LF and HF by the total power minus VLF. The LF/HF ratio was considered an index of autonomic balance (20-22).

Quantification of plasma non-transferrin bound iron (NTBI)

NTBI concentration was measured using the nitrilotri acetic acid disodium salt (NTA) chelation/flow cytometry method established by Ma and coworkers (23). Plasma was incubated with NTA solution (a final concentration of 80 mM) pH 7.0 for 30 minutes at room temperature to produce an Fe^{3+}-$(NTA)_2$ complex. Afterwards, the Fe^{3+}-$(NTA)_2$ was separated from plasma proteins by spinning the plasma mixture through a membrane filter (NanoSep®, 30-kDa cut off, polysulfone type; Pall Life Sciences, Ann Arbor, MI USA). Concentration of the Fe^{3+}-$(NTA)_2$ representing NTBI in the ultrafitrate was determined using a chelatable fluorescent beads based on flow cytometry (Guava Easy Cyte HT, Merck Millipore, Germany). Ferric nitrate was used as a standard, and was prepared at the final concentrations 0-10 μM. NTBI concentration was calculated from a standard curve using Graph Pad Prism software.

Cardiac iron determination

At the end of experiments, the hearts were removed and homogenized in deionized water. Then, the heart tissue homogenates were precipitated in precipitation solution (1 N HCl and 10% trichloroacetic acid) and heated at 95 ˚C for 1 hour. Then, the tubes were cooled down at room temperature, mixed with vortex, and then centrifuged at 8200 x g for 10 minutes. The supernatants were mixed with chromogen solution (0.508 mmol/l ferrozine, 1.5 mol/l sodium acetate and 0.1% or 1.5% (v/v) thioglycolic acid) and incubated at room temperature for 30 minutes. After incubation, the absorbance was measured at 562 nm and the cardiac iron concentration was compared with the iron standard curve (24).

Preparation of isolated ventricular cardiomyocytes

Cardiomyocytes were isolated from the hearts in all groups of mice. The hearts were removed and cannulated rapidly through the aorta. The cannulated hearts were perfused on a modified Langendorff perfusion apparatus with oxygenated normal Tyrode's solution, at 37 ºC in the first column at constant flow (~2 ml/mins) for 10 minutes. After that, the hearts were switched to the second column and perfused with oxygenated Ca^{2+}-free Tyrode's solution for 4 minutes. Then, the hearts were switched to the third column (enzymatic buffer), where they were digested with the 30 ml normal Tyrode's solution containing 100 µM $CaCl_2$ and 1 mg/ml of collagenase type II (Worthington Biochemical Corp., Lakewood, NJ, USA) for 7 minutes. After that, the hearts were cut off the cannula, and the aorta and atria were removed from the hearts. The remaining ventricular tissues were cut into several pieces and filtered through nylon mesh into a plastic tube. After that, the cardiomyocytes were left to settle down by gravity for 10 minutes. The supernatant was aspirated and the cell pellets were resuspended and incubated in the warm oxygenated Tyrode's solution containing 1 mM $CaCl_2$. The cardiomyocytes were ready for Ca^{2+} transient study (25, 26).

Intracellular Ca^{2+} transient measurement

The intracellular Ca^{2+} transient was measured by a fluorimetric ratio technique (27, 28) The isolated cardiomyocytes were incubated with 25 µM of Fura-2/AM (Sigma Chemical, St. Louis, MO, USA), the fluorescent Ca^{2+} indicator, at room temperature for 30 minutes to allow for intracellular deesterification of the Fura-2. Then, the cardiomyocytes were then perfused with a normal bath solution at 35±1ºC for 1 minute to wash out the

extracellular indicator. Ultraviolet light at the wavelengths of 340 and 387 nm with a monochromator were used for the excitation of the Fura-2 from a xenon arc lamp controlled by a microfluorometry system (OSP 100-CA, Olympus, Tokyo, Japan). The excitation light was directed into an inverted microscope (IX-70, Olympus, Tokyo, Japan). The emitted fluorescent intensity from Fura-2 was detected at wavelength 500 nm. The ratio of fluorescent emission of two wavelengths were recorded as the index of intracellular Ca^{2+}. The Ca^{2+} transient amplitude was measured during a 1-Hz field-stimulation with 10-ms twice-threshold strength square-pulses wave. The ratio of fluorescent emission of two wavelengths were processed and stored in a computer using Xcellence imaging software (Olympus, Tokyo, Japan).

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). The data were processed using SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) release 22.0 for Windows. Differences between groups were tested for via One-way ANOVA analyses. Significant difference between groups was assumed if P values < 0.05.

Results

Effects of the pharmacological interventions on plasma NTBI

The level of plasma NTBI were significantly higher in FE mice than ND control mice, suggesting that an iron overload conditions occurred in the FE-fed mice **(Figure 1).** On the other hand, plasma NTBI level was significantly decreased in iron-overloaded mice treated with DFP, NAC and combined DFP plus NAC treatment for 1 month and there were no significant differences among the groups of treatments **(Figure. 1)**.

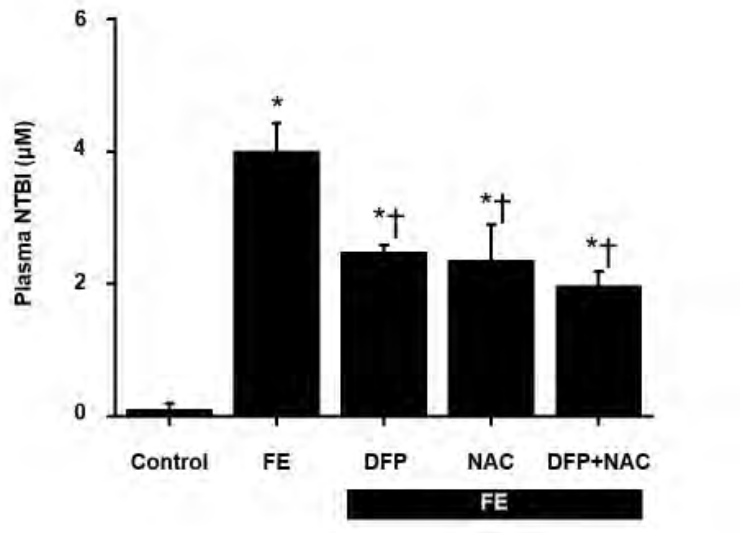

Figure 1. Effects of pharmacological interventions on plasma non-transferrin bound iron (NTBI) level in iron-overloaded mice (n=4/group). *p<0.05 vs control, †p<0.05 vs. FE.

Effects of the pharmacological interventions on cardiac iron concentration

The cardiac iron status showed that cardiac iron concentration was significantly increased in FE mice when compared with ND control mice **(Figure 2)**. Although DFP and NAC showed similar efficacy in reducing cardiac iron concentration, combined DFP plus NAC exerted the greater efficacy in reducing cardiac iron accumulation than monotherapy **(Figure. 2)**.

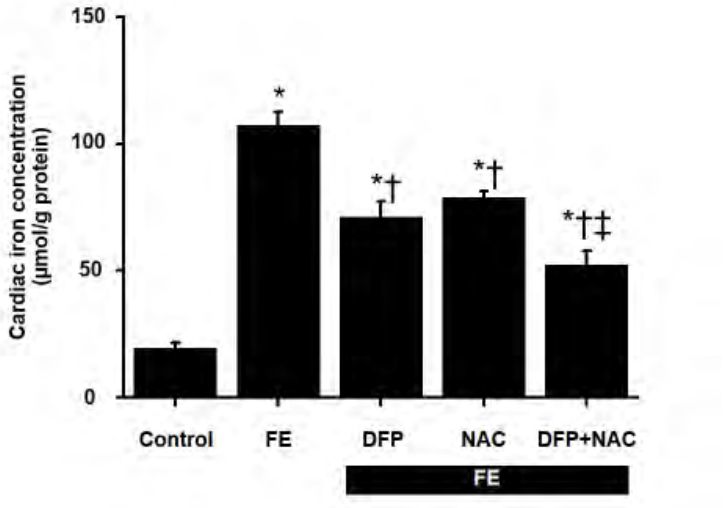

Figure 2. Effects of pharmacological interventions on cardiac iron concentration in iron-overloaded mice (n=4/group). *p<0.05 vs control, †p<0.05 vs FE, ‡p<0.05 vs. monotherapy.

Effects of the pharmacological interventions on heart rate variability

The LF/HF ratio represented to cardiac autonomic balance. LF/HF ratio was significantly higher in FE mice than ND control mice, indicating that iron-overloaded conditions impaired cardiac autonomic regulation **(Figure 3)**. DFP, NAC and combined DFP plus NAC significantly decreased LF/HF ratio when compared with FE group, suggesting that all pharmacological interventions could improve cardiac autonomic balance in iron-overloaded mice **(Figure 3)**.

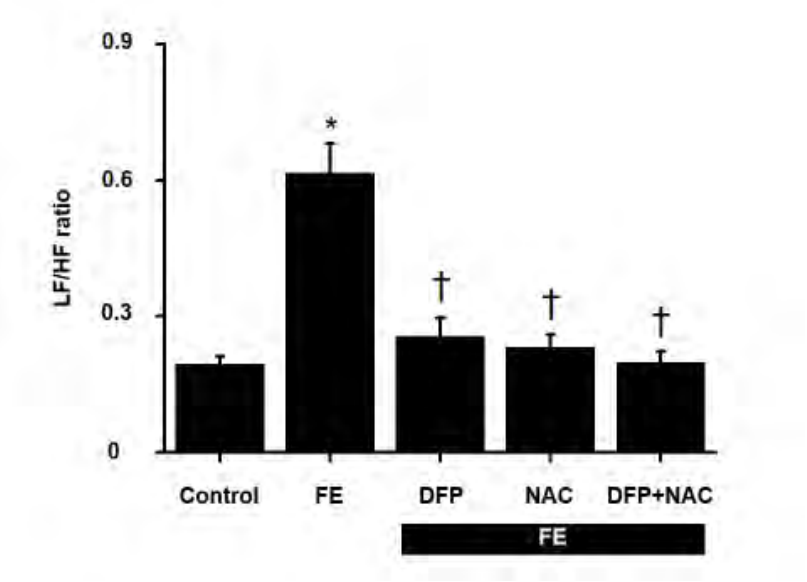

Figure 3. Effects of pharmacological interventions on heart rate variability (HRV) in iron-overloaded mice (n=4/group). Normalized low frequency power (LF), normalized high frequency power (HF). *p<0.05 vs control, †p<0.05 vs FE.

Effects of the pharmacological interventions on intracellular Ca^{2+} transient amplitude

The result found that the level of cardiac $[Ca^{2+}]i$ transient amplitude was significantly decreased in FE mice when compared with ND control mice **(Figure 4)**. After 1 month of treatment, DFP or NAC alone could not improve cardiac $[Ca^{2+}]i$ transient amplitude, whereas only combined DFP plus NAC restored cardiac $[Ca^{2+}]i$ transient amplitude in iron-overloaded mice back to normal level similar to ND control mice **(Figure 4)**.

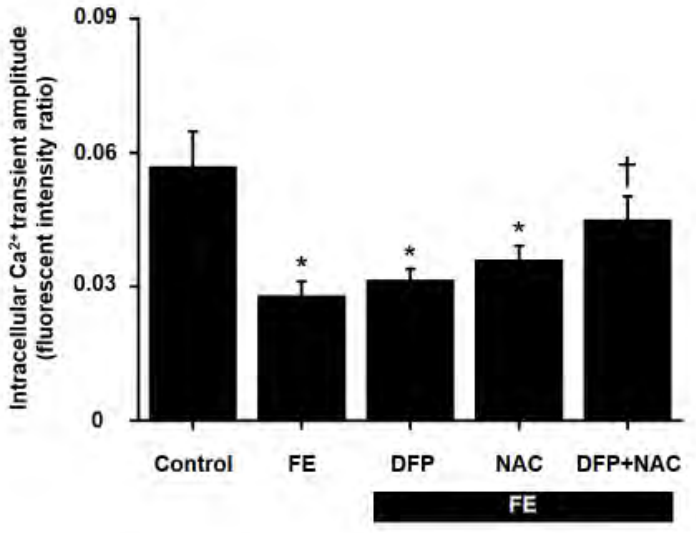

Figure 4. Effects of pharmacological interventions on intracellular Ca^{2+} transient amplitude in iron-overloaded mice (n=4/group). *p<0.05 vs control, †p<0.05 vs FE.

Discussion

The major findings in the present study are that 1) iron loading in mice led to significantly increased the levels of plasma NTBI, cardiac iron concentration, impaired cardiac autonomic balance as shown by increased LH/HF ratio and altered cardiac $[Ca^{2+}]i$ transient amplitude when compared with ND control mice, 2) DFP or NAC monotherapy could decrease plasma NTBI and CIC leading to improve cardiac autonomic balance, but not attenuated cardiac $[Ca^{2+}]i$ transient amplitude. 3) a combination therapy of DFP plus NAC could greatly reduce CIC, and restore cardiac $[Ca^{2+}]i$ transient amplitude when compared with monotherapy.

Consistent with previous study (11), our iron-overloaded mice showed increased level of NTBI and CIC that could generate reactive oxygen species (ROS), leading to cardiac damage due to impaired cardiac autonomic balance. Previous study indicated that Fe^{2+} competed with Ca^{2+} at the activating sites on RyR2 leading to decreasedCa^{2+}-induced Ca^{2+} release in rat hearts which might contribute to decrease cardiac $[Ca^{2+}]i$ transient amplitude, and caused impairment of cardiac contractility under iron overload condition (29).Our present study also showed the decreased cardiac $[Ca^{2+}]i$ transient amplitude in iron-overloaded mice which is consistent with previous study(11). Treatment withDFP or NAC alone showed similar efficacy in reducing plasma NTBI and CIC which led to improved cardiac autonomic balance in the present study. However, only combined DFP plus NAC could greatly reduce CIC

resulting in restoration of the cardiac $[Ca^{2+}]i$ transient amplitude in iron-overloaded mice. The mechanisms regarding the combination of DFP and NAC in reducing CIC and restoration of cardiac $[Ca^{2+}]i$ transients amplitude could be clarified by the following reasons. Firstly, DFP is an iron chelator that can effectively chelate the intracellularfree irons from heart tissue (17). Secondly, NAC also provides an iron chelating activity, which can effectively get rid of the excessive free iron in the heart (17, 30). Moreover, NAC is an antioxidant that can reduce ROS-mediated iron overload injury which lead to improved cardiac autonomic balance (31). Therefore, these two drugs havesynergistic effects in reducing CIC and restoration of cardiac $[Ca^{2+}]i$ transient amplitude in iron-overloaded mice in the present study.

In conclusion, treatment with DFP, NAC or DFP plus NAC exerted beneficial effect in reducing plasma NTBI and removal of CIC resulting inimprovement of cardiac autonomic balance in iron-overloaded mice. However, only combined DFP plus NAC could reduce CIC greater than monotherapy, resulting in restoration of the cardiac $[Ca^{2+}]i$ transient amplitude in iron-overloaded mice. Therefore, the combined DFP plus NAC mightimprove cardiac contractility in iron-overloaded mice.

Acknowledgements

This work is supported by grants from the Thailand Research Fund MRG5980222 (SK) and RTA6080003 (SCC), the NSTDA Research Chair Grant from the National Science and Technology Development Agency Thailand (NC), and a Chiang Mai University Center of Excellence Award (NC).

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Probiotic attenuates kidney dysfunction by reducing hypercholesterolemia and insulin resistance in high-fat diet-induced obese rats

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Abstract

There is an evidence indicating that dysbiosis of intestinal microbiota is associated with the development of obesity and insulin resistance, a condition which could affect to kidney function. This study aimed to investigate whether a probiotic intervention has a helpful effect on obesity, insulin resistance and kidney injury in high-fat diet-induced obese rats. Obesity was induced in male Wistar rats by high-fat diet giving for 12 weeks. After that, *Lactobacillus paracasei* HII011x10^8 CFU/ml were daily administered for 12 weeks by oral gavage. Energy intake, body weight, plasma total cholesterol, fasting blood glucose, microalbuminuria and oral glucose tolerance test (OGTT) were analyzed. Elevated body weight associated with increased plasma total cholesterol was observed in the obese rats. High-fat diet-induced obese rats also demonstrated kidney dysfunction as indicated by the significant increase in microalbuminuria.

The supplementation of *Lactobacillus paracasei* HII01 to the obese rats resulted in the reduction of plasma total cholesterol and microalbuminuria without the change in energy intake and body weight. Moreover, *Lactobacillus paracasei* HII01 also attenuated insulin resistance as shown by the significant decrease in total area under the curve of OGTT. Overall, these findings indicate that probiotic supplementation by *Lactobacillus paracasei* HII01 in the high-fat-diet-induced obese rats can restore hypercholesterolemia and insulin resistance which could lead to the improvement in kidney function.

Keywords: Probiotic: Insulin resistance; Obesity; Kidney function; Microalbuminuria

Introduction

Prolonged high-fat diet consumption is the cause of obesity, a common worldwide health problem. This condition leads to insulin resistance that can promote cardiovascular disease and diabetes mellitus. Moreover, obesity and insulin resistance also act as a risk factor for developing kidney injury and dysfunction(1). It is, therefore, reason able to elucidate the cause of obesity for management of obesity-related renal impairment. Interestingly, the studies in the past few years have documented that high-fat diet consumption is shown as a factor inducing gut microbiota dysbiosis which can lead to obesity and insulin resistance(2). Therefore, the increasing interest in the strategies to maintain gut microbiota composition, including probiotics, has been suggested.

Probiotic are identified as “live microorganisms which when administered in adequate amounts confer a health benefit to the host” (3). *Lactobacillus* and *Bifidobacterium* are the certain probiotic strains that have been used to attenuate obesity and insulin resistance in a model of high-fat-diet-induced obese animals (4-6). Also, there are the studies reported that probiotics also have the effect to improve renal function in animal models of kidney injury and dysfunction and the maintenance of CKD patients (7-9).However, the effect of probiotics on the kidney function in case of obesity is still ambiguous. The present study aimed to examine the effect of probiotic *Lactobacillus paracasei* HII01 on obesity, insulin resistance and kidney function in high-fat-diet-induced obese rats. We hypothesized that supplementation of *Lactobacillus paracasei* HII01 in the obese rats could attenuate obesity and insulin resistance, leading to delay the progression of kidney dysfunction.

Materials and Methods

Animals

Male Wistar rats were obtained from the National Animal Center, Mahidol University, Bangkok, Thailand. The animal facilities and protocols were approved by the Laboratory Animal Care and Use Committee at Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. All rats were housed in a room temperature 25 °C and at a light-dark cycle of 12/12 h. After 1 week of adaptation, rats were randomly divided into two groups: normal diet group (ND, n=6) and high-fat diet group (HFD, n=6). HFD-fed rats have received the diet composes of 59.28% energy from fat, and ND rats were received a standard chow (19.77% fat). The animals were given free access to drinking water. After 12 weeks of obesity induction, blood samples and urine 24 hours were collected to determine metabolic and kidney function parameters. Rats in each group were then randomly divided into vehicle group (ND or HFD, n=3/group) and probiotic-treated group (ND-Lac or HF-Lac, n=3/group).

Lactobacillus paracasei HII01, a non-human origin-isolated strain of lactic acid-producing bacteria (obtained from Assistant Professor Chaiyavat Chaiyasut and his colleagues from the Faculty of Pharmacy, Chiang Mai University), which has been approved by Thailand Food and Drug Administration (FDA) was administered to the rat by oral gavage at $1x10^8$ CFU/ml/day. The supplementation period was continued for 12 weeks. At the end of the experiment, oral glucose tolerance test (OGTT) were performed, and blood and urine were collected.

Metabolic parameters

After fasting, the rats were anesthetized by isofuran. A blood sample was collected from a lateral tail vein. The plasma glucose and total cholesterol levels were determined using commercially colorimetric diagnostic kits (ERBA Diagnostic Mannheim GmBH, Germany). The body weight and energy intake were recorded.

Renal function assessment

The microalbuminuria was estimated to investigate the renal injury and dysfunction. The rats were placed in metabolic cages for 24 hours to collect the urine. Microalbumin in the urine was measured using an automatic biochemical analyzer at the Clinical Laboratory, Maharaj Nakhon Chiang Mai Hospital, Chiang Mai, Thailand.

Oral glucose tolerance test (OGTT)

OGTT was performed after 12 weeks of treatment. After fasting for 12 hours, the rats have orally received glucose solution (2 g/kg body weight). The blood samples were collected to determine plasma glucose levels at 0, 15, 30, 60 and 120 min intervals. Total area under the curve (AUC) of OGTT was calculated to determine insulin resistance.

Statistical analysis

Results were presented as mean ± SEM. The differences between two and four groups were examined using independent-samples t-test and One-way ANOVA followed by Tukey post-hoc test, respectively. *P*-values less than 0.05 were considered significant.

Results

Metabolic profiles and kidney function before probiotic treatment

The parameters after 12 weeks of obesity induction by high-fat diet are shown in Table 1. The HFD group showed significant increases in energy intake, body weight and the level of plasma total cholesterol when compared to the control ND group ($p<0.05$). In contrast, there was no significant difference in fasting blood glucose level between HFD and ND groups. Moreover, the significant increase in microalbuminuria was found in the HFD when compared to the ND rats ($p<0.05$). These results indicated that providing a high-fat diet for 12 weeks to the rats was able to induce obesity and impair the function of the kidney.

Table 1 The parameters before probiotic treatment

Parameters	ND	HFD
Energy intake (kcal/day)	82.14 ± 0.81	128.83 ± 2.51*
Body weight (g)	436.67 ± 10.22	616.67 ± 9.19*
Total Cholesterol (mg/dl)	52.23 ± 3.59	101.10± 3.54*
Fasting blood glucose (mg/dl)	130.10 ± 2.85	136.11 ± 4.05
Microalbuminuria (mg/g creatinine)	17.26 ± 3.07	44.10 ± 5.06*

Data are mean ± SEM with six rats in each group. *$p<0.05$ compared with ND.

Metabolic profiles and kidney function after probiotic treatment

The changes in metabolic and kidney function parameters after 12 weeks of probiotic intervention are shown in Table 2. At week 24, there were significant increases in energy intake, body weight and plasma total cholesterol in HFD when compared to the ND rats (p<0.05). *Lactobacillus paracasei* HII01 administration to the obese rats (HF-Lac group) significantly reduced plasma total cholesterol (p<0.05), but could not decrease energy intake and body weight when compared to the HFD rats. There was no significant difference in fasting blood glucose levels among four groups. In addition, increased microalbuminuria in the obese rats was significantly attenuated by *Lactobacillus paracasei* HII01 treatment (p<0.05). These results demonstrated that probiotic l*actobacillus paracasei* HII01 could reduce hypercholesterolemia and restore the kidney dysfunction in the high-fat-diet-induced obese rats.

Table 2 The parameters after probiotic treatment

Parameters	ND	ND-Lac	HFD	HF-Lac
Energy intake (kcal/day)	80.22 ± 1.98	80.40 ± 1.70	130.36 ± 1.03*,†	130.43 ± 0.55
Body weight (g)	77.96 ± 1.43	80.40 ± 1.70	131.11 ± 0.38*,†	129.19 ± 0.89
Total cholesterol (mg/dl)	66.08 ± 4.97	71.62 ± 8.33	123.85 ± 4.78*,†	77.12 ± 3.76‡
Fasting blood glucose (mg/dl)	137.25 ± 5.00	138.17 ± 5.45	141.95 ± 11.42	141.67 ± 2.07
Microalbuminuria (mg/g creatinine)	14.89 ± 0.78	25.23 ± 9.45	82.43 ± 25.88*	17.00 ± 7.00‡

Data are mean ± SEM with three rats in each group. $^{*}p$<0.05 compared with ND. $^{\dagger}p$<0.05 compared with ND-Lac. $^{\ddagger}p$<0.05 compared with HFD.

Effect of probiotic supplementation on insulin sensitivity in high-fat-diet-fed rats

As shown in Figure 1A, HFD-fed rats showed a higher level of fasting blood glucose 60 and 120 min after glucose loading than those of the ND and ND-Lacrats (p< 0.05). Interestingly, oral supplementation with *Lactobacillus paracasei* HII01 in HF-Lac rats could

restore these increasing blood glucose levels when compared with HFD rats ($p< 0.05$).Along with the results shown in Figure 1A, the total area under the curve (AUC)of the HFD rats was significantly larger than that of the ND and ND-Lac rats ($p<0.05$), indicating that insulin resistance was presented. After *Lactobacillus paracasei* HII01 supplementation, the total AUC was significantly reduced when compared with HFD rats ($p<0.05$). These data demonstrated that probiotic *Lactobacillus paracasei* HII01 affects decreasing insulin resistance in high-fat-diet-induced obese rats.

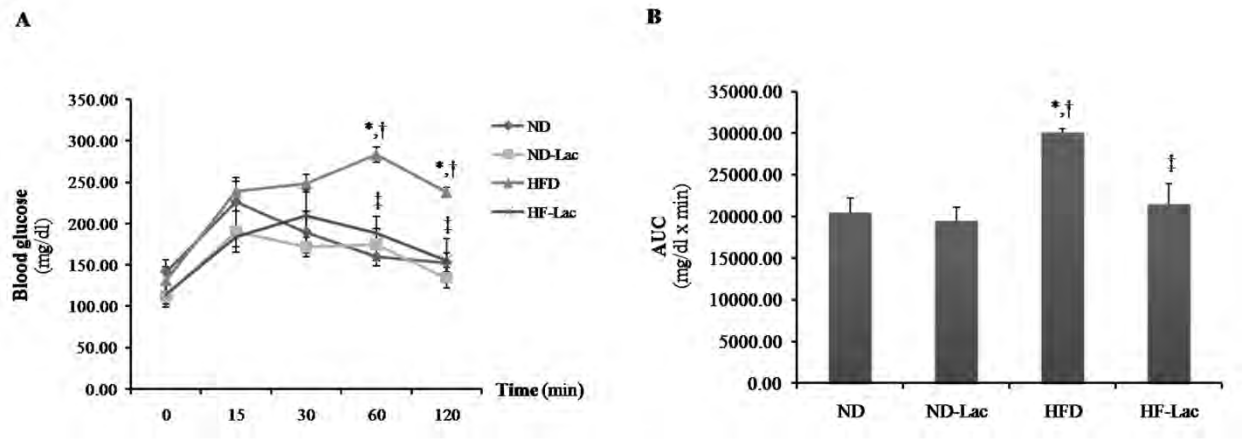

Figure 1 Insulin sensitivity in the obese rats and the effect of probiotic treatment. (A)Oral glucose tolerance test (OGTT) and (B)total area under the curve (AUC) are shown. Data are mean ± SEM with three rats in each group. The significant difference is presented by $^{*}p<0.05$ compared with ND, $^{\dagger}p<0.05$ compared with ND-Lac, and$^{\ddagger}p<0.05$ compared with HFD.

Discussion

The present study demonstrates that prolonged consumption of high-fat diet induces obesity and promotes kidney dysfunction. The intervention with probiotic *Lactobacillus paracasei* HII01 attenuated the kidney dysfunction associated with the decreases in hypercholesterolemia and insulin resistance in high-fat-diet-induced obese rats. The rats fed with high-fat diet exhibited obesity which is represented by the increases in energy intake, body weight, and plasma total cholesterol level. These results were demonstrated along with the increase in microalbuminuria, a marker of kidney injury and dysfunction. Recently, the previous study has shown that obesity is related to the modulation of renal function by the

releasing of adipocyte-derived factors that play a role in podocyte injury (10). Our study also showed that there was no significant difference in fasting blood glucose level. However, the OGTT and total AUC showed the significant difference in the high-fat-diet-induced obese rats as compared to ND rats. These findings indicate that the insulin resistance was developed in obese rats in this study. The previous study reported that insulin resistance is associated with the leakage of albumin into the urine and promotes the progression of chronic kidney disease (CKD) (11). It could suggest that prolonged high-fat diet consumption leading to obesity and insulin resistance is the factor promoting renal impairment.

Using probiotic *Lactobacillus paracasei* HII01 for the managing of obesity-induced kidney dysfunction in this study resulted in the reduction of plasma total cholesterol level along with the decrease in micro albumin uria although the energy intake and body weight were not affected. The previous study has been reported that Lactobacilli has a hypocholesterolemic effect. They could modulate the lipid metabolism by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an enzyme responsible for cholesterol synthesis, and also by enhancing bile salt hydrolase activity (12, 13). It is recently recognized that gut dysbiosis is involved in the development of obesity and low-grade systemic inflammation which could promote organ injury (14).There was a study demonstrated that treatment with *Lactobacillus plantarum* could decrease body weight and plasma lipid profiles in high-fat-diet-induced obese rats. These findings were accompanied by the attenuation of systemic inflammation and a reduction in kidney injury and dysfunction (15). In addition, a recent study has shown that probiotic treatment in diabetic hemodialysis patients can improve insulin sensitivity, inflammation and oxidative stress (16). Taken together, it could probably propose that *Lactobacillus paracasei* HII01treatment in the present study could alter the intestinal environment which would then reduce low-grade systemic inflammation and insulin resistance, and resulted in the attenuation of kidney dysfunction.However, the possible mechanism effects of *Lactobacillus paracasei* HII01 on the improvement of kidney function that involved in low-grade systemic inflammation, kidney inflammation, and oxidative stress are the topics that the authors plan to explore.

The results obtained from this study conclude that probiotics may play a role in attenuating the hyperlipidemia as well as insulin resistance that contribute to the improvement of kidney function in the high-fat-diet-induced obese condition.

Acknowledgement

The authors acknowledge National Research Council of Thailand, Thailand Research Fund RSA6080015, the Faculty of Medicine Research Fund, Chiang Mai University and Chiang Mai University World Class University (CMU-WCU) for a grant supporting this project.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Testosterone replacement therapy attenuates hippocampal oxidative stress in testosterone-deprived obese rats

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Abstract

Obesity has been associated with testosterone deficiency in animals and humans. Previous studies demonstrated that either testosterone deprivation or obesity could increase brain oxidative stress, which led to impaired cognitive function. In addition, testosterone replacement improved cognitive function in testosterone-deprived rats. However, the effects of obesity followed by testosterone deprivation on metabolic parameters, brain oxidative stress, and cognitive function are still unclear. In addition, the effects of testosterone replacement on brain oxidative stress and cognitive function in this condition have never been investigated. We hypothesized that testosterone deprivation aggravates peripheral insulin resistance, brain oxidative stress, and cognitive dysfunction in obese rats, and testosterone replacement therapy decreased these impairments in testosterone-deprived obese rats. Thirty-two male rats received either a normal diet (ND) or a high-fat diet (HF) for a total duration of 28weeks.At week 13th, ND rats received sham-operation (NDS). In HF group, rats were divided into 2 operation groups, sham operation (HFS) or orchiectomy (HFO). At week 25th, rats were randomly assigned to the treatment groups: (1) NDS treated with vehicle (NDSV), (2) HFS treated with

vehicle(HFSV) , (3) HFO treated with vehicle (HFOV), (4) HFO treated with testosterone replacement (HFOT; 2 mg/kg/day). Rats were treated for 4 weeks, cognitive function and metabolic parameters were determined. Rats were then sacrificed, and hippocampi were used to determine hippocampal oxidative stress. The result demonstrated that high-fat fed rats exhibited obese-insulin resistance, cognitive impairment, and increased hippocampal oxidative stress. Testosterone deprivation aggravated hippocampal oxidative stress and cognitive dysfunction in the obese rat. In addition, testosterone replacement reduced hippocampal oxidative stress; however, it did not affect cognitive function in testosterone-deprived obese rats. These data indicated that testosterone deprivation aggravated hippocampal oxidative stress and cognitivedys function in obese rats. Testosterone replacement therapy decreased hippocampal oxidative stress, but not cognitive function, in testosterone-deprived obese rats.

Keywords Testosterone replacement therapy; Obese-insulin resistance; Cognitive function; Testosterone deprivation; Hippocampal oxidative stress

Introduction

Obesity is a major public health problem that has become epidemic worldwide. It is a major risk factor of many diseases including neurodegenerative disorders(1). Obesity is directly related to long-term high-fat diet (HFD) consumption (2, 3). Several previous studies demonstrated that long-term HFD consumption caused both peripheral and brain insulin resistance, as indicated by increased plasma insulin levels, increased HOMA index and impaired brain insulin sensitivity (4, 5). In addition, 12-week HFD consumption in rats could also lead to brain oxidative stress, which subsequently resulted in cognitive impairment (6-8).

Testosterone not only plays an important role in the reproductive system but also involves in metabolic control and cognitive function (9).There are reports showing that testosterone levels were declined in obese subjects (10, 11). In addition, previousstudiesdemonstrated that either testosterone deprivation or obesity increased the oxidative stress level, and led to the development of insulin resistance and cognitive impairment (6, 12). Recently, Pintana and colleagues also reported that both obesity and testosterone deprivation increased brain oxidative stress and impaired hippocampal synaptic plasticity, which caused cognitive impairment, and obesity did not aggravate these impairments in testosterone deprived-rats (19). However, the effects of obesity followed by testosterone

deprivation on metabolic parameters, brain oxidative stress, and cognitive function have never been investigated. Furthermore, previous studies found that testosterone replacement therapy could decrease cognitive decline in testosterone-deprived rats (13, 14) and hypogonadal men with Alzheimer's disease (15). However, the effects of testosterone replacement therapy on brain oxidative stress and cognitive function in testosterone-deprived obese condition have never been investigated. Therefore, we hypothesized that 1) testosterone deprivation aggravated peripheral insulin resistance, brain oxidative stress and cognitive dysfunction in obese rats, and 2) testosterone replacement therapy improved these impairments in testosterone-deprived obese rats.

Materials and Methods

Animal models and experimental protocols

All experimental protocols were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care, and Use Committee, in compliance with NIH guidelines. Thirty-two male Wistar rats weighing 180-200 g were obtained from the Nomura Siam International Co., Ltd., Bangkok, Thailand. The rats were housed in a room with controlled temperature (25 °C) and humidity. Rats were divided into 2 dietary groups, receiving either a normal diet (ND; 19.77%E fat, n=8) or a high-fat diet (HF; 59.28%E fat, n=24) for total 28 weeks. At week 13,ND rats received sham-operation (NDS) and HF rats received either sham-operation (HFS) or orchiectomy (HFO). At week 25, sham rats received vehicle (castor oil; NDSV; HFSV) and orchiectomized rats received either vehicle (castor oil; HFOV) or testosterone replacement (2 mg/kg/day of testosterone enanthate (Bayer Schering, Berlin, Germany); s.c.; HFOT) for 4 weeks. At the end of the experiment, all animals were taken to have their learning and memory tested with Morris water maze test (MWM). Then, blood samples were also collected from the rats after a period of 5-hour fasting in order to measure glucose and insulin levels. Finally, all animals were deeply anesthetized with isoflurane and decapitated. The brains were rapidly removed for hippocampal oxidative stress determination.

Bilateral orchiectomy procedure

Rats were anesthetized and maintained using 2% isoflurane, and the scrotal area was shaved under a sterile technique. Orchiectomy was performed by the scrotal approach

technique. The rats were carefully monitored to prevent them from chewing their sutures and also to observe for any other complications.

Metabolic parameters determination

Body weight, visceral fat weight, plasma glucose and insulin levels were determined. Fasting plasma insulin levels were measured using the sandwich enzyme-linked immunosorbent assay (ELISA) kit (Millipore, MI, USA). Fasting plasma glucose levels were measured using a colorimetric assay kit (ERBA Mannheim, Mannheim, Germany).

Hippocampal reactive oxygen species (ROS) assay

The details of ROS measurement were previously described(8).Briefly, hippocampi were rapidly removed from the brain and transferred into 1 ml of ice-cold MSE solution.Dichloro-hydrofluoresceindiacetate (DCFH-DA) dye was used to determine hippocampal ROS production. Fluorescence intensity was determined at the excitation wavelength of 485 nm and the emission wavelength of 530 nm by a microplate reader (Bio-tek Instrument, Inc. Winooski, Vermont USA).

Morris water maze test (MWM)

The protocol of MWM was modified from a previous study (16). This experiment determined learning and memory are abilityusing two different types of MWM, acquisition test and probe trial test as described in our previous study (17). In the acquisition test, rats were trained to navigate a route to the hidden platform in 4 trials/day for 5 days, with an inter-trial interval of 15 s. For each trial, rats were given 120 s to find the platform, and the time taken to reach the platform was recorded. In the probe trial test, the platform was removed, and the time spent in the target quadrant was recorded. After the test was completed, time taken to reach the platform, time spent in the target quadrant, and swimming speeds were calculated usingSmart 3.0 software (Panlab, Harvard Apparatus, and Barcelona, Spain).

Statistical analysis

Data from each experiment were expressed as mean ± S.E.M. For all multiple comparisons; data were analyzed using three-way ANOVA followed by post-hoc least

significant difference analysis. For the behavioral test, the significance of the differences of acquisition test results was calculated using repeated three-way ANOVA followed by post-hocleast significant difference analysis. $P < 0.05$ was considered as statistically significant.

Results

Effect of testosterone replacement therapy on metabolic changes in testosterone-deprived obese rats

High-fat fed rats developed obesity and insulin resistance as indicated by increased body weight, increased visceral fat weight and increased fasting plasma insulin level with euglycemia (Table1, $p<0.05$). Testosterone deprivation did not aggravate obesity or insulin resistance in obese rats. Testosterone replacement therapy did not decrease obesity or insulin resistance in testosterone-deprived obese rats.

Table 1. Metabolic parameters after 4 weeks of testosterone replacement therapy.

Parameter	Treatment			
	NDSV	HFSV	HFOV	HFOT
Body weight (g)	513±19	673±32*	600±15*,†	605±11*,†
Visceral fat (g)	31.1±2.9	66.6±3*	55.9±2*,†	54.7±3*,†
Plasma glucose (mg/dl)	145.16±5	147.40±4.4	147.40±4.4	138.80±4.2
Plasma insulin (ng/ml)	3.43±0.3	5.63±0.53*	5.57±0.2*	5.42±0.42*

*p<0.05 vs. NDSV, †p<0.05 vs. HFSV. NDSV; sham ND-fed rats with vehicle treatment, HFSV; sham HF-fed rats with vehicle treatment, HFOV; orchiectomized HFD-fed rats with vehicle treatment, HFOT; orchiectomized HFD-fed rats with testosterone treatment.

Effect of testosterone replacement therapy on hippocampal oxidative stress and cognitive function in testosterone-deprived obese rats

High-fat fed rats increased hippocampal oxidative stress as indicated by increased ROS production (Fig.1, $p<0.05$). In addition, high-fat fed rats also had impaired cognitive function as indicated by increased time to reach the platform and decreased time in the target quadrant (Fig.2A and B, $p<0.05$). Testosterone deprivation aggravates hippocampal oxidative stress and

cognitive dysfunction in obese rats. Testosterone replacement therapy decreased hippocampal oxidative stress but failed to improve cognitive impairment in testosterone-deprived obese rats.

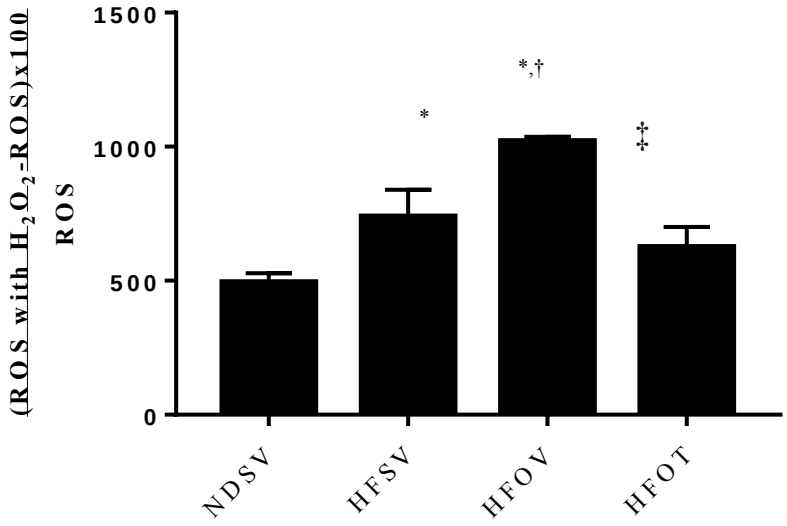

Fig.1. The effects of testosterone replacement therapy on hippocampal oxidative stress levels in testosterone-deprived obese rats.

*p<0.05 vs. NDSV, † p<0.05 vs. HFSV, ‡ p<0.05 vs. HFOV. NDSV; sham ND-fed rats with vehicle treatment, HFSV; sham HF-fed rats with vehicle treatment, HFOV; orchiectomized HFD-fed rats with vehicle treatment, HFOT; orchiectomized HFD-fed rats with testosterone treatment

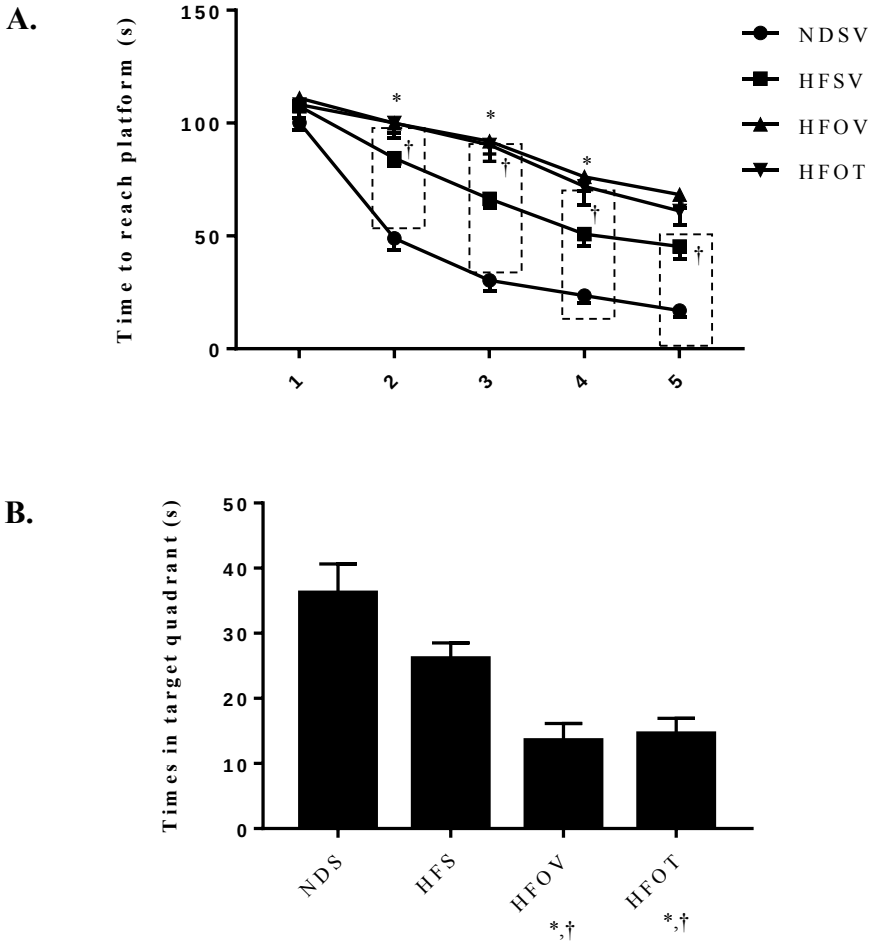

Fig.2. The effects of testosterone replacement therapy on cognitive function in testosterone-deprived obese rats. (A)Acquisition trial in MWM test (B) Probe trial in MWMtest

* p<0.05 vs. NDSV, † p<0.05 vs. HFSV, ‡ p<0.05 vs. HFOV. NDSV; sham ND-fed rats with vehicle treatment, HFSV; sham HF-fed rats with vehicle treatment, HFOV; orchiectomized HFD-fed rats with vehicle treatment, HFOT; orchiectomized HFD-fed rats with testosterone treatment

Discussion

The major findings of the present study were as follows:1) the testosterone deprivation aggravatedhippocampaloxidative stress and cognitive dysfunction in obese rats; 2) testosterone replacement therapyrestoredhippocampal oxidative stress, but failed to improve cognitive impairment in testosterone-deprived obese rats.

Our findings confirmed the effects of high-fat diet consumption on obesity and peripheral insulin resistance as shown in several previous studies (6, 18). Interestingly, we found that testosterone deprivation did not aggravate obesity or peripheral insulin resistance in obese rats. Moreover, testosterone replacement did not restore peripheral insulin insensitivity in testosterone-deprived obese rats. In contrast, a previous study found that testosterone replacement improved peripheral insulin sensitivity in these testosterone-deprived obese rats(14).The possible explanation of this controversy could be due to the different durations of testosterone replacement therapy.We found that high-fat fed rats increased hippocampal oxidative stress and impaired cognitive function. Testosterone deprivation aggravated hippocampal oxidative stress, which leads to aggravate cognitive dysfunction in obese rats. Similarly, previous studies reported that either testosterone deprivation or obesity increased brain oxidative stress, which leads to impaired hippocampal synaptic plasticity, and caused cognitive impairment (14, 19). Interestingly, we found that testosterone replacement therapy decreased hippocampal oxidative stress in testosterone-deprived obese rats. The consistent with previous study,testosterone treatmentreduced brain oxidative stress and increased brain antioxidants levels in testosterone-deprived rats (14). Regarding to the cognitive function, previous studies found that testosterone replacement restored cognitive function in testosterone-deprived rats possibly through the antioxidant effect (12, 20).However, testosterone treatment in this study failed to restore the cognition. This contrary might be due to the difference in the dose of testosterone that given to the rats. Thus, our data indicated that although 2 mg/kg of testosterone exerted its beneficial effect on hippocampal oxidative stress, it was insufficient to restore cognitive function in testosterone-deprived obese rats. Therefore, a higher dose of testosterone or the combination of testosterone with other drugs may improve cognitive function in testosterone-deprived obese rats. In conclusion, the present study indicated that testosterone deprivation aggravated hippocampal oxidative stress and impairment of cognitive function. Testosterone replacement therapy successfully decreased hippocampal oxidative stress, but not cognitive function, in testosterone-deprived obese rats.

Acknowledgements

This work was supported by Thailand Research Fund Grants TRF- RTA6080003 (SCC), TRG6080005 (NA), MRG5980198 (WP); a NSTDA Research Chair Grant from the National Science and Technology Development Agency Thailand (NC); a Faculty of Medicine, Chiang Mai University Endowment Fund (NA), and Chiang Mai University Center of Excellence Award (NC).

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
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Testosterone replacement therapy and dipeptidyl peptidase 4 inhibitor shared similar cardioprotective effects in obese-insulin resistant rats with testosterone deprivation.

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Abstract

Obese-insulin resistance and testosterone deprivation are related to cardiac dysfunction. Testosterone replacement therapy (TRT) could restore circulating testosterone levels in subjects with hypogonadism. However, long-term TRT showed an adverse effect to the heart such as increasing the risk of myocardial infarction. Our previous study showed that vildagliptin, a dipeptidyl peptidase 4 inhibitor, reduced cardiac ischemia/reperfusion injury in obese orchiectomized rats. Although vildagliptin exerted favorable effects on the heart, vildagliptin could not restore testosterone levels. Therefore, we hypothesized that combined low-dose TRT and vildagliptin would improve metabolic parameters and cardiac function better than single regimen in obese-insulin resistant rats with testosterone deprivation. In this study, male Wistar rats were fed with either normal diet (ND) or high-fat diet (HFD) for 12 weeks. HFD rats were subdivided into 2 operation groups: sham operationand orchiectomy (ORX). HFD with ORX rats were subdivided into 5 intervention groups, namely, vehicle, physiological dose of TRT (2 mg/kg/day), vildagliptin (3 mg/kg/day), and combined low-dose

TRT (1mg/kg/day) and vildagliptin (3 mg/kg/day). Rats received their assigned intervention for 28 days. Metabolic parameters, systolic and diastolic blood pressure (SBP and DBP), cardiac function, and cardiac mitochondrial oxidative stress levels were determined. Our data showed that HFD rats had obese-insulin resistance to cardiac dysfunction, and ORX aggravated cardiac dysfunction in obese-insulin resistant rats. Moreover, body weight was decreased after ORX. Treatment with vildagliptin and combined drugs reduced plasma insulin levels, and all interventions did not affect body weight and plasma glucose levels. All interventions effectively reduced SBP and DBP and improved cardiac function. Moreover, all interventions shared similar efficacy in attenuating cardiac mitochondrial oxidative stress levels. Therefore, our data suggested that physiological dose of TRT and vildagliptin attenuated cardiac dysfunction by reducing cardiac mitochondrial oxidative stress, and the only vildagliptin improved insulin resistance in obese-insulin resistant rats with testosterone deprivation.

Keywords: Testosterone replacement therapy; Dipeptidyl peptidase 4 inhibitors; Obese-insulin resistance; Mitochondria; Heart

Introduction

Long-term high-fat diet (HFD) consumption could induce obesity, peripheral and myocardial insulin resistance (1, 2). In pre-diabetic models, obesity coincided with hyperinsulinemia and euglycemia (3, 4). However, hyperglycemia was observed later at an advanced stage of metabolic syndrome(5, 6). In the HFD-induced obese-insulin resistant rat model, an increased lipid accumulation caused cardiac sympathovagal imbalance and cardiac dysfunction (3-5, 7). Additionally, cardiac mitochondrial dysfunction has been implicated as another mechanism associated with cardiac dysfunction (8-10). Previous studies suggested that mitochondrial oxidative stress levels were increased in the cardiac tissue from HFD-fed rats compare to normal rats (8-11).

Besides obesity, sex hormone deprivation is another factor that associated with cardiac dysfunction, hypertension, atherosclerosis, and all of which could progress to heart failure (12, 13). It is interesting that recent study showed a positive correlation between obesity, they suggested that people with high BMI had a greater decline in sex hormone levels (12). Therefore, the severity of cardiac dysfunction would be markedly increased in subjects who have both obesity and testosterone deprivation.

Several studies reported that testosterone replacement therapy (TRT) could restore circulating testosterone levels back to the physiological levels (14,15). In addition, TRT also provided the cardioprotective effects such as improved cardiac function, and attenuated cardiac mitochondrial dysfunction (14, 15). Furthermore, TRT could reduce plasma triglyceride, cholesterol, and inflammatory biomarker levels in patients with ischemic heart disease and patients with hypogonadism (16, 17). Moreover, TRT protected the heart against ischemia/reperfusion injury (14), chronic myocardial infarction (18), and HFD-induced cardiomyopathy (17, 19). Although TRT exerted several beneficial effects, long-term TRT could stimulate the growth of prostate cancer, worsen benign prostatic hypertrophy, cause liver toxicity, and exacerbate sleep apnea.

To minimize the adverse effects of TRT, several alternative medications have been used in testosterone-deprived subjects. Our previous studies reported that a dipeptidyl peptidase-4 (DPP-4) inhibitor, vildagliptin, exerted beneficial effects on the heart and brain functions in obese-insulin resistant rats with testosterone deprivation (20, 21). The DPP-4 inhibitors are oral anti-diabetic drugs used to treat patients with type 2 diabetes mellitus (T2DM). DPP-4 inhibitors help to increase insulin sensitivity and normalize blood sugar levels without a hypoglycemic effect (11, 22). In addition to its beneficial effects on metabolic parameters, vildagliptin also showed beneficial effects on the heart such as improving cardiac function in adult rats with insulin resistance induced by high-fat diet and in heart failure models (11, 23-26). Moreover, vildagliptin also reduced myocardial death during ischemia/reperfusion injury and reduced left-ventricular hypertrophy in animals with heart failure (26). However, vildagliptin could not increase circulating testosterone levels in subjects with testosterone deprivation (14). Therefore, the combination therapy consisting of low-dose TRT and the DPP-4 inhibitor potentially exerts beneficial effects on the metabolic and cardiac functions in obese-insulin resistant rats with testosterone deprivation. Although there are studies that demonstrate the beneficial effects of the DPP-4 inhibitor and TRT on the heart, the effects of combined DPP-4 inhibitor and TRT on the heart of obese-insulin resistant rats with testosterone deprivation have never been investigated. In this study, we hypothesized that the combination therapy with low-dose TRT and DPP4 inhibitor could improve metabolic parameters, blood pressure, cardiac function, and cardiac oxidative stress better than the single regimen.

Materials and Methods

Animal models and experimental protocols

This experiment protocol was approved by the Faculty of Medicine, Chiang Mai University, Institutional Care and Use Committee, in compliance with NIH guidelines. Male Wistar rats (6 weeks old, n=5/group) were purchased from Nomura Siam International Co., Ltd., Bangkok, Thailand. Rats were allowed to acclimatize for 1 week after their arrival, and they were housed in a room with controlled temperature and humidity. Then, rats were divided into 2 dietary groups: normal diet (ND) and high-fat diet (HFD) groups. Rats were fed with their assigned diet for 12 weeks to induce obese-insulin resistance, and orchiectomy or sham operations were performed afterward to induce testosterone deprivation. ND rats, received only a sham operation, whereas HFD rats were divided into 2 operation groups:sham operation (n=5) and orchiectomy (n=20). Twelve weeks after the operation, HFD rats with orchiectomy were subdivided into 4 subgroups including vehicle (castor oil), vildagliptin (3 mg/kg/day), physiological dose of testosterone (2 mg/kg/day), and combined low-dose testosterone (1 mg/kg/day) and vildagliptin (3 mg/kg/day). For ND and HFD rats with the sham operation, castor oil was administered. All rats received their assigned intervention for 4 weeks. After the treatment, metabolic parameters, blood pressure, and cardiac function were determined. Moreover, cardiac mitochondria were isolated to determine mitochondrial ROS levels.

Metabolic parameters determination

Body weight, visceral fat weight, plasma glucose and insulin levels were determined. Plasma glucose level was determined using a commercial colorimetric assay kit (Erba, Germany). Plasma insulin level was determined using a commercial ELISA kit (Merck, USA).

Blood pressure determination

Blood pressure was determined using a CODA2 channel non-invasive system (Kent Scientific Corporation). Systolic BP and diastolic BP were used to represent blood pressure.

Cardiac function determination

Cardiac function was determined using echocardiography (vivid i, GE, USA). % Left ventricular ejection fraction (%LVEF) was used to represent a cardiac function.

Cardiac mitochondrial reactive species (ROS) levels determination

Cardiac mitochondria were isolated using a differential centrifugation technique. Cardiac mitochondria (0.4 g/ml) were incubated with 2μM DCFH-DA at 25 °C for 25 min. The levels of fluorescence intensity were used to determine cardiac mitochondrial ROS levels.

Statistical analysis

Data from each experiment were expressed as a mean ± standard error of the mean (SEM). For all multiple comparisons, data were analyzed using three-way ANOVA, followed by post hoc least significant difference analysis. The statistical significance was accepted at a P value <0.05.

Results

Effects of testosterone replacement therapy (TRT) and vildagliptin on metabolic parameters

We found that body weight was increased in HFD-fed rats with the sham operation (HFS), compared with normal diet-fed rats with the sham operation (NDS). In ORX group, HFD-fed rats with orchiectomy treated with vehicle (HFOV) had lowered body weight than HFS rats. Treatment with physiological dose ofTRT, vildagliptin, and combined low-dose TRT and vildagliptin did not affect body weight in HFO rats (Figure 1A).

In addition, plasma insulin level was increased in HFS rats, compared with NDS rats. These finding suggested that HFS rats exhibited obese-insulin resistance following long-term HFD consumption. Treatment with vildagliptin and combined drugs reduced plasma insulin levels in HFO rats. However, TRT did not affect plasma insulin levels (Figure 1B). Furthermore, plasma glucose levels did not look different among groups (Figure 1C).

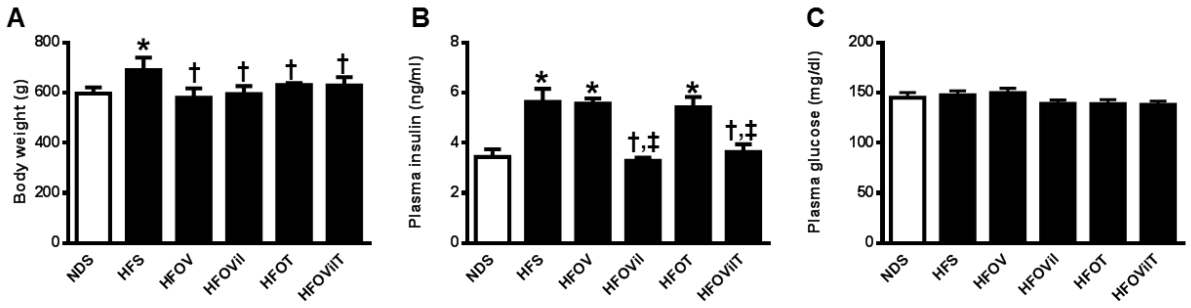

Figure 1 Effects of physiological dose ofTRT, vildagliptin, and combined low-dose TRT and vildagliptin on metabolic parameters. (A) Body weight, (B) Plasma insulin level, (C) Plasma glucose level. *p<0.05 vs. NDS, †p<0.05 vs. HFS, ‡p<0.05 vs. HFOV. NDS; normal diet-fed rats with sham operation, HFS; high-fat diet-fed rats with sham operation, HFO; high-fat diet-fed rats with orchiectomy, HFOV; HFO rats treated with vehicle, HFOVil; HFO rats treated with vildagliptin, HFOT; HFO rats treated with physiological dose of TRT, HFOVilT; HFO rats with combined low-dose TRT and vildagliptin.

Effects of testosterone replacement therapy (TRT) and vildagliptin on blood pressure and cardiac function

For blood pressure, in rats with the sham operation, HFS rats had increased SBP and DBP, compared with NDS rats (Figure 2A-B). In ORX group, SBP and DBP were significantly increased in HFOV rats, compared with HFS rats. Treatment with a physiological dose of TRT, vildagliptin, and combined low-dose TRT and vildagliptin effectively returned SBP and DBP to the normal level (Figure 2A-B).

For cardiac function, in sham operation group, %LVEF was increased in HFS rats, compared with NDS rats (Figure 2C). In ORX group, %LVEF markedly decreased in HFOV rats, compared with HFS rats (Figure 2C). Treatment with a physiological dose of TRT, vildagliptin, and combined low-dose TRT and vildagliptin increased %LVEF in HFO rats, compared with HFOV rats (Figure 2C).

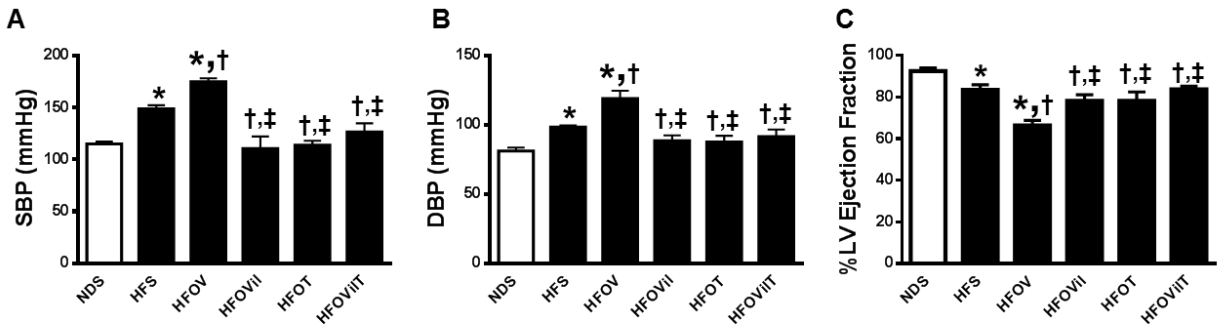

Figure 2 Effects of a physiological dose of TRT, vildagliptin, and combined low-dose TRT and vildagliptin on blood pressure and cardiac function. (A) SBP, (B) DBP, (C) %LVEF. *p<0.05 vs. NDS, †p<0.05 vs. HFS, ‡p<0.05 vs. HFOV. NDS; normal diet-fed rats with sham operation, HFS; high-fat diet-fed rats with sham operation, HFO; high-fat diet-fed rats with orchiectomy, HFOV; HFO rats treated with vehicle, HFOVil; HFO rats treated with vildagliptin, HFOT; HFO rats treated with physiological dose of TRT, HFOVilT; HFO rats with low-dose TRT and vildagliptin.

Effects of testosterone replacement therapy (TRT) and vildagliptin on cardiac mitochondrial oxidative stress

Regarding the oxidative stress in isolated cardiac mitochondria, our data showed that mitochondrial ROS was increased in HFS rats, compared with NDS rats (Figure 3). Mitochondrial ROS level was further increased in HFOV rats, compared with HFS rats (Figure 3). Treatment with a physiological dose of TRT, vildagliptin, and combined low-dose TRT and vildagliptin reduced mitochondrial ROS level in HFO rats, compared with HFOV rats (Figure 3), and the levels of mitochondrial ROS were not different among the treatment groups (Figure 3).

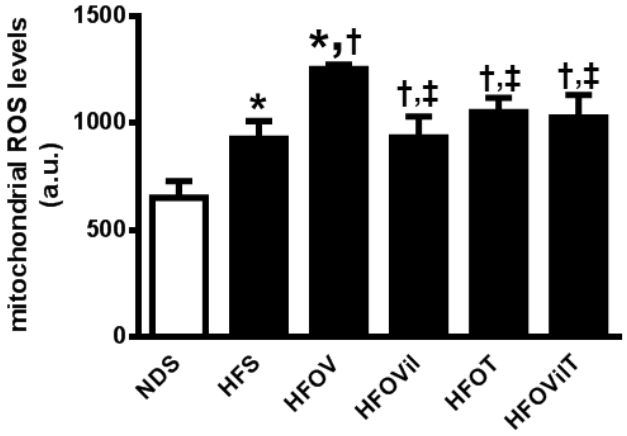

Figure 3 Effects of a physiological dose of TRT, vildagliptin, and combined low-dose TRT and vildagliptin on mitochondrial oxidative stress. *p<0.05 vs. NDS, †p<0.05 vs. HFS, ‡p<0.05 vs. HFOV. NDS; normal diet-fed rats with sham operation, HFS; high-fat diet-fed rats with sham operation, HFO; high-fat diet-fed rats with orchiectomy, HFOV; HFO rats treated with vehicle, HFOVil; HFO rats treated with vildagliptin, HFOT; HFO rats treated with physiological dose ofTRT, HFOVilT; HFO rats with low-dose TRT and vildagliptin.

Discussion

Data from this study demonstrated that testosterone deprivation aggravated cardiac dysfunction in HFD-induced obese-insulin resistant rats. The physiological dose of TRT, vildagliptin, and combined low-dose TRT with vildagliptin improved cardiac function in obese-insulin resistant rats with orchiectomy. Moreover, all interventions effectively reduced systolic and diastolic in obese-insulin resistant rats with orchiectomy. However, only the regimens with vildagliptin, either given alone or combined with low-dose TRT, could reduce plasma insulin levels in obese-insulin resistant rats. All interventions did not affect body weight and plasma glucose levels.

Several studies demonstrated that long-term high-fat diet consumption could lead to obesity and metabolic impairments such as hyperinsulinemia and impaired insulin sensitivity (8, 11, 13, 27, 28). Consistent with those findings, our HFD-fed rats had obesity and increased plasma insulin while plasma glucose levels were unchanged, these data suggested that our HFD-fed rats exhibited obese-insulin resistance. We found that body weight was reduced in testosterone deprived rats. The previous study also showed that testosterone deprivation could reduce food intake in Otsuka-Long-Evans-Tokushima Fatty rats (29). Although testosterone

deprivation decreased body weight in obese-insulin resistant rats, it did not affect plasma glucose and insulin levels. Our data showed that plasma insulin level was reduced in vildagliptin-treated and combined-drug-treated obese-insulin resistant rats with testosterone deprivation. Therefore, our data indicated that vildagliptin attenuated insulin resistance in obese-insulin resistant rats, and TRT had no effects on peripheral metabolic parameters. Vildagliptin is a DPP-4 inhibitor that is used to treat type 2 diabetes mellitus. Moreover, vildagliptin also improved peripheral metabolic impairments in a model of pre-diabetes (30-32). However, TRT could not reduce insulin resistance in obese-insulin resistant rats with testosterone deprivation. In contrary to our findings, there is a clinical study reporting that TRT improved fasting insulin sensitivity in hypogonadal men with type 2 diabetes (33).

In this study, we found that testosterone deprivation increased SBP and DBP, and exacerbated cardiac dysfunction in obese-insulin resistant rats when compared to the obese-insulin resistant rats with sham operation. All interventions could bring SBP and DBP back to the normal level. In addition, both TRT and vildagliptin provided similar efficacy in attenuating cardiac dysfunction, and combined low-dose TRT and vildagliptin did not enhance the cardioprotective effects of TRT and vildagliptin. Thus, these data indicated that TRT would exert the beneficial effects while administrated at the physiological dose (2 mg/kg/day). Also, previous studies suggested that both TRT and vildagliptin attenuated cardiac dysfunction in testosterone deprived rats via ameliorating cardiac mitochondrial dysfunction (21, 34, 35).

Mitochondria have a central role in generating adenosine triphosphate (ATP) to supply the cellular function (36). Moreover, they are a major intracellular source of reactive oxygen species (ROS). An excessive amount of ROS directly damages cellular contents and leads to cellular dysfunction and cell death (37, 38). Several studies suggested that an increased cardiac mitochondrial ROS level is an underlying mechanism for cardiac dysfunction (6, 33, 39). Our data demonstrated that physiological dose of TRT and vildagliptin could reduce mitochondrial ROS production in obese-insulin resistant rats with testosterone deprivation. Therefore, our data suggested that TRT and vildagliptin attenuated cardiac dysfunction via reducing cardiac mitochondrial ROS production.

In conclusion, our data suggested that physiological dose of TRT and vildagliptin attenuated cardiac dysfunction by reducing cardiac mitochondrial oxidative stress, and the only vildagliptin improved insulin resistance in obese-insulin resistant rats with testosterone deprivation.

Acknowledgements

This work was supported by Thailand Research Fund Grants TRF-RTA6080003 (SCC), TRG6080005 (NA), MRG5980198 (WP); a NSTDA Research Chair Grant from the National Science and Technology Development Agency Thailand (NC); a Faculty of Medicine, Chiang Mai University Endowment Fund (NA), and Chiang Mai University Center of Excellence Award (NC).

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Anti-diabetic activity of *gymnema inodorum* extract in high-fat diet and streptozotocin-induced diabetic rats: preliminary study

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Abstract

*Gymnema inodorum*or Chiang, a plant species in genus Gymnema, has been widely consumed as food in the Northern of Thailand. Although numerous studies recognized the hypoglycemic activity of *Gymnema sylvestre* which belongs to the same genus as the *G.inodorum*butthe activity of *G.inodorum* on diabetes mellitus (DM) had been few studied. Therefore, this study aimed to evaluate the anti-diabetic activity of *G.inodorum* extract (GE) in diabetic rats induced by high-fat diet and streptozotocin (STZ) injection. The rats were divided into 4 groups (n=5) as follow: 1) NC: normal control, 2) DMC: diabetic control, 3) DM-GE: diabetic rats supplemented with GE at a dose of 150 mg/kg body weight and 4) DM-M: diabetic rats treated with metformin at a dose of 50 mg/kg body weight as a positive drug. The experimental rats have received vehicle, GE or metformin for 8 weeks. At the end of the experiment, GE supplement exerted the anti-hyperlipidemic activity by reducing plasma total cholesterol and triglyceride levels. The fasting plasma glucose levels of diabetic rats were restored after supplementation with GE, suggesting the anti-hyperglycemic activity of GE. Moreover, the results from oral glucose tolerance test (OGTT) showed that impaired glucose tolerance was attenuated in the diabetic rats supplemented with GE. In conclusion, this preliminary study demonstrated that GE supplement had anti-diabetic activity in high-fat diet and STZ induced diabetic rats.

Keywords: *Gymnema inodorum*, Anti-hyperglycemic activity, Anti-hyperlipidemic activity, Diabetes Mellitus

Introduction

Diabetes mellitus (DM) is an important chronic disease that is one of four priority non-communicable diseases (NCDs)[1]. The prevalence of diabetic patients is increasing in every country in the world and also in Thailand [2,3]. DM is characterized by the chronic hyperglycemia resulting from a defect in insulin secretion and/or insulin resistance [4]. The chronic hyperglycemia leads to various complications and can increase the risk of death [1]. Although several oral anti-diabetic drugs have been used in diabetic patients, treatment has undesirable side effects such as weight gain and hypoglycemia[5]. Therefore, treatment of DM without any side effect is still a challenge to the medical system. The use of natural products may be an adjuvant in the treatment of DM.

Gymnema inodorum, a plant species in the genus Gymnema, commonly known as "Chiangda" has been found ubiquitously in the South-Eastern Asia including Thailand and widely used as food in the Northern of Thailand[6]. The major phytoconstituents of Gymnemaaregymnemic acid, gymnemasaponins, and gymnemasides[7]. Previous studies reported the effects of *G.inodorum*such as anti-hyperglycemic and anti-oxidant activities[8-10]. The anti-hyperglycemic activity of *G. inodorum*may is associated with inhibition of glucose absorption in isolated intestinal tract[9,10]. However, the anti-hyperglycemic action of *G.inodorum* extract (GE) in diabetic rats has not been investigated yet. In the present study, we aimed to evaluate the anti-hyperglycemic and anti-hyperlipidemic activities of GE in the high-fat diet and streptozotocin (STZ)induced type 2 diabetic rats.

Materials and Methods

Gymnema inodorum extract (GE)

*Gymnema inodorum*was obtained from Chiang Mai Province. The plant materials were extracted by water. The extract was then dried using the spray drying technique to obtain the extract powder.

Animals

Adult male Wistar ratsweighing 200-250 g obtained from the National Laboratory Animal Center, Mahidol University.The animal facilities and protocols were approved by the Laboratory Animal Care and Use Committees at Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. All rats were housed in a controlled temperature (22±2°C) and 12 hr light/dark cycle. The animals were allowed to acclimatize for at least 1 week before the beginning of the experiment.

Induction of DM

After acclimatization, diabetic rats were induced by feeding with high-fat diet for 2 weeks, and then the rats fasted before intraperitoneal injection with STZ at a dose of 40 mg/kg body weight. The rats with the fasting glucose level ≥ 250 mg/dl were considered as diabetes. Normal rats were fed with normal chow diet (C.P. Mouse Feed Food no. 082; 20% energy from fats) whereas diabetic rats were fed with high-fat diet (54% energy from fats) throughout the experiment.

Experimental design

A total of 20 rats were random divided into 4 groups (5animals in each).

Group I: Normal control (NC)

Group II: Diabetic control (DMC)

Group III: Diabetic rats supplemented with GE at a dose of 150 mg/kg body weight (DM-GE)

Group IV: Diabetic rats treated with metformin at a dose of 50 mg/kg body weight (DM-M)

The animals were received the vehicle, GE or metformin for 8 weeks. The dose of GE intervention was modified from previous studies [11, 12]. All rats had free access to water and food. Blood samples were collected for measurement of plasma glucose, total cholesterol and triglyceride concentrations. Glucose tolerance status was assessed by oral glucose tolerance test (OGTT) at week-7 and all animals were sacrificed 1 week later.

Study of Oral Glucose Tolerance Test

Blood samples were collected from the overnight fasted rats as baseline value (min-0), and then a glucose solution (2g/kg body weight) was administrated by gavage feeding. After that, blood samples were collected at 15, 30, 60 and 120 minutes after glucose loading. The blood samples were centrifuged to separate the plasma for analysis of glucose concentrations. The increment of plasma glucose concentrations was presented in the term of the area under the curve (AUC) for glucose, using trapezoidal rule [13].

Animal sacrification

After 8 weeks of treatment, the overnight fasted rats were sacrificed by Nembutal®overdose injection. Blood samples were collected from the inferior vena cava, and then plasma was separated by centrifugation and stored at -20°C until being assayed glucose, total cholesterol and triglyceride levels. Finally, the visceral fat was collected and weighed.

Blood biochemical analysis

The plasma glucose, total cholesterol, and triglyceride levels were determined by the enzymatic colorimetric method using a commercial kit (Biotech, Bangkok, Thailand).

Statistical Analysis

Data were expressed as a mean ± standard error of the mean (SE). The statistics were evaluated by one-way analysis of variance (ANOVA) followed by Turkey's*post-hoc* analysis, using the computer program SPSS Advanced Statistics software (version 22 SPSS Inc, Chicago, IL, USA). $P< 0.05$ was accepted as statistically significant.

Results

Effect of GE on body weight and visceral fat weight in high-fat diet and STZ induced diabetic rats

As shown in table 1, the baseline values of body weight in the NC, DMC and the DM-GE groups were comparable. The initial body weight was significantly lowered in the DM-M group than in the NDC group. At the end of the experiment, the body weight was significantly higher in the DMC group than the NC group. Neither GE supplement nor metformin treatment for 8 weeks had any effect on body weight in diabetic rats. As expected, feeding with high-fat

diet significantly increased the visceral fat weight of the rats in DMC group when compared with the rats in NC group. There was no significant difference in the body weight between the DMC and DM-GE groups. Compared with the DMC group, the visceral fat weight markedly decreased in the DM-M group.

Effect of GE on plasma lipid levels in high-fat diet and STZ induced diabetic rats

To evaluate the anti-hyperlipidemic effect of GE supplement, the fasting plasma total cholesterol and triglyceride levels were measured. Both plasma total cholesterol and triglyceride levels were significantly elevated in the DMC group respected to the NC group (table 1). The lipid-lowering effect of GE supplement was shown in this study as shown by significant decreases in the total plasma cholesterol (-32%) and triglyceride (-53%) levels in the DM-GE group compared with the DMC group. A reduction in plasma total cholesterol (-29%) and triglyceride (-63%) levels were also found in the DM-M group. This study showed that GE supplement had anti-hyperlipidemic effect in high-fat diet and STZ induced diabetic rats.

Table 1. Effect of GE supplement on body weight, visceral fat weight and plasma lipid levels in high-fat diet and STZ induced diabetic rats

	NC	DMC	DM-GE	DM-M
Body weight wk-0 (g)	382.86 ± 8.08	364.29 ± 8.41	380.00 ± 7.07	350.00 ± 8.85 *
Body weight wk-8 (g)	468.57 ± 10.56	516.43 ± 15.53 *	505.00 ± 2.89	483.33 ± 10.85
Visceral fat weight (g)	19.29 ± 10.56	37.00 ± 2.93 *	30.25 ± 1.11 *	29.50 ± 1.26 * †
Plasma total cholesterol level (mg/dl)	74.04 ± 4.38	112.66 ± 7.61 *	76.03 ± 5.03 †	79.33 ± 5.83 †
Plasma triglyceride level (mg/dl)	43.46 ± 4.72	74.35 ± 6.11 *	35.75 ± 3.19 †	27.33 ± 0.85 †

Values are means ± SE for 5 animals per group. *p< 0.05 vs. NC group; † p< 0.05 vs. DMC group.

Effect of GE on glycemic control in high-fat diet and STZ induced diabetic rats

The effect of GE supplement on the fasting plasma glucose levels was shown in figure 1. Compared with the NC group, the fasting plasma glucose levels were significantly greater in the DMC group than in the NC group throughout the experiment. Supplementation with GE successfully attenuated the fasting plasma glucose levels in diabetic rats since week-4 (-44%) to week-8 (-51%) respected to the DMC group. Also, the fasting plasma glucose levels of the DM-M group significantly decreased both in week-4 (-42%) and week-8 (-45%) respected to the DMC group. These findings demonstrated that GE supplement had anti-hyperglycemic effect in high-fat diet and STZ induced diabetic rats.

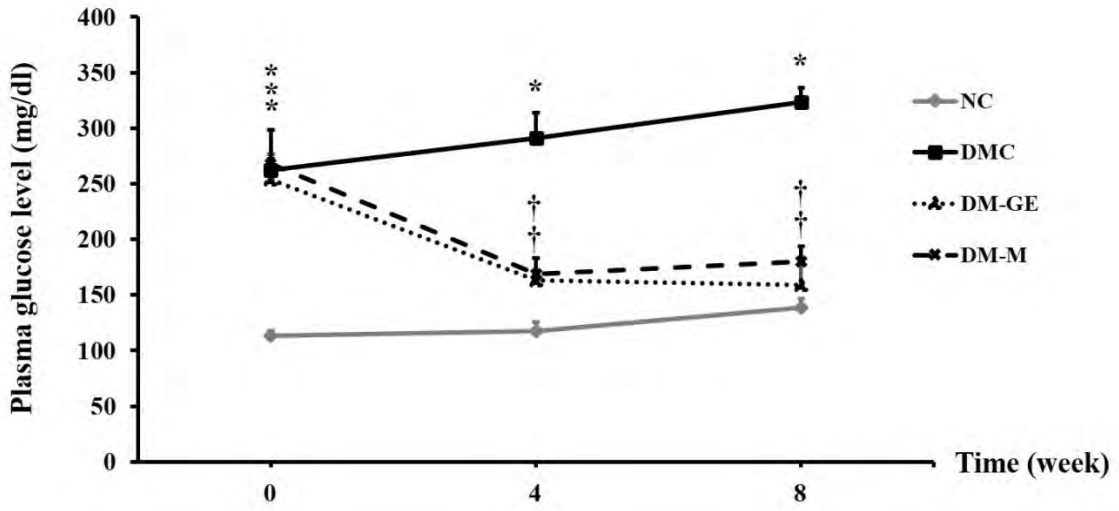

Figure 1. Effect of GE supplement on glycemic control in high-fat diet and STZ induced diabetic rats.Values are means ± SE for 5 animals per group. *p< 0.05 vs. NC group; † p< 0.05 vs. DMC group.

Effect of GE on glucose tolerance in high-fat diet and STZ induced diabetic rats

The OGTT was used to determine whether GE supplement could improve glucose tolerance status which indicating the whole body insulin sensitivity in diabetic rats. The glucose response from OGTT was shown in figure 2. The plasma glucose levels of the DMC group were markedly increased in min-0, 30, 60 and 120 when compared with the NC group, suggesting these diabetic rats had impaired glucose tolerance. Compared with the DMC group,

the plasma glucose levels of the DM-GE group were significantly decreased in min-0, 30, 60 and 120. While the plasma glucose levels of the DM-M group were significantly reduced only in min-0 and 120 when compared with the DMC group. The results from glucose response were calculated to the total area under the curve (TAUC) for glucose as shown in figure 3. In accordance with the results from glucose response, the TAUC of the diabetic rats was significantly elevated respected to the normal rats. Interestingly, only GE supplement, but not metformin treatment, significantly reduced the TAUC in diabetic rats. The results from OGTT indicated that GE supplement significantly improved glucose tolerance in high-fat diet and STZ induced diabetic rats.

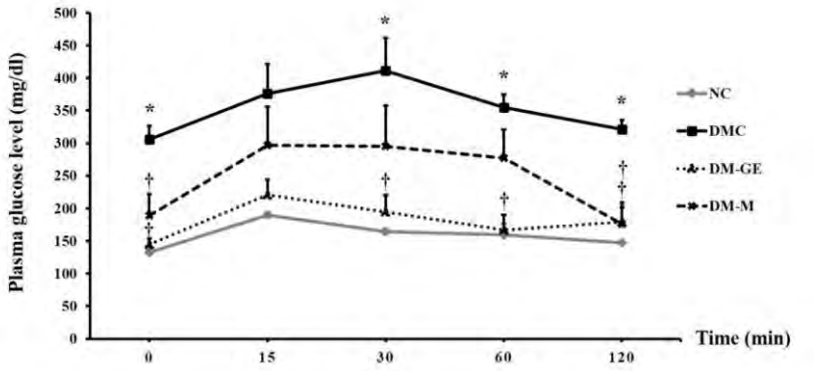

Figure 2. Effect of GE supplement on glucose response from OGTT in high-fat diet and STZ induced diabetic rats. Values are means ± SE for 5 animals per group. *p< 0.05 vs. NC group; † p< 0.05 vs. DMC group.

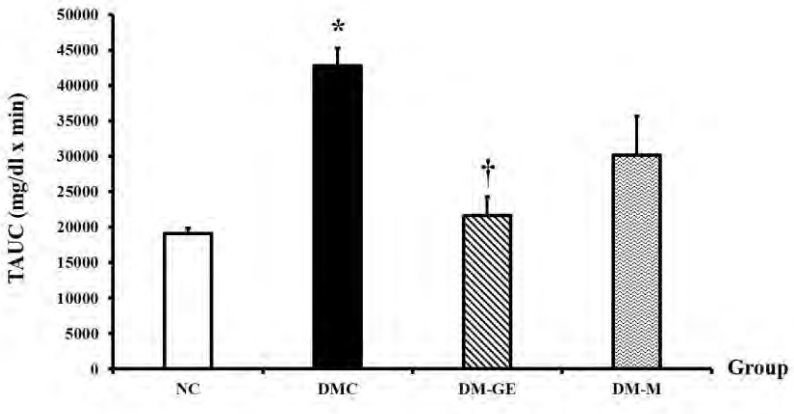

Figure 3. Effect of GE supplement on TAUC in high-fat diet and STZ induced diabetic rats. Values are means ± SE for 5 animals per group. *p< 0.05 vs. NC group; † p< 0.05 vs. DMC group.

Discussion

The thispreliminary study demonstrated that GE supplement at a dose of 150 mg/kg body weight successfully decreased the fasting plasma glucose levels in the high-fat diet and STZ induced diabetic rats indicating the anti-hyperglycemic effect of GE supplement. Furthermore, supplementation with ineffectively improved glucose tolerance in diabetic rats. In addition to that anti-hyperglycemic activity, GE supplement also exerted a lipid-lowering effect as shown by reducing both plasma total cholesterol and triglyceride levels in diabetic rats.

Gymnema has been used in traditional Asian medicine for a long time[14]. The major of chemical constituents of Gymnema include gymnemic acid, gymnemasaponins and gymnemasides[7]. *G. sylvestre*, a medicinal woody climber found in central and southern India, is well known for treatment of DM[14-16]. Several studies reported that the hypoglycemic effect of *G. sylvestre* is due to gymnemic acid[6,14-16]. The possible mechanisms underlying the hypoglycemic effect of gymnemic acid are associated with increased insulin secretion, regeneration of islet cells, increased glucose utilization and an inhibition of glucose absorption from intestine[17,18]. According to the principle of chemotaxonomy which *G. inodorum* and *G. sylvestre*are classified into the same genus. Hence, *G. inodorum* might contain gymnemic acid as phytochemicals and could exhibit the anti-hyperglycemic effect via the similar mechanisms. Supported with previous studies, they demonstrated that *G. inodorum* inhibited intestinal glucose absorption of guinea pig[8,9]. Recently, the study of Chiabchalard et al. reported that *G. inodorum*reduced postprandial glucose peak after meal or glucose loading[19]. Whereas, long-term consumption of *G. inodorum* had no effect on fasting plasma glucose levels, insulin secretion or α-glucosidase enzyme activity in healthy human[19]. Supplementation of GE for 8 weeks also improved glucose tolerance in diabetic rats. Notably, GE supplement seems to be more effective than metformin treatment in improving glucose tolerance of diabetic rats.

Supplementation with GE also exerted anti-hyperlipidemic activity in high-fat diet and STZ induced diabetic rats. Study of Wang et al. revealed that gymnemic acid effectively inhibited lipid absorption in the intestine of rats[20].Supported with the study of Bishayee and Chatterjee, they found that administration of *G. sylvestre* extracts exhibited the reductions of elevated serum triglyceride, total cholesterol, very low-density lipoprotein and low-density lipoprotein in hyperlipidemic rats[21]. Also, a recent study demonstrated that gymnemic acid administration at a dose of 100 and 500 mg/kg body weight decreased serum total cholesterol

and triglyceride levels in alloxan-induced diabetic rats[22]. Therefore, the lipid-lowering effect of GE might be associated with gymnemic acid.

In conclusion, this study firstly revealed the anti-diabetic and anti-hyperlipidemic effect of *G. inodorum* extract in diabetic rats induced by high-fat diet and STZ injection. These effects may be partly mediated through improvement of whole-body insulin. However, the mechanisms underlying anti-hyperglycemic and anti-hyperlipidemic effects of GE supplement should be further studied.

Acknowledgements

This study was partly supported by a grant from the Industrial Research and Technology Capacity Development Program (IRTC), Maejo University, Chiang Mai, Thailand.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
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Elimination of cellular senescence in neuronal cell by 5, 6, 7, 4'- tetramethoxyflavanone

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Abstract

Alzheimer's disease (AD) has been widely known as the neurodegeneration that has high production of amyloid beta(Aβ). Aβ-induced neuronal toxicity mediated by several pathways, including excessive of ROS production, intracellular-enzymatic antioxidant deficiency, inflammatory responses, cellular senescence promotion, and apoptosis before losing synaptic plasticity. Recently, the reduction of Aβ toxicity in neuronal tissues by an antioxidant compound such as flavonoid has been widely studied both *in vitro* and *in Vivo* for retrieving the primary production of Aβ toxicity especially, ROS contents.In this study, we interest to investigate the effects of 5, 6, 7, 4'- tetramethoxyflavanone (TMF), the derivative of flavonoid isolated from *Chormolaela odorata* in $A\beta_{25-35}$ -induced toxicity in SK-N-SH Cells whether reduced cellular senescence and promoted synaptic plasticity. Herein, we demonstrated that TMF exhibited potent of antioxidant by using DPPH assay and it significantly increased cell viability and decreased the ROS production in a dose-dependent

manner using MTT and ROS assay, respectively. Furthermore, the superoxide dismutase (SOD) activity which is the intracellular enzymatic antioxidant was also upregulated in TMF Treatment prior treated with Aβ which assessing by SOD assay kit. Importantly, TMF protects against $A\beta_{25-35}$-induced cellular senescence in SK-NSH cells by attenuated β-galactosidase expression which evaluating by β-galactosidase assay kit. Thus, this study showed the pivotal role of TMF in the reduction of negative responses by Aβ exposing to neuronal cells, and the TMF may be usefully for AD treatmentinthefuture.

Keywords: Alzheimer's disease, Antioxidant, Cell stress, Senescence, Synaptic plasticity

Introduction

Alzheimer's disease (AD) is the memory deficit that correlated with the high production of amyloid beta (Aβ) and the phosphorylation-tau protein in brain tissue. The pathological hallmark of the AD was mainly decreased the short-term memory by the induction of loss synaptic plasticity and neuronal cell death in hippocampus both *in vitro* and *in vivo*[1]. The high percentage of neuronal cells death and dysfunction was widely reported that it closely associated with the increasing of cellular senescence response in the cell [2]. Recently findings were reported that Aβ-induced cellular senescence in a neuronal cell by promoting the β-galactosidase (β-Gal) activity together with inhibition of p-retinoblastoma(p-Rb) expression and induction of p-21 and p53 expression before underwent apoptosis and loss of synaptic plasticity both *in vitro* and *in vivo*[3]. Importantly, several studies demonstrated that the major factor that caused negative responses was mainly by the excessive amount of reactive oxygen species (ROS). According to previous studies suggested that the toxicity of Aβ to brain cells could promote the excessive production of ROS and this event was mainly found in the early stage before undergoing senescence, dysfunction, and death of neuronal cells in AD patients [4, 5].

As a rule, the cellular defense system became active when exposing to stress response and then mediated and released antioxidant enzymes for scavenging the free radicals. Nuclear factor erythroid 2-related factor 2(Nrf2) mechanism has been commonly known as antioxidant regulator for producing antioxidant enzymes such as superoxide dismutase (SOD), and catalase (CAT) etc[6]. Recently reviews demonstrated that the excessive stress stimulation in neuronal cells by Aβ caused disturbance of Nrf2 mechanism that leads stress

progression, dysfunction and death in neuronal tissue both *in vitro* and *in vivo* [7, 8]. Finally, entire negative responses promoted cell death and synapse loss by decreasing synaptic protein associated with the increasing of acetylcholine esterase (AchE) in neuronal tissue and then become memory deficit [9].

5, 6, 7, 4'- Tetramethoxyflavanone (TMF) is a flavonoid compound from *Chormolaela odorata* that commonly known as Siam weed and this pure compound also found in plant and vegetable that contain essential oil. The plant is commonly used in traditional medicine in Thailand for wound healing and stop bleeding [10]. Importantly, it had high antioxidant property and abounded in Thai forest [11]. Nevertheless, it has not been understood on its effect and the mechanistic evidence for retrieving Aβ toxicity in neuronal cells. Thus, this study is the primary finding of TMF effects and we hypothesized that TMF might protect against Aβ-mediated cell stress, senescence, death, and synaptic plasticity in neuronal cells.

Materials and Methods

Reagents and Chemicals

5, 6, 7, 4'- Tetramethoxyflavanone (TMF) was obtained from Professor. Dr. Apichart Suksamrarn, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, $A\beta_{25-35}$, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH)were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′, 7′-dichlorofluorescin diacetate were purchased from Merck Millipore, BetaRed™ β-Gal AssayKit (Millipore, MA, USA). The superoxide dismutase assay kit was purchased from Cayman Chemical Company (Cayman, MI, USA).

Cell culture

SK-N-SH cells were grown in minimum essential medium (MEM) supplemented with 10%FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO-BRL, Gainthersburg, MD) at 37°C in humidified 5% CO_2 and 95% air. The cells were cultured to 80% confluence and passaged every 3 days by trypsinization with 0.025% Trypsin/EDTA.

DPPH antioxidant assay

Briefly, various concentrations of TMF (0.1, 1, 10, and 100 µM) were incubated with 0.3 mM DPPH solution in the dark at 30ºC for 30 min. The qualification analysis was conducted to measure the absorbance at 517 nm by using a spectrophotometer.

Cell viability assay

A density of the 2×10^4 cells/ml were seeded in 96-well microplates at 37°C for 24 h, followed by treatment with various concentrations of TMF (0.1, 1, and 10 µM) for 2 h prior treated with 10µM of $A\beta_{25-35}$ for 24 h. Afterwards, 10 µg/ml of the MTT solution was added to cell culture and the cells were maintained further for 1 h at 37°C. Finally, the assay solution was discarded and added DMSO for dissolved formazan. The absorbance was measured at 570/600 nm by using a spectrophotometer.

DCFDA Assay

After the cells exposed to the conditions, 20 µM of H2DCF-DA in 1x PBS was added in each well at 37ºC for 2 h in the dark. The fluorescence values were then measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm by using a Synergy H4 microplate reader (Biotek, VT, USA).

Superoxide Dismutase Activity Assay

After the cells were treated following to the condition, the cells were lysed with chilled-lysis buffer and the supernatant was assessed by the SOD assay kit. According to the manufacturer's protocol, the assay utilized a tetrazolium salt to detect the superoxide radicals generated by xanthine oxidase and hypoxanthine. The qualification analysis was conducted to measure the absorbance at 440-460 nm by using a spectrophotometer.

β-galactosidase enzyme assay

After the following treatment conditions, the cells were incubated with Beta Red reaction buffer at 37ºC for 2 h and finally stopped the activity with the stop reaction buffer. Then, the positive β-gal cells were evaluated under the converted microscopy (Olympus, Japan).

Statistical Analysis

All data are expressed as mean ± SEM of three independent experiments. The statistical differencewas analyzed using one-way analysis of variance (ANOVA) followed by Post Hoc Dunnett's test for comparing the significance between the individual groups ($p<0.05$).

Results

TMF promoted SK-N-SH cell viability by its antioxidant property

The antioxidant property of TMF in various concentrations (0.1, 1, 10, and100μM)was also assessed by DPPH assay. The data showed that TMF at 0.1-100 μM significantly inhibited DPPH free radical($p<0.001$) compared to untreated group (Fig. 1A). In accordant to the results, the optimal concentration of TMF at 0.1, 1, and 10 μM were then used in the subsequent experiment. To investigate the protective effect of TMF on $A\beta_{25-35}$ –induced toxicity in SK-N-SH cells, cells were pretreated with TMF (0.1, 1, and 10 μM) for 2 h, followed by treated with $A\beta_{25-35}$ for 24 h. The results showed that TMF significantly increased cell viability in a dose-dependent manner compared to $A\beta_{25-35}$ treatment (Fig. 1B). As the results, we also found that the significantly increased of cell viability was related with the reduction of ROS production (Fig. 1C) and the increasing of SOD activity (Fig. 1D) in a dose-dependent manner. Importantly, TMF at 10 μM was the effective dose in $A\beta_{25-35}$ treatment for protecting negative responses. Thus, this concentration was then used in the subsequent experiment.

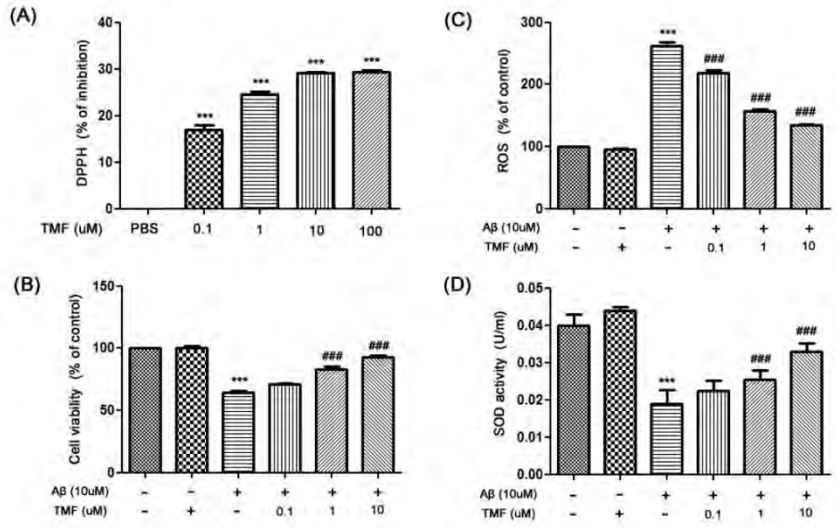

Fig. 1 TMF promoted cell viability in $A\beta_{25\text{-}35}$treated cells. DPPH assay was used to determine the effect of TMF-scavenged DPPH free radical (A). The cell viability in TMF pretreatment with $A\beta_{25\text{-}35}$treatmentwere analyzed by using MTT assay(B). The level of ROS production was determined by ROS assay(C). The activity of SOD was determined by SOD assay kit (D). The values present the mean ± SEM from 3 independent experiments. ***p< 0.001, in comparison with the control treatment.$^{\#\#\#}p$<0.001, in comparison with Aβ treatment.

TMF protected against $A\beta_{25\text{-}35}$–mediated cell senescence in SK-N-SH cells

According to previous results, we found that TMF protect against $A\beta_{25\text{-}35}$–induced cell death and stress responses. Then the cellular senescence in SK-NSH cells was then examined. The data showed that $A\beta_{25\text{-}35}$ significantly modulated β-galactoxidase expression (Fig. 2) in SK-N-SH cells (p<0.001) compared to control treatment. The data of TMF pretreatment on $A\beta_{25\text{-}35}$ toxicity showed significantly reverse of cell senescence response when compared to $A\beta_{25\text{-}35}$ treatment alone (Fig. 2)(p<0.001).The TMF treatment alone did not showed any effects on SK-N-SH cells. Therefore, in this experiment showed that TMF could protect the cellular senescence in neuronal cells-treated with Aβ.

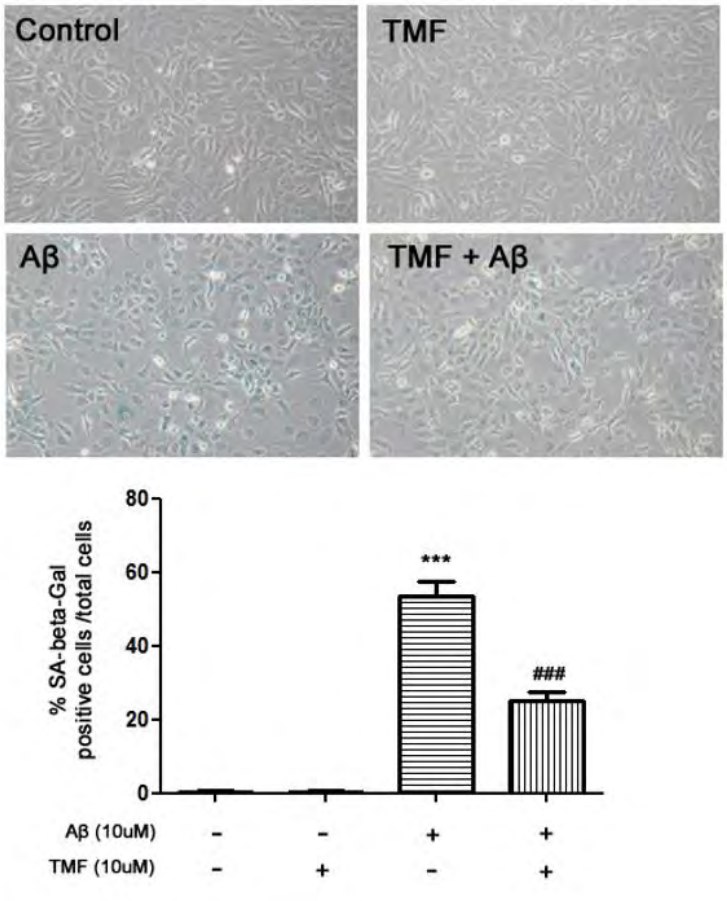

Fig. 2TMF protected against cell senescence by $A\beta_{25-35}$toxicity in SK-N-SH cells. β-galactosidase the assay was used to investigate the activity of the β-galactoxidase enzyme in SK-N-SH cells. The values present the mean ± SEM from 3 independent experiments. ***$p<$ 0.001, in comparison with the control treatment. $^{###}p<0.001$, in comparison with Aβ treatment.

Discussion

The production of Aβ peptides in hippocampus and cerebral cortex has been widely considered as the common cause of memory loss [12]. The neuropathology was by Aβ generated toxicity-induced negative responses which are characterized by (1) free radical generation and cellular stress induction (2) cell senescence (3) imbalance of cellular defense system, prior to the loss of cells and synapse [13-15]. In this study, we evaluated the neuroprotective TMF in SK-N-SH cell and found that at the dose of 10 µM showed the neuroprotective property (Fig.1A), which closely associated with the significantly decreased of intracellular ROS (Fig. 1C)and increasing of SOD activity (Fig. 1D). Cellular senescence

has been widely known as the cellular aging process before undergoing cellular dysfunction and apoptosis [16]. Several studies reported that the exposing to a toxin such as lipopolysaccharide (LPS) or $A\beta_{25-35}$ could promote cellular senescence prior mediated apoptosis in neuronal tissue [17-19]. The up-regulation of senescence in toxic exposure was by increasing of p21 and p53 together with the decreasing of Sirt1 that closely related with the increasing of β-Gal activity, the biomarker of cell aging [20]. In this study showed that the increasing of ROS and decreasing of SOD activity were closely related with the increasing of cellular senescence which confirmed by the upregulation of β-gal expression in SK-N-SH cells treated $A\beta_{25-35}$ when compared to control ($p<0.001$).

Nowadays, the protection and treatment of AD have been concerned over a natural or herbal extraction which has been widely accepted as a diversity effect with low adverse effect. Interestingly, they found that these herbal extractions showed the antioxidant property for protecting against the toxicity from toxic exposure in neuronal cells by ameliorated negative mechanisms [21-23]. In according to previous studies, we then investigated the extraction namely 5, 6, 7, 4'- tetramethoxyflavanone (TMF) from *Chormolaelaodorata*which showed the high potent of antioxidant property as presented in Fig. 1A. In this study We found that TMF could prevent SK-N-SH cell viability in $A\beta_{25-35}$ treatment in a dose-dependent manner (0.1, 1, and 10 μM). The protection of TMF on $A\beta_{25-35}$ treatment was correlated with the decreasing of intracellular ROS production (Fig. 1E) along with increasing of % scavenging DPPH-free radical (Fig. 1C) and SOD activity (Fig. 1D). Importantly, we found that the antioxidant property of TMF showed the reduction of cellular senescence by decreasing the β-gal expression. Thus, the decreasing of negative responses by TMF could inhibit the senescence response in neuronal cells.

In conclusion, our study demonstrates the TMF alleviated negative responses including cell stress response and cell senescence in $A\beta_{25-35}$–treated SK-N-SH cells. Therefore, TMF might be usefully for reducing $A\beta_{25--35}$ toxicity in the AD.

Acknowledgements

This work was supported by the Faculty of Medical Science, Naresuan University, Faculty of Medicine, Chiang Mai University, as well as by a research grant from the Thailand Research Fund (TRF) (DPG5780001).

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
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Lovastatin extracted from *A. sclerotiorum PSU-RSPG178* ameliorates hepatic glucose production, insulin resistance, and hepatic steatosis in obese insulin resistance rats

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Introduction:

Non-alcoholic fatty liver disease (NAFLD) is characterize by increasing lipid accumulation in the liver. It has been known that NAFLD associated with insulin resistance and dysregulation of lipid metabolism, leading to progressive decline of liver function. Statin is known as 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitor which is the most widely used as anti-hyperlipidemia agent. The major effect of statin is to inhibit the rate-limiting step in cholesterol biosynthesis resulted in reducing plasma total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C). More recently, lovastatin was firstly extracted from a novel *A.sclerotiorum PSU-RSPG178* by Phainuphong P. et. al. Thus, this study aimed to investigate the action of lovastatin extracted from a novel *A.sclerotiorum PSU-RSPG178* and identified the possible mechanisms involved in lipid-lowering action in high-fat diet induced obese insulin resistance rats.

Materials and Methods:

Male wistar rats were divided into 3 groups (n=6/group) and fed with either standard diet (control) or high-fat diet (HFD) for 12 weeks. Subsequently, high-fat diet rats were treated with lovastatin at the dose of (10 mg/kg/day) (HFD+L) for 5 weeks. Hepatic gluconeogenesis was determined using alanine tolerance test. The levels of serum lipid profile, plasma glucose, insulin, and the level of hepatic lipid contents were determined using colorimetric assay kits. Hepatic lipid accumulation was also observed by H&E staining

Results:

Administration of lovastatin extracted from *A.PSU-RSPG178* significantly reduced serum TC and TG levels, increased HDL-C, and improved hepatic insulin resistance and lipid contents. Moreover, lovastatin also shows pleiotropic effect on glucose metabolism by ameliorating hepatic glucose production.

Conclusions:

This study suggest that lovastatin extracted from a novel *A.PSU-RSPG178* was able to ameliorate hyperlipidemia, hepatic glucose production, and insulin resistance, leading to improved non-alcoholic fatty liver.

Keywords: Lovastatin; Obese insulin resistance; hepatic gluconeogenesis; non-alcoholic fatty liver; insulin resistance

Introduction

Obesity becomes a major worldwide health problem. The incidence of obesity is rising continuously and association with morbidity and mortality. Obesity has been known to become major risk factors for several diseases, including type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), hypertension, cancer, and non-alcoholic fatty liver disease (NAFLD) (1,2). High fat diet has been shown to be the primary factor contributed for obesity and hyperlipidemia. Several studies demonstrated that high-fat diet fed in rodents induced oxidative stress, increased triglycerides (TG), total cholesterol (TC) contents and increased cholesterol biosynthesis in the liver, leading to hepatic lipid accumulation (3). Increasing of

hepatic lipid accumulation leads to generate more reactive oxygen species resulting in increased peroxidation which is also the cause of progressive hepatic insulin resistance (4).

Lovastatin was the first 3-hydroxy-3-methylglutanyl coenzyme A (HMG-CoA) reductase inhibitor and known as statin. It lowers TC and low-density lipoprotein cholesterol (LDL-C) in patients with dyslipidemia (5,6). The main effect of lovastatin is to inhibit HMGR enzyme which is a rate limiting step in cholesterol synthesis (7). Previous *in vivo* studies showed that lovastatin not only decreased the TC, TG, and LDL-C, but also increased HDL-C significantly (8,9). Furthermore, lovastatin also significantly decreased hepatic fatty acid and TG synthesis and improved hepatic lipid dysregulation (10). Recently, lovastatin exhibited several pleiotropic effects, including improved insulin sensitivity, insulin resistance, antioxidant, and anti-inflammation (11,12). However, the information concerning its effects on hepatic glucose metabolism is limited.

Recently, lovastatin was firstly extracted from a novel *A.sclerotiorum PSU-RSPG178* (13). However, the lipid-lowering action of lovastatin extracted from a novel *A.sclerotiorum PSU-RSPG178* and its pleiotropic effects *in vivo* have not been investigated yet. Thus, the present study aimed to investigate the therapeutic effects of lovastatin extracted from *A.sclerotiorum PSU-RSPG178* on hyperlipidemia, insulin resistance, hepatic glucose production, and hepatic steatosis in obese insulin resistance rats.

Methodology/Experimental design

Chemicals

CelLytic™ MT mammalian Tissue Lysis/Extraction reagent were purchased from Sigma Aldrich (St. Louise, MO, USA). Protease inhibitors were purchased from Roche Diagnostics Ltd (IN, USA). Lovastatin was kindly provided by Prof. Vatcharin Rukachaisirikul from Department of Chemistry, Faculty of Science, Prince of Songkla University (Songkhla, Thailand). All other chemicals with high purity were obtained from commercial sources.

Animals

Male Wistar rats (150-190 g) were obtained from Nomura Siam International (Bangkok, Thailand). The animal facilities and protocols were approved by the Laboratory Animal Care and Use Committee at Faculty of Medicine, Chiang Mai University, and Chiang

Mai, Thailand. All experimental rats were housed in a room maintained at 25±1 °C on a 12:12 h dark–light cycle. Eighteen animals were randomized and equally divided into 3 groups: normal control (ND), high-fat diet (HFD) and high-fat diet treated with lovastatin at doses of 10 mg/kg BW (HFD+L), respectively. Obese insulin resistance rats was induced by a combination of high-fat diet for 12 weeks. Lovastatin was subsequently administered daily by oral gavage for 5 weeks.

Hepatic gluconeogenesis

The alanine tolerance test (ATT) was determined at the week 17. The test was performed in each animal to determine hepatic gluconeogenesis. Briefly, alanine (100 mg/kg BW) was administered in each rat. The blood sample was subsequently collected from tail vein at 15, 30, 60, 90 and 120 min after injection of alanine. Blood glucose in each time point was then determined and total area under the curve was calculated and analyzed.

Biochemical blood analysis

After overnight fasting, the rats were anaesthetized by pentobarbital, and sacrificed. The blood samples were collected. The plasma glucose, TC, TG, and HDL-C levels were determined using commercially colorimetric diagnostic kits (Biotechnical Nology, Bangkok, Thailand). The plasma insulin concentrations were measured using a Sandwich ELISA assay kits from LINCO Research (Millipore, MA, USA). Insulin resistance was calculated and expressed as the homeostasis assessment (HOMA index).

Histopathological analysis

To assess liver morphology and lipid accumulation, the liver was excised and fixed in 10% buffered formalin solution for 24 hr. The sample was then filtrated with xylene embedded in paraffin and cut into 5 µm thick sections. Subsequently, the liver was stained by hematoxylin and eosin (H&E) to evaluate liver morphology. A microscopic examination was performed and photographs were taken under a regular light microscope.

Statistical analysis

Data were expressed as mean ± S.D. and compared among experimental groups using One-way ANOVA followed by post-hoc test. The difference was considered if the significant of $p < 0.05$.

Results

Effect of lovastatin on plasma parameters

As shown in Table 1, HFD rats had significant increase in body weight, liver weight per body weight, plasma TC, TG, LDL-C, but decrease in HDL-C. Moreover, HFD rats also showed hyperinsulinemia and insulin resistance as reflected by a markedly increase in HOMA index when compared to control. On the other hand, plasma TC, TG, LDL-C, and HOMA index were decreased in HFD+L while HDL-C was increased when compared with HFD alone. Nonetheless, there was no significant different in fasting blood glucose level among experimental groups. This result indicated that lovastatin extracted from *A.sclerotiorum PSU-RSPG178* was able to improve lipid profile and restored insulin resistance in high-fat diet fed rats.

Table 1. Effects of lovastatin extracted from *A.sclerotiorum PSU-RSPG178* on metabolic variables.

Parameters	NDV	HFV	HFD+L
Body weight (g)	540.83±2.29	637.50±20.24*	595.83±18.73
Liver weight (g)	13.12±0.78	29.48±2.61*	21.63±2.37
Plasma cholesterol (mg/dl)	74.42±3.54	115.25±7.98*	77.17±2.71#
Plasma triglyceride (mg/dl)	48.82±3.26	82.92±4.32*	62.44±6.55#
Plasma HDL (mg/dl)	28.95±1.37	11.71±1.73*	30.38±1.88#
Plasma LDL (mg/dl)	36.90±4.16	86.99±7.13*	34.29±1.76#
Plasma glucose (mg/dl)	117.65±10.90	146.13±10.12	125.35±14.15#
Plasma insulin (ng/ml)	1.57±0.48	4.36±0.49*	1.76±0.28#

*p<0.05 compared with ND, #p<0.05 compared with HFD

Effect of lovastatin on hepatic gluconeogenesis

At the end of experiment, animals were assessed whether lovastatin improved hepatic glucose production using the alanine tolerance test. The results show that plasma blood glucose after alanine injection were greatly increased up to 120 min in HFD group when compared with ND. Consistent with this impairment, the total area under the curve (AUC) of blood glucose response was significantly increased in HFD group when compared with ND. Nonetheless, blood glucose response and total AUC of HFD+L rats had markedly declined similar to ND group. This data indicates that lovastatin was able to abolish hepatic insulin resistance through inhibiting hepatic glucose production process which, in turn, improved hepatic glucose metabolism

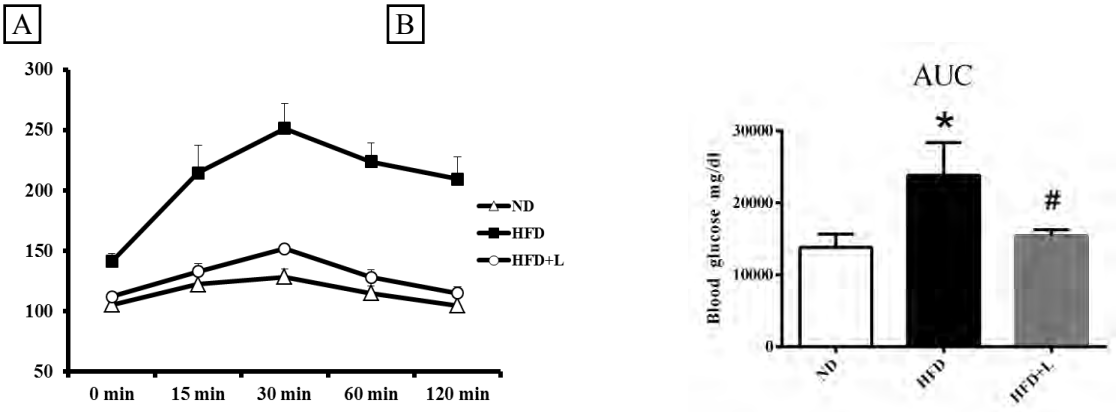

Fig.1 The effect of lovastatin on hepatic gluconeogenesis.

Alanine tolerance test was used to determine hepatic glucose production. (A) Blood glucose levels in response to alanine administration in each time point. (B) The total area under the curve (AUC) of blood glucose levels. ND indicates a normal diet group, HFD indicates a high fat diet group, and HFD+L indicates high fat diet treated with lovastatin at the dose of 10 mg/kg. Values are expressed as means ± S.D (n = 6). *$p<0.05$ compared with ND, $^{\#}p<0.05$ compared with HFD.

Lovastatin improved hepatic steatosis

Since lovastatin had potential to improve insulin resistance, the liver morphology was subsequently determined. As shown in Fig 2B, HFD rats markedly increase in hepatic lipid droplet with the enlargement in size when compared to control (Fig. 2A), indicating lipid deposition was observed in obese insulin resistance rats. Interestingly, the liver morphology of HFD+L rats had shown a significant decrease in both numbers of lipid droplets and the

droplet size (Fig. 2C). Therefore, this data demonstrate that administration of lovastatin was able to reduce hepatic steatosis.

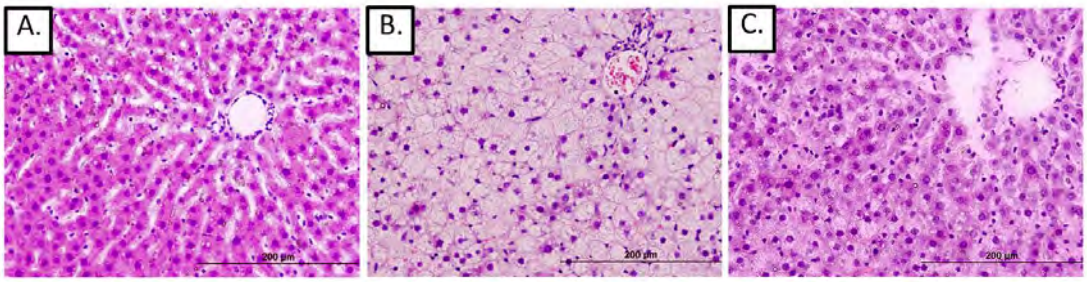

Fig.2 Micrographs of conventional hematoxylin and eosin staining of rat liver.

A sagittal half of liver from each experimental group was removed, fixed, embedded, cut and stained by hematoxylin and eosin dyes. The data were repeated at least 3 times from separate animals. The results were analyzed using bright field microscopy. (A) Control or ND, (B) HFD and (C) HFD+L, respectively.

Discussion

This study demonstrated that high-fat diet induced the accumulation of total lipid contents and increased hepatic lipid biosynthesis, leading to hepatic lipid accumulation. Previous study found that high-fat diet up-regulated Sp1 gene mediated SREBP-2 expression, leading to increased expression of HMG-CoA reductase (HMGR), which is the rate limiting step enzyme for de novo cholesterol synthesis (14). This would lead to excessive levels of cholesterol and its oxysterol metabolites, which, in turn, activate liver X receptor-α (LXRα) which also additively up-regulated SREBP1 gene, resulting in promote lipogenesis process and progressively developed hepatic steatosis (15). In addition, fatty acid accumulation also initiated ROS formation by enhancing mitochondrial β-oxidation in the liver of both human and rodents (16,17). Increased ROS could, in turn, decrease ATP and nicotinamide dinucleotide concentrations, produce DNA and protein damage, impaired membrane structure and function through lipid peroxidation, and increased the release of pro-inflammatory cytokines, which then trigger hepatic insulin resistance (18,19). In this study clearly show that lovastatin mainly reduced serum lipid profile and improved hepatic lipid accumulation. As a result, reduced ROS production and hepatic insulin resistance. Recently, the correlation between oxidative stress and insulin resistance have been extensively investigated. For instance, hyperglycemia, elevated FFA levels, and the production of cytokines have been

shown to induce serine phosphorylation of insulin receptor substrate (IRS) (20). These factors can also activate multiple stress-sensitive serine/threonine (Ser/Thr) kinase signaling cascades, such as, IKK-ß, p38 MAPK and JNK/SAPK, leading to increased serine phosphorylation of IRS and decreased insulin-stimulated tyrosine phosphorylation (21,22). Consequently, the association of impaired downstream insulin signaling molecules, resulting in reduced insulin action that can cause insulin resistance and promote hepatic glucose production process (23). Moreover, over reaction of inflammatory response also leads to multiple side effects, such as, tissue injury and organ dysfunction. Thus, hepatic insulin resistances define as reduced sensitivity of the liver to insulin causes of promote gluconeogenesis and hyperglycemia. In this study found that lovastatin was able to decrease both numbers and size of lipid droplets along with improving hepatic insulin resistance and gluconeogenesis. Therefore, this study suggest that lovastatin not only improves hepatic lipid metabolism, but also attenuates hepatic glucose metabolism in obese insulin resistance rats. Further studies is needed to demonstrated the molecular mechanisms by which lovastatin modulates this process.

Conclusion

These findings indicate that Lovastatin extracted from a novel *A.PSU-RSPG178* was able to ameliorate hyperlipidemia and improved hepatic insulin resistance, hepatic steatosis, and glucose dysregulation. These findings would lead to promote the use of local lovastatin produced in Thailand as an option for obesity and dyslipidemia.

Acknowledgement

This research was supported by the Faculty of Medicine, Faculty of Graduate studies, Chiang Mai University, Chiang Mai, Thailand, and the NSTDA Chair Professor grant (the Fourth Grant) of the Crown Property Bureau Foundation and the National Science and Technology Development Agency to Professor Dr. Vatcharin Rukachaisirikul.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Hexahydrocurcumin modulates amyloid-β precursor protein processing in stress-induced cognitive impairment in mice

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Abstract

Stress is an important factor that triggers neuronal cell injury involved with a pathophysiological of neurodegenerative disorder. A high dose of dexamethasone, glucocorticoid receptor agonist, increases the expression of beta-secretase cleavage enzyme and amyloid precursor protein that lead to the overproduction of amyloid beta peptide which cause neuronal injury and cognitive impairment in Alzheimer's disease. Accordingly, interventions that attenuate neuronal cell injury may reduce the severity of Alzheimer's disease. In this study, we interest to investigate the effect of hexahydrocurcuminto ameliorate neuronal cell injury in dexamethasone-induced mice. The results show that hexahydrocurcumin attenuates dexamethasone-induced neuronal cell injury by reducing the expression of the beta-secretase cleavage enzyme and amyloid precursor protein by RT-PCR. Furthermore, it also improves the cognitive impairment. Our finding suggests that hexahydrocurcumin is an alternative treatment for dexamethasone-induced neuronal cell injury that is one of the causes of Alzheimer's disease.

Keywords: Hexahydrocurcumin, Dexamethasone, Beta-secretase, Amyloid precursor protein, Alzheimer's disease

Introduction

Alzheimer's disease is rapidly growing in elders known as the most common type of dementia that is one of neurodegenerative disorder. The pathology of AD includes of amyloid beta (Aβ) production, tau protein hyperphosphorylation and decrease of acetylcholine level. Several evidences found that the major hall mark of pathology is to increase of Aβ that exert neuronal injury. Aβ accumulation associate with increase of production and decrease of clearance (1, 2). When it occurrence in the brain are highly associated with synaptic dysfunctions involves activation of several receptors at the membrane for example N-Methyl-D-aspartate (NMDA) and alpha-amio-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) lead to calcium overload that generate neuronal excitotoxicity. Moreover, binding with another receptor are modulate neuronal loss, synaptic failure and promote long term depression(LTD) of memory (15).

One of the factors that lead to Aβ accumulation is the elevations of glucocorticoids hormone (GC) known as stress (5), which regulates by the hypothalamic-pituitary-adrenal (HPA) axis. Importantly, increase of an activity of HPA axis lead to alter structure and function of the brain, especially in hippocampus, which mainly found glucocorticoid receptor (3, 4).

Dexamethasone (DEX), which is glucocorticoids receptor agonist. Generally, low dose of DEX were used for anti-inflammation and immune suppression. Growing evidence suggest that a high dose of DEX exert neuronal injury via decreasing levels of glutathione, increasing of oxidative stress, activating inflammation and promoting hippocampal cell death (5, 6). Moreover, DEX directly activate beta (β)-secretase cleavage enzyme lead to increase Aβ production which cause of AD pathology (7). An effect of neurotoxicity depends on times and dose of DEX exposure (6).

Currently, treatment of AD pathology is still lacking (1). However, many studies focus on the main target of Aβ involve in oxidative stress, inflammation and neuronal apoptosis. Consequently, our study is interested in effective agents that reverse there effect. Hexahydrocurcumin (HHC) is one of major metabolites of curcumin that belonging to a group of curcuminoids which extracted from turmeric (16). Previous studies showed that curcumin could reduce inflammation, furthermore, neurodegeneration also reduced Aβ plague and peptide (8), but its chemical is instability. In addition, recently investigation found the comparative scavenging activities of HHC have a higher number than curcumin (9). However, the effect of HHC on DEX exposure and the underlying mechanisms have not been investigated

in this study, we examined the effect of HHC to attenuate dexamethasone-induced neuronal injury.

Materials and Methods

Experimental animals

Male Swiss Albino mice (35-45g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand and kept under the controlled conditions: 12 h light-dark cycle, temperature (25±1 ºC) and free access of food and water. The mice were randomly divided into 6 groups including: 1) Normalsaline control; 2) Dexamethasone (DEX) 60 mg/kg BW 3) DEX received hexahydrocurcumin (HHC) 20 mg/kg BW; 4) DEX received HHC 40 mg/kg BW; 5) DEX received HHC 80 mg/kg BW; 6) DEX received acethylcholinesterase inhibitor (AChEIn) 5 mg/kg BW.

Behavioral test (Morris water maze)

The water maze consists of diameter 120 cm. diameter, temperature at 25 °C and the platform always locate in northeast quadrant then the mice is released in southwest quadrant of the maze. First 5 days are for training and the last day after the training period will be test. Each day of the training, the mice is tested 4 times within 60 s per trial to find the platform. On the testing day, the platform is removed. All activities were recorded with overhead camera**.**

Reverse transcription Polymerase chain reaction (RT-PCR) analysis

After the animals were euthanized, the brain was collected and keptat -80°C. The brain tissue were extracted of the RNA by RNA isolation kit (NucleoSpin® RNA). Total RNA was converted into complementary DNA (cDNA) with (ReverTra Ace®) then cDNA was amplified via reverse transcriptase-PCR assay (PerfeCTa® SYBR® Green FastMix®). Sequence specific primer of GAPDH, APP and beta-secretase were used.

Statistical analysis

All data were expressed as mean ± SD. Analysis of one-way analysis of variance (ANOVA) to compare the result between groups and then followed by Post Hoc Dunnett's test. Statistically significant is considered when the p value less than 0.05 ($p< 0.05$).

Results and Discussion

HHC treatment ameliorates DEX-induced cognitive impairment.

To investigate the effects of HHC on memory impairment induce by DEX, We evaluated the spatial memory of mice by Morris water maze test. We found that the times to spend in target quadrant were decrease in DEX group when compared with control group. However, these times were significantly increased after treatment with 40 and 80 mg/kg/d of HHC for 4 weeks (Figure 1). AChEIn also significantly improved cognitive impairment

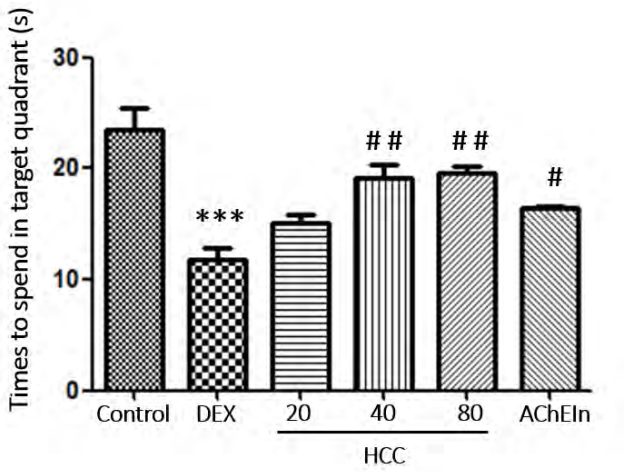

Figure 1. Effect of HCC on DEX-induced cognitive function by Morris water maze test.***p < 0.001 compared with normal group; #p< 0.05; ##p< 0.01 compared with DEX group.

HHC attenuated APP expression and β-secretase enzyme expression.

To investigate the effects of HHC on DEX-induced amyloid-β production. The mRNA levels of amyloid precursor protein and β-secretase enzyme were determined by qRT-PCR. We found that the mRNA levels of APP and β-secretase enzyme were significantly increased in DEX group when compared with control group. However, these levels were significantly decreased after treatment with 40 and 80 mg/kg/d of HHC and AChEIn for 4 weeks (Figure 2).

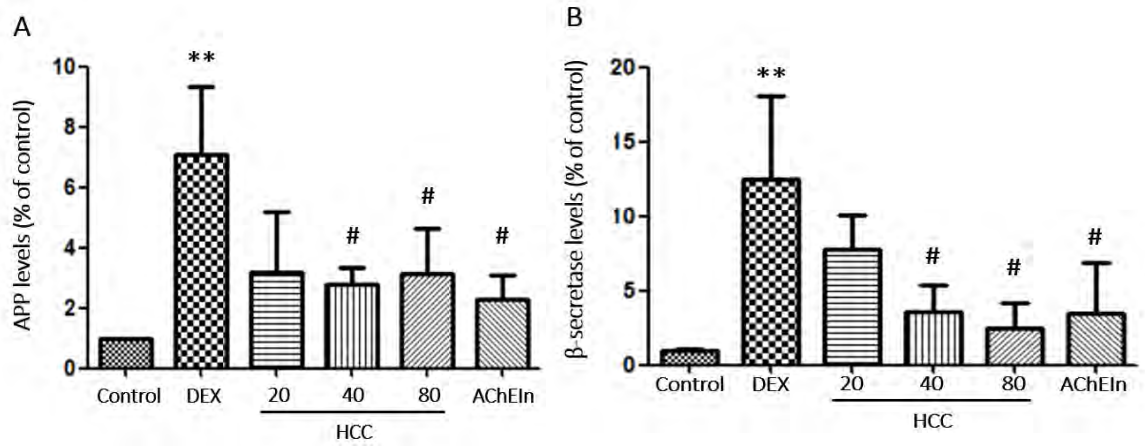

Figure 2 Effect of HHC on DEX-induced mRNA expression of APP and β-secretase by RT-PCR. $^{**}p < 0.01$ compared with normal group; $\#p < 0.05$ compared with DEX group.

The present study is provide evidence that HHC attenuates DEX-induced memory impairment, amyloid-β production. Injection of 60 mg/kg DEX for 28 days induced memory impairment by increasing the expression of both APP and β-secretase enzyme which is an important for amyloid-β production. We demonstrated that HHC treatment ameliorates the effects of DEX on alteration behavior, indicating a reduction of degree of spatial memory impairment. Thus, we suggest that the HHC treatment demonstrates the neuroprotective effect of HHC against DEX-induced neuronal degeneration.

Amyloid-β accumulation in the brain has been implicated in neuronal loss and cognitive impairment during AD progression. β-secretase enzyme plays an important role to generate amyloid-β accumulation. APP is the precursor of amyloid-β production. In the present study showed increased levels of β-secretase enzyme and APP in DEX treated group. According to, Aβ hypothesis, an accumulation of Aβ by increasing of both APP and β-secretase enzyme is the major influence driving to AD pathology (17). However, the levels of these proteins were attenuated by HHC treatment.

Interestingly, the result HHC shows the consistent with curcumin (12). Previous study reported that curcumin completely suppressed the β-secretase mRNA levels and it decreased expression of both APP and beta-secretase (10). Furthermore, curcumin potently attenuated the maturation of APP (11). Moreover, curcumin also promote the increasing of glutathione levels and the enhancing of hippocampal neurogenesis (13, 14). Taken together, this study demonstrates that DEX-induced cognitive declined by increasing the mRNA levels of APP and β-secretase in mice. Our result demonstrates that HHC attenuates the mRNA levels of APP and

β-secretase similar to AChEIn lead to cognitive improvement in dexamethasone-induced neuronal cell injury. Accordingly, HCC may have therapeutic potential in the treatment of Alzheimer's disease.

Acknowledgements

This study was supported by a research grant from the Thailand Research Fund (TRF) (DPG 5780001), Faculty of Medicine, Chiang Mai University is gratefully acknowledged.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
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Impairment of renal transport function mediated by oxidative stress in acute myocardial ischemic injury in rats

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Introduction: Acute kidney injury (AKI) is one of phenomenon which is decreased renal blood flow related to acute myocardial infarction (AMI). Kidney plays an importance role in the renal clearance by renal transporters, such as, organic anion transporter 1 (Oat1), organic anion transporter 3 (Oat3), and organic cation transporter 2 (Oct2). These transporters handle several substrates, including, endogenous organic anions and cations, drug metabolites, and uremic toxin. This study aims to determine the effects of acute myocardial ischemic injury on renal transport function, and identify the mechanisms involved in the impairment of this renal transport function.

Materials and Methods: Acute myocardial ischemic injury was induced in rats by left anterior descending coronary artery occlusion for 30minute. The renal hemodynamic parameters, renal morphology, serum blood urea nitrogen, serum and urine creatinine were subsequently determined along with the level of plasma malondialdehyde and renal mitochondrial reactive oxygen species.

Results: The results indicate that acute myocardial ischemic injury led to transient renal hypo perfusion, renal tubular damage, increased renal mitochondrial reactive oxygen species and plasma malondialdehyde. These consequences led to impaired renal organic secretion.

Conclusions: This study suggests that acute myocardial ischemic injury impaired renal transport function mainly by oxidative stress, which could potentially worsen AKI. Thus, any anti-oxidant properties might be considered as therapeutic choice for reversing such injury.

Keywords: Acute myocardial ischemic injury, renal transport function, oxidative stress, hypoperfusion

Introduction

Acute kidney injury (AKI) is frequently associated and defined to be the highest risk factor following acute myocardial infarction (AMI) (1-4). Moreover, short- and long-term hospitalization of the patients suffering from these consequences would mostly lead to cardio renal syndrome (5), resulting in a difficulty and failed managements (2). Therefore, the understanding of pathophysiological consequences is necessarily required to seek potential targets for early intervention of myocardial infarction induced kidney injury which eventually could improve the clinical outcomes and reduced mortality.

Renal dysfunction has been implicated to be a significant public health concern because of its frequent occurrence with other clinical conditions, including AMI, congestive heart failure, cardio angiography and percutaneous coronary intervention. The mechanisms involved in the consequences of AKI after AMI have been extensive elucidated; the most common ones are systemic hypo-perfusion, renal hemodynamic alteration, glomerular and tubular damage, and increased oxidative stress (4). Oxidative stress has been proposed to be a major mediator of worsening renal diseases after ischemic condition (6) which is characterized by an imbalance between the levels of reactive oxygen species (ROS) and antioxidants (4, 6). During renal occlusion, oxygen deprivation leads to a rapid degradation of ATP to ADP and, AMP, and a final metabolite hypoxanthine. In addition, the conversion of accumulated hypoxanthine to uric acid by xanthine oxidase generates ROS (7), which resulted in DNA damage (7).

Kidney is known to play an important role in the elimination of several xenobiotics, including endogenous metabolites, drugs, and toxins (8). The active secretion of organic substances to the tubular lumen appears to be restricted to the basolateral membrane of proximal tubule via several transporters. At the present, renal transporters involved in the tubular secretion have been extensively characterized (8). Among these, organic anion transporters 1 (Oat1), organic anion transporters 3 (Oat3) and organic cation transporters 2 (Oct2) have been shown to play a major role in the cellular uptake of organic substances across the basolateral membrane of renal proximal tubules due to their high expressions (8).

Previous studies reported that renal ROS overproduction induced by renal ischemic injury has been demonstrated to directly alter pharmacokinetic of anionic and cationic drugs that excrete via renal transporters in rats (9, 10). However, there is limited information regarding to the effects and mechanisms involvement of acute myocardial ischemic condition induced ROS overproduction on renal transporter functions which could potentially generate acute kidney injury.

Methodology/Experimental design

Animals

Adult male Wistar rats were obtained from the Nomura Siam international, Bangkok, Thailand and cared in the Laboratory Animal center facilities of Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. The experimental protocols were approved by the Laboratory Animal Care and Use Committees at Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. All experimental rats were housed in a room and maintained at 25± 1 °Con a 12: 12 h dark-light cycle and allowed to acclimatize at least 1 week before the beginning of the experiments. The rats were divided into sham and cardiac ischemic groups. Before experiment, rats were fasted overnight with free access to water. At the end of study, the animals were sacrificed, urine, blood, and tissue samples were collected for subsequent experiments.

Determination of renal hemodynamic parameters

The non-invasive flow assessment of the right renal artery was carried out using slightly modified protocol of pulsed wave (PW) viewed ultrasound system (11). The animals were

measured renal hemodynamic parameters at baseline and ischemic phase. This data was presented as meter/second (m/s).

Renal morphology examination

To assess renal morphology, the kidneys were excised and one half of the kidney was fixed in 4% neutral formalin buffer for 12-24 hrs, and then embedded in paraffin. Each slide was cut into 5-7 µm-thick sections and subsequent stained by hematoxylin and eosin (H&E). The tissue morphological change was determined by using bright-field microscopic evaluation.

Determination of blood urea nitrogen, plasma creatinine, urine creatinine and plasma malondialdehyde

The quantitative total blood urea nitrogen, plasma creatinine and urine creatinine was determined by commercial enzymatic colorimetric assays. Plasma malondialdehyde was also determined using commercial TBARS assay kit purchased from Cayman Chemical (Ann Arbor, MI, USA).

Renal mitochondrial isolation

Renal mitochondrial isolation was modified and carried out as previously described (12, 13). The renal cortical tissues were removed, cut into small pieces, and homogenized in ice-cold isolation buffer 1 (I1-buffer) (mM: 215 mannitol, 75 sucrose, 1 EGTA, 20 HEPES, and 0.1% BSA, pH 7.2) and centrifuged at 3,700 rpm for 8 min at 4°C. The supernatant was collected and re-centrifuged at 11,800 rpm for 10 min at 4°C. The pellet was re-suspended in ice-cold I1-buffer and the sample was layered on 15% percoll. The sample was centrifuged at 15,400 rpm for 9 min at 4°C. The pellet was re-suspended in the same buffer and re-centrifuged at 15,400 rpm for 13 min at 4°C to remove percoll. The pellet was re-suspended in ice-cold I2-buffer (mM: 215 mannitol, 75 sucrose, 20 HEPES, and 0.1% BSA, pH 7.2) and centrifuged at 11,800 rpm for 13 min at 4°C and the pellet from this step was designated as mitochondrial fraction.

Renal mitochondrial ROS production

Renal mitochondrial ROS was measured using 2', -7'-dichlorodihydro fluorescein diacetate (H_2DCFDA, Invitrogen) as modified by previous study (13). The renal isolated mitochondria was washed 3 times with PBS and 10 uM H_2DCFDA at 37°C was incubated for 30 min. Excess H_2DCFDA was then removed by PBS for 4 times, and the mitochondrial fluorescence of dichlorofluorescein (DCF) was determined at 485 nm excitation and 528 nm emission in a fluorescent microplate reader.

Statistical analysis

Data were expressed as mean ± S.E. Statistical differences was assessed using t-test. Differences was considered to be significant when $p<0.05$.

Results

Effects of acute cardiac ischemic condition on body weight, kidney weight, and kidney index

As shown in Table 1, the body weight, kidney weight, and kidney index (kidney weight per body weight and multiply by 1000) were not significant between sham and cardiac ischemic groups, indicating that cardiac ischemic did not alter body weight and kidney weight.

Table 1 General characteristics of body weight, kidney weight and kidney index.

Parameters	Sham	Ischemic
Body weight (BW, g)	336.00 ± 3.67	335.00 ± 5.00
Kidney weight (KW, g)	2.24 ± 0.03	2.14 ± 0.02
Kidney index	6.68 ± 0.11	6.40 ± 0.15

Effect of acute cardiac ischemic conditions on renal blood flow

As shown in Figure 1, the renal blood flow in ischemic condition was significantly decreased to 0.34±0.03 m/s during ischemic phase when compared with sham group (0.60±0.05 m/s). This data suggest that complete cardiac occlusion resulted in partially renal hypoperfusion.

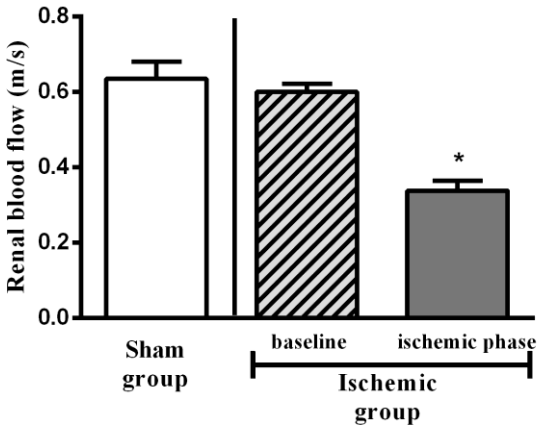

Figure 1 Effect of cardiac ischemic condition on renal blood flow was recorded during baseline and onset of ischemia phase in ischemic group. *p<0.05 vs. sham group.

Effect of cardiac ischemic condition on renal morphology.

The renal morphology after cardiac occlusion was also assessed. The data show that sham rat kidney had normal renal structures, including glomerulus, Bowman's capsule space, and proximal convoluted tubules (Figure 2A). On the other hand, acute cardiac ischemic rat kidney markedly showed glomerular infiltration with a wide Bowman's capsule space (line arrow) and tubular lumen space (dot arrow) (Figure 1B), indicating that acute cardiac ischemic condition affected in both glomerular structural infiltration and tubular damage which might lead to impaired renal function.

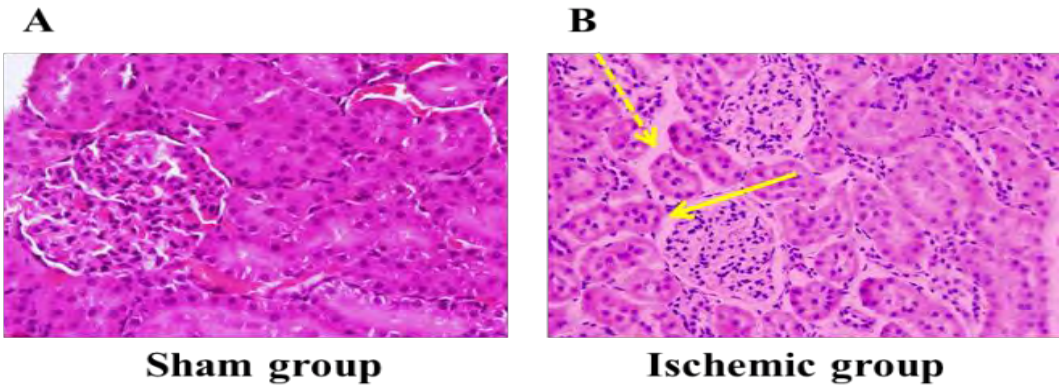

Figure 2. Hematoxylin and Eosin (H&E) staining of rat kidneys. A sagittal half of kidney from (A) sham, and (B) ischemic group was removed, fixed, embedded, cut, and stained by H&E. The results were analyzed using bright-field microscopy.

Effect of acute cardiac ischemic condition on kidney function biomarkers

Acute cardiac ischemic condition significantly increased blood urea nitrogen (BUN) (Figure 3A) and plasma creatinine (Figure 3B) when compared with sham. However, urine creatinine level was no significant difference between sham and ischemic group (Figure 3C). This data indicate that acute cardiac ischemic leads to progressive decline of kidney function reflected by impaired creatinine excretion by renal transporters.

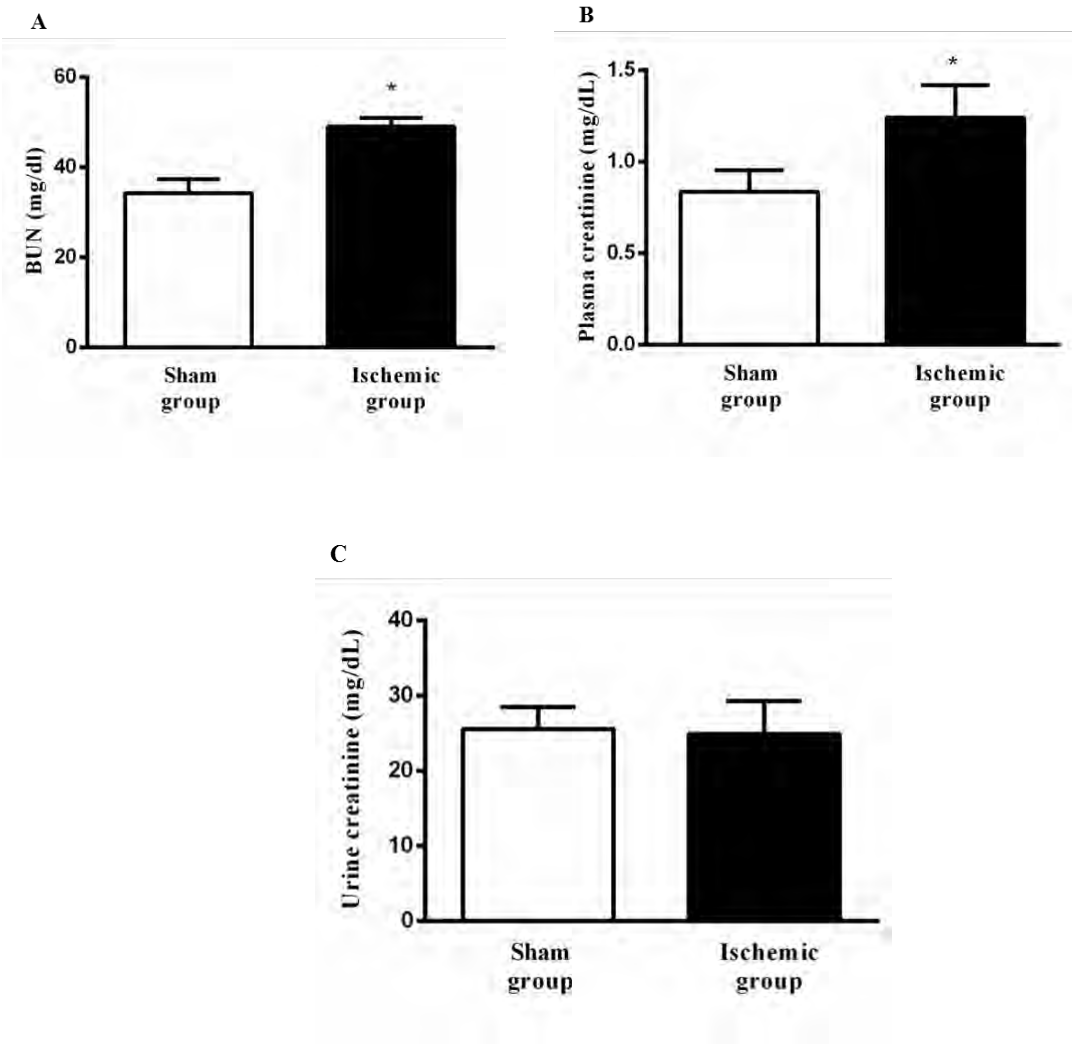

Figure 3. Effect of acute cardiac ischemic condition on kidney function biomarkers. (A) blood urea nitrogen (BUN), (B)plasma creatinine, and (C) urine creatinine. The data were analyzed and represented as mean ± S.E (N=6). *p<0.05vs. sham.

Effect of acute cardiac ischemic condition on lipid peroxidation and renal mitochondrial reactive oxygen species.

As shown in Figure 4, plasma MDA levels in cardiac ischemic group significantly increased when compared with sham operation, indicating that acute cardiac ischemic condition lead to increased systemic oxidative stress. Moreover, the renal mitochondrial ROS level was markedly increased in ischemic group when compared with sham group (Figure 4B). Therefore, this data suggest that acute cardiac ischemic condition aggravated renal tubular damage through mitochondrial ROS production.

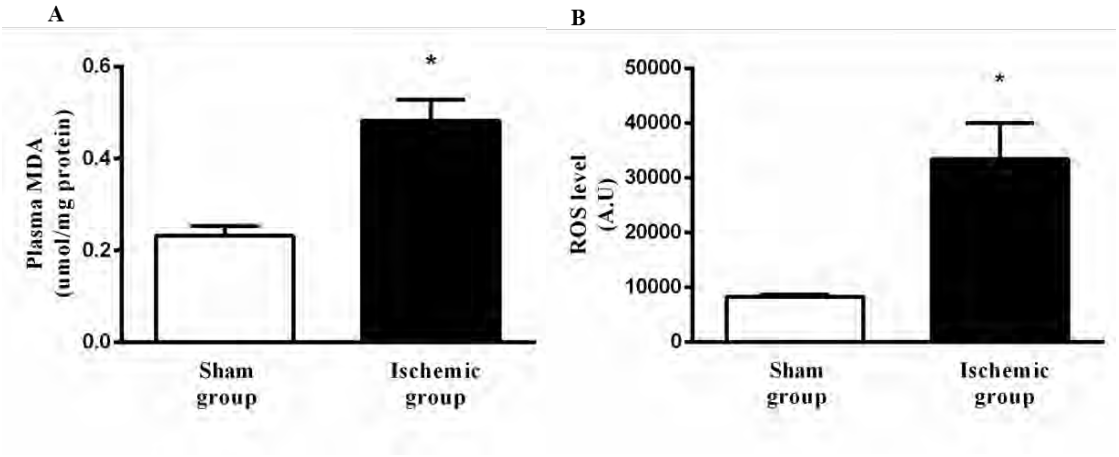

Figure 4 Effect of acute cardiac ischemic condition on (A) plasma MDA and (B) renal mitochondrial reactive oxygen species. The data were analyzed and represented as mean ± S.E. (N=6). *$p<0.05$vs. sham group. #$p<0.05$ vs ischemic group.

Discussion

In the present study, we have shown that acute cardiac ischemic injury led to impair renal hemodynamic (14), resulting in hypoperfusion (15). This condition is attributed to decreased oxygen and ATP supply, leading to systemic lipid peroxidation in which a consequence of renal mitochondrial damage and ROS production (16, 17), resulting in renal tubular damage.

Organic anion transport 1 (Oat1) and Organic anion transport 3 (Oat3) are rate-limiting step of renal secretory transport of organic anions and highly expression on basolateral membrane in renal proximal cells. Oat1 and Oat3 are known as tertiary active transport (18) which depend on sodium and α-ketoglutarate gradient (14, 19) Previous study showed that the

lack of ATP due to renal ischemic/reperfusion led to reduced Oat1/3 expression and function, resulting in impaired anionic secretion (14). This consequence was partially involved with several molecular mechanisms, including the production of prostagladin E2, up-regulation of iNOS and generation of total nitric oxide, and recruitment of macrophage ED1 cells (14). Consistently, this study demonstrate that acute cardiac occlusion partially affected on renal perfusion which, in turn, produced systemic ROS, resulting in renal mitochondrial dysfunction. This consequence impaired the clearance of substrate, particularly, creatinine, via renal organic anion transporters, particularly Oat1/Oat3. Thus, any anti-oxidant agents which could act against oxidative stress might be considered as therapeutic choice for reversing acute kidney injury induced acute myocardial infarction.

Conclusion

This study indicate that acute myocardial ischemic injury impaired renal transport function mainly by oxidative stress, which could potentially worsen of acute kidney injury (AKI). Thereby, any anti-oxidant drugs might be considered as therapeutic choice for reversing such injury

Acknowledgement

This research was supported by the Thailand Research Fund (RSA5980009 to CS), Faculty of Medicine Endowment Fund, Faculty of Medicine, Faculty of Graduate studies, Chiang Mai University, Chiang Mai, Thailand.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets ***January 22nd-24th, 2018***
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Comparison of anti-inflammatory and antioxidant activities of water and hydrolysed extracts *Hibicus sabdariffa* L.

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Abstract

Hibicus sabdariffan L. (Roselle) has been used in treating myriad disorders. Plenty researches of anthocyanins are antioxidants and also reduced on inflammatory processes. The main ingredients of Roselle are anthocyanin. The objective of this study was to compare anti-inflammatory and antioxidant activities of water and hydrolysed roselle extract. Water extracts and acid-chloroform extracts or hydrolysed form of *Hibicus sabdariffa* L. were prepared. The extracts were tested anti-inflammatory activity by inhibitory effect on nitric oxide production with using lipopolysaccharide (LPS) activated on RAW264.7 macrophages and antioxidant activity used by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay. The inhibitory effect on nitric oxide production showed that hydrolysed extracts of *Hibicus sabdariffa* L. showed high inhibitory effect on nitric oxide production than water extract with IC_{50} values 42.22 ±1.56and>100 µg/ml. For antioxidant activities, hydrolysed extracts also showed higher antioxidant activity than water extract (IC_{50} value as 22.89± 1.99 and 50.40 ± 1.41 µg/ml). This study demonstrated that hydrolysed extraction of *Hibicus sabdariffa* L. had more anti-inflammatory and antioxidant activities than water extract. Therefore, it can concluded that hydrolysed form of water roselle extract which imitated in stomach showed higher both activities than water extract.

Keywords : *Hibicus sabdariffa* L., Nitric oxide inhibitory, DPPH radical scavenging, anti-inflammatory, antioxidant

Introduction

Hibicus sabdariffa L. (HS)also called Roselle and Kra-Jeab by Thai name. It has been used in Thai traditional medicine for a long time. Nowadays, the consumption of herbal is increasing in wellness trend. HS use as ingredient, colorant in foods and beverages [1,2]. Previous study demonstrated that HS showed anti-hypertensive, anti-hyperlipidemia, diuretics, digestive and anti-inflammatory activity [2,3,4,5]. HS calyces contain a natural edible brilliant red color which rich of antioxidant include anthocyanin [6,7]. Many reports revealed that anthocyanin inhibit inflammation process and display antioxidant activity [8,9]. However, bioactive compound in HS may be loss in stomach digestion. Therefore, the objective of this study was to compare anti-inflammatory and antioxidant activity of water extract and hydrolysed extract of *Hibicus sabdariffa* L.

Methods

Extraction

Water extract of *Hibicus sabdariffa* L. was obtained from Center of Excellence on Applied Thai Traditional Medicine Researchs, Faculty of Medicine, Thammasat University.

Hydrolysed

Water extract of *Hibicus sabdariffa* L. (50 g) was boiled with 1% hydrochloric acid for 15 min. Then, chloroform was added into water extract with ratio 1:1. The hydrolysed of water extract was obtained from chloroform part which was evaporated and stored at -20°C.

Nitric oxide inhibitory assay [10,11]

Murine macrophage cell line (RAW264.7) was cultured in RPMI 1640 (Gibco, USA) medium contained with 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were maintained at 37°C incubator with 5% CO_2 atmosphere and 95% humidity. Concisely, cultured RAW264.7 cell line was seeded in 96 well microplate with $1x10^5$ cells/well for 24 hours at 37°C. After incubation, 100 µl of RPMI in each well was removed and replaced with 100 µl fresh RPMI medium contained 2 ng/ml of lipopolysaccharide (LPS)in

A-D row of 96 well plate and RPMI medium without LPS in E-H row. Various sample concentration (1, 10, 30, 50 and 100 µg/ml) was added into each well and incubated at 37°C for 24 hours. After that, 100 µl of supernatant was removed to another 96 well microplate and added 100 µl of Griess reagent for determined nitric oxide production. The absorbance was measured by microplate reader at wavelength of 570 nm. The percentage of inhibition was calculated by the formulae below and IC_{50} was calculated by Prism program.

$$\%\text{Inhibition} = \frac{\text{ODcontrol} - \text{ODsample}}{\text{ODcontrol}}$$

Cytotoxicity was also determined by using MTT colorimetric method. MTT (10 µl/well) was added and incubated at 37°C for 2 hours. Then, supernate was removed and replaced with isopropanol containing 0.04 M HCl to dissolved formazan in the cells. Formazan production was measured at wavelength of 570 nm by microplate reader.

DPPH radical scavenging assay [12,13]

The antioxidant activity determined by 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH solution was prepared at $6x10^{-5}$ M in absolute ethanol. Butylatedhydroxytoluene (BHT) was used as positive control. Various concentrations (1, 10, 50 and 100 µg/ml) of sample were added into 96 well microplate. After that, DPPH solution 100 µl was added to each well and incubated under dark room for 30 minutes at room temperature. The absorbance was measured at wavelength 520 nm of microplate reader. The percentage of activity was calculated by the formulae below and IC_{50} was calculated by Prism program.

$$\%\text{Inhibition} = \frac{\text{ODcontrol} - \text{ODsample}}{\text{ODcontrol}}$$

Results

Nitric oxide inhibition of water extract and hydrolysed extract of HS was presented in Table1. The results demonstrated that water extract of HS had no inhibitory effect on nitric oxide production whereas hydrolysed extract had potentially effect on nitric oxide inhibition

with IC_{50} value of 42.22 ±1.56µg/ml. Prednisolon, as positive control, suppressed nitric oxide production with IC_{50} value of 0.12 ±0.01 µg/ml.

The results of antioxidant activity were shown in Table1. Hydrolysed extract and water extract of HS showed antioxidant activity with IC_{50} value of 22.89± 1.99 µg/ml and 50.105 ± 1.238 µg/ml, respectively. However, hydrolysed extract indicated antioxidant effect better than water extract.

Table 1. Table showed the %yield, antioxidant activities and anti-inflammatory activities of *Hibicus sabdariffa* L. extract

Extraction	% yield	Anti-oxidant activities (IC_{50} µg/ml)	NO inhibitory activities (IC_{50} µg/ml)
Water extract	-	50.40 ± 1.41	>100
Hydrolysed extract	4.76	22.89± 1.99	42.22 ±1.56

Discussion

This study demonstrated that hydrolysed extraction of *Hibicus sabdariffa* L. had more anti-inflammatory and antioxidant activities than water extract. Therefore, it may be concluded that hydrolysed form of water roselle extract which imitated in stomach showed higher both activities than water extract**.**

Acknowledgements

We would like to thanks Center of Excellence in Applied Thai Traditional Medicine Research and Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University for suggestion and helpful.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Antimicrobial activity of pineapple peel extract

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Abstract

Thailand is the number one exporter of pineapple in the world, both, fresh and processed pineapples. In the industry, pineapple peel, contributing to approximately 30-35 % of the whole fruit, is normally used as animal feed. However, pineapple peel has been reported to still have some bioactive compounds. Some works already reported biological activities of those compounds, however, limited works reported on its antimicrobial activity. This work aimed to investigate the antimicrobial activity of pineapple peel (PP) extract (PPE) to observe possibility of using it as a natural antimicrobial agent in food. The PP was dried at the temperature of 70 C by tray drier and ground to approximately 100-mesh size. Then, the dried PP was macerated in methanol at the PP-to-Methanol ratio of 1:10 on a shaker for 4 hrs. Subsequently, it was filtered and the filtrated was evaporated under reduced pressure to get a crude PPE. The antimicrobial activity of the PPE was performed against some food pathogens (*Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Escherichia coli*) by disc diffusion and broth dilution assays. It was found that the PPE could inhibit all the tested bacteria. In addition, the gram positive bacteria tended to be more susceptible to the PPE than the gram negative one. In disc diffusion assay, *B. cereus* exhibited the most sensitive strain with minimum inhibition concentration (MIC) of 0.0675 g/mL, followed by *S. aureus and E. coli* (0.1349 g/mL) and *S. typhimurium* (0.2699 g/mL), respectively. In addition, the minimum bactericidal concentration (MBC) against all the tested bacteria in broth dilution assay was 0.0675 g/mL, except *B. cereus*, in which the lethally effect was not detected. The results suggested that the PPE is a potent natural antimicrobial agent for food application.

Keywords: Gram positive bacteria, Gram negative bacteria, pineapple peel powder

Introduction

Pineapple (*Ananas comosus* (L) Merr.) is a tropical fruit and Thailand is one of the pineapple exporter in the world. It is also used as a raw material in food industry in which pineapple peel is the major by-product, contributing to approximately 30-35 % of the whole fruit (*1*). The pineapple peel is normally used as an animal feed and some have applied it as a raw material for various products production, including single cell protein, biomass, ethanol, etc. (2-5) However, some researchers have reported that pineapple peel was a potential source of bioactive compounds, such as vitamin C, carotenoid, phenolic compounds, flavonoids etc. (*6-9*). Those compounds have been reported to have various biological activities, particularly, antioxidant activity (*10-12*). However, those bioactive compounds are varied depending on many factors, including cultivar, pre-and postharvest treatment and particularly method for raw material and extract preparation, etc. (13-17) Some works already reported biological activities of those active compounds in pineapple peel, however, limited works reported its antimicrobial activity. This work, therefore, was aimed to investigate the antimicrobial activity of pineapple peel (PP) extract (PPE) in order to observe possibility of using it as a natural antimicrobial agent for food application.

Materials and Methods

Pineapple peel(PP) preparation was obtained from Siam Food Products Public Co., Ltd., Chonburi, Thailand. The PP was sliced to small pieces and dried at 70 C, which is the condition provided the highest antioxidant activity from our previous study until the moisture content of ≤ 13 % was reached (*18*). Then, the dried PP was ground to approximately 100-mesh size and stored at 4 C until used.

PP extract (PPE) preparation

The dried PP was macerated in methanol (Me) at the PP-to-Me ratio of 1:10 on a shaker for 4 hrs. Subsequently, the mixture was filtered and the filtrate was evaporated under reduced pressure in order to get a crude PPE. The PPE was collected and put into a vial closed with a perforated paraffin film in a desiccator until constant weight was obtained and used for % yield calculation. The PPE was stored at 4 C for further used in the experiment.

Microorganisms

Four potent foodborne pathogens used in the experiment including gram positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 6633) and gram negative bacteria (*Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922) were obtained from Department of Microbiology, Faculty of Science, Burapha University. Microbial suspension of approximately 1×10^8 CPU/mL was prepared via 0.5 McFarland standard solution for further antimicrobial activity testing.

Antimicrobial testing

Agar disc diffusion assay

The antimicrobial activity of the PPE was tested against four foodborne pathogens using agar disc diffusion method according to the method of Clinical and Laboratory Standards Institute (*19*). Briefly, within 15 min after the bacterial suspension preparation, a sterile cotton swab was dipped into the suspension and swabbed on Mueller-Hinton agar (MHA). The PPE at different concentrations (0.0337, 0.0675, 0.1349, 0.2699 and 0.5398 mg/L) was prepared. Then, the PPE or the standard positive control (20 µL) of each microorganism or negative control (methanol) was individually impregnated onto a disc (6 mm diameter) and dried. The discs were placed on the microorganism lawn on MHA and incubated at 37 C for 18 hrs. Then, the inhibition zone was measured (mm.)

Broth dilution assay

The broth dilution assay was applied in order to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the PPE regarding to the method of Clinical and Laboratory Standards Institute (*19*). The PPE was mixed with the microbial suspension in order to get the desired final concentrations (0.5398-0.0042 g/mL) in the test tubes (2-8) and the tube (1 and 10) were the sterile control and the growth control, respectively. The MIC (the concentration where the first inhibition was observed) and MBC (the concentration where no growth was observed) were observed after incubation at 37 C for 24 hrs.

Results and discussion

From the disc diffusion assay, the extract could inhibit all the tested bacteria as shown in Figure 1. The results also showed that *B. cereus* was the most sensitive bacteria compared to the others as indicated by the lowest minimum inhibitory concentration (MIC) of the extract (0.0675 g/mL) and the largest inhibition zone of 10.50 ± 0.24 mm when the highest concentration of the PPE was applied. Whereas, the lowest concentration that could inhibit *E. coli* and *S. aureus* was the same (0.1349 g/mL) and the highest MIC was observed for *S. typhimurium* (0.2699 g/mL). These results suggest that *S. typhimurium* is the most resistant bacteria which is consistent with some previous reports (*20*). It was also found that the inhibitory effect of the extract tended to increase with the increasing concentration ($p \leq 0.05$) as indicated by the larger inhibition zone (Table 1). In addition, the gram positive bacteria tends to exhibit more susceptible to the PPE than the gram negative ones when the same concentration of PPE was applied.

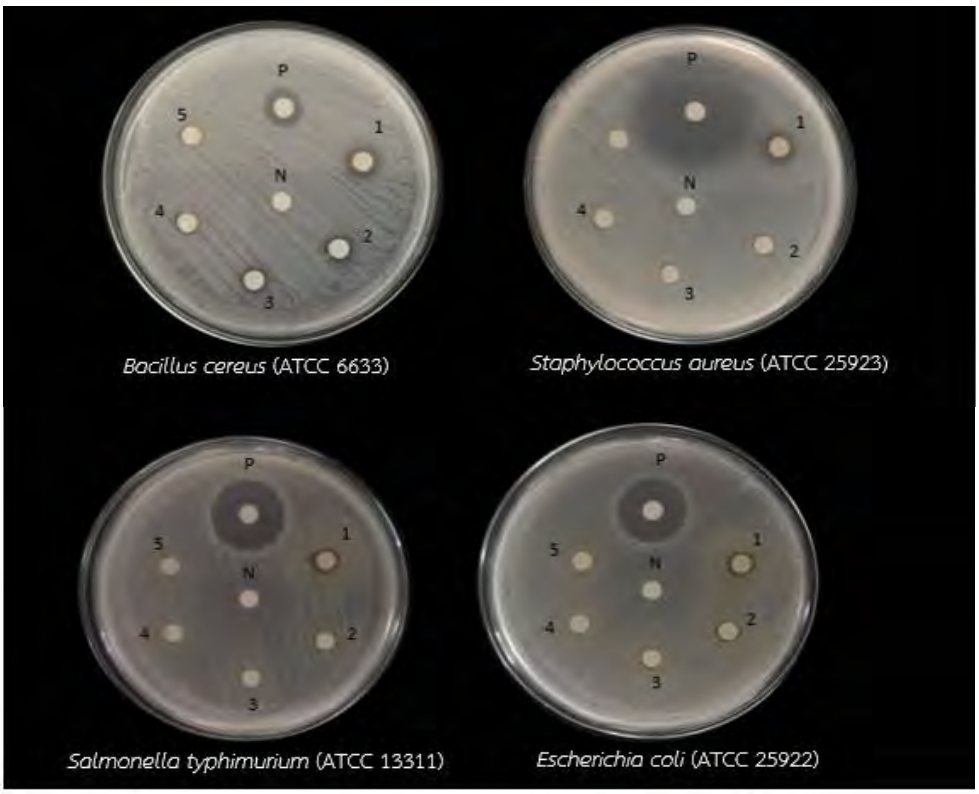

Figure 1 Inhibitory effect of pineapple peel extract at the concentration of 1) 0.5398 2) 0.2699 3) 0.1349 4) 0.0675 5) 0.0337 g/mL and P) Positive control, N) Negative control

Table 1 Antimicrobial activity of pineapple peel extracts against some pathogens

Pineapple extract concentration (g/mL)	Inhibition zone (mm)			
	Gram negative bacteria		Gram positive bacteria	
	Escherichia coli ATCC 25922	*Salmonella typhimurium* ATCC 13311	*Staphylococcus aureus* ATCC 25923	*Bacillus cereus* ATCC 6633
0.0337	ND	ND	ND	ND
0.0675	ND	ND	ND	7.00 ± 0.00^{a}
0.1349	$7.00 \pm 0.00^{b,B}$	ND	$7.50 \pm 0.24^{a,B}$	$7.00 \pm 0.19^{b,C}$
0.2699	$7.50 \pm 0.24^{b,B}$	$7.67 \pm 0.00^{b,B}$	$7.00 \pm 0.00^{c,B}$	$8.17 \pm 0.24^{a,B}$
0.5398	$8.33 \pm 0.00^{c,A}$	$9.33 \pm 0.00^{b,A}$	$10.33 \pm 0.47^{a,A}$	$10.50 \pm 0.24^{a,A}$

Note: Means with the same uppercase letter (A,B,C, …) in column and the same lowercase letter (a, b, c, …) in row indicate no significant difference ($p > 0.05$), ND: not detected.

In broth dilution assay, different concentrations of the pineapple peel extract (0.5398–0.0042 g/mL) were applied in order to observe the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of the PPE. It seems that the gram positive bacteria was more susceptible to the PPE than the gram negative ones indicating by the lowest MIC of 0.0084 g/mL which is similar to those results obtained from the disc diffusion assay. These results is also consistent to some researches which reported that the gram positive bacteria was more sensitive to some antimicrobial agents than the gram negative ones. These results from the different cell structure of those bacteria (*21*). In addition, lethally effect was observed in all the tested bacteria with the MBC of 0.0675 g/mL, except *B. cereus*, where the bactericidal effect was not detected (Table 2). The lethally effect might be due to effect from some bioactive compounds in the pineapple peel, particularly phenolic compounds that causes changes in permeability of the cell structure. Those changes could lead to leakage of some intracellular cell contents or permission of those bioactive compounds to the cell causing to the cell death (*22, 23*).

Table 2 Minimum inhibition concentration (MIC, g/mL) and Minimum bactericidal concentration (MBC, g/mL) of pineapple peel extracts against some pathogens.

Microorganisms	MIC (g/mL)	MBC(g/mL)
Gram negative bacteria		
Escherichia coli ATCC 25922	0.0168	0.0675
Salmonella typhimurium ATCC 13311	0.0084	0.0675
Gram positive bacteria		
Staphylococcus aureus ATCC 25923	0.0084	0.0675
Bacillus cereus ATCC 6633	0.0084	ND*

*ND: Not detected.

These results were different from the results obtained by the disc diffusion assay in which *B. cereus* was found to be the most sensitive bacteria and *S. typhimurium* was the most resistant bacteria. This might due to the nature of the assay itself. In broth dilution assay, the extract was allowed to be in contact with the bacterial cell in the solution, consequently injuring the bacterial cell, whilst inducing bacterial spore forming, then the spore germinated afterward in the new media for the *B. cereus* case. Whereas, the others which were not able to produce spore, consequently, no survival cell was detected.

Conclusions

It was found that gram positive bacterial tended to be more susceptible than the gram negative bacteria. Broth dilution assay revealed that the most resistant bacteria was *B. cereus*. The concentration that lethally effect against the tested bacteria, except *B. cereus*, was 0.0675 g/mL. Therefore, it could be concluded that the pineapple peel extract is a potential source of antimicrobial agent and could be applied for food application.

Acknowledgements

Authors would like to thank for Burapha University for financial support and also would like to thank Siam Food Products Public Co., Ltd. for providing pineapple peel for the experiment.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
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Toxicity study of Ma KhangDaeng (*Dioecerciserythroclada* (Kurz) Tirveng.) and Ma KhangKhao (*Tamilnadia uliginosa* (Retz.) Tirveng. & Sastre) in rats.

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Abstract

The acute toxicity and subacute toxicity test of Ma kang Dang (*Dioecerciserythroclada*) Kurz (Triveng) and Ma KangKhaw (*Tamilnadiauliginosa*) was determined to evaluate of methanolic extract form Ma kang Dang and Ma KangKhawin adult male and female Sprague-Dawley rats. For the study of acute toxicity, the methanol extract of barks of Ma kang Dang and Ma KangKhaw was given separately in various doses (2,5 and 10 g/kg) by oral administration. The results showed no significance of toxicity such as general behavior change, mortality, or change in the gross appearance of internal organs. Subacute toxicity was studied by daily oral doses 500 mg/kg (intermediate dose) orally for 28 days. The results showed no abnormalities in treated groups as compared to the controls. Although significantly different, all of the values were within normal limits. Neither gross abnormalities nor histopathological changes were observed.

Keywords: Ma KhangDaeng (*Dioecerciserythroclada*), (Ma KhangKhao) *Tamilnadia uliginosa*, Toxicity, Rats

Introduction

A plant in Rubiacea such as *Dioecresciserythroclada* Kurz (Triveng) (Ma KhangDaeng) and *Tamilnadia uliginosa* (Ma KhangKhao) are widely distributed in Thailand [1]. Ma KhangDaeng and Ma KhangKhao are used as a local medicine and various aliment [1]. But well before any such studies were undertaken, local people knew how to use the fresh leaves of Ma KhangDaeng and Ma KhangKhao to treat illness such as eye pathogens, diarrhoea, dysentery, skin pathogen, diuretic, tonic properties, biliousness, aphrodisiac, their extract also showed antioxidant and antimicrobial activities the fruit used as astringent in cosmetic . A greenish Rubiacea has long been popular among Thai people of all ages. There have been many other scientific studies regarding traditional applications of Ma KhangDaeng and Ma KhangKhao [2], and another recent study has shown that Ma KhangDaeng and Ma KhangKhao extracts exhibit antioxidant activity and antimicrobial activity [4]. Ma KhangDaeng and Ma KhangKhao extract were found many active compounds such as flavones, glycosides, saponins [2,3], fatty acids and phytols [6], tannins and alkaloids [7]. There have been many other scientific studies regarding traditional applications of Ma KhangDaeng and Ma KhangKhao extracts [4], and another recent study has shown that Ma KhangDaeng and Ma KhangKhao extracts exhibit antioxidant activity and showed hypoglycemic effects [2]. There were many studies of Ma KhangDaeng and Ma KhangKhao extract with an organic solvent such as ethanol. But there was no scientific report of Ma KhangDaeng and Ma KhangKhao extract with methanol, dichoromethane, and hexane.

The objectives of our study were observed the Ma KhangDaeng and Ma KhangKhao extracts for acute toxicity and subacute toxicity in the rat.

Materials and Methods

Experimental procedures using rats were performed at the University of Phayao. Male and female Sprague Dawley (SD) rats were received in good health from National Laboratory Animal Center, Thailand and acclimated for 16 days, including a 7-day quarantine period. Animals were housed individually in stainless steel wire mesh cages (29W × 22D × 21H cm) in an animal room designed to maintain a temperature range of 21.0–25.0 °C, relative humidity of 40.0–70.0%, clean air changes per hour and 12-h light-dark cycle (fluorescent lighting from 7:00 to 19:00). Animal was maintained by the “Guide for the car and Use of Laboratory animal” (National Research Council, 2011) . The study protocol was reviewed and approved by the

University of Phayao Animal Care and Use Committee (IACUC) (UP-AE550104). Animals were allowed free access to the feed and drinking water and were 6-weeks of age at initiation of dosing.

Ma KhangDaeng and Ma KhangKhao extracts and dextrin (a powder excipient contained in Ma KhangDaeng and Ma KhangKhao powder extracts at 90%) were mixed with powdered feed at 3% and provided to 10 rats/sex/group ad libitum for 6-weeks (42 days) to achieve dose levels of 0 (dextrin only), 6, 20 and 60 mg/kg/day as geniposide. Dose levels were determined based on a previously conducted 2-week oral dose study. In that study, increased total bilirubin and decreased triglycerides in plasma, dark to blackish brown discoloration of the liver and kidneys and increased liver weights were evident in animals receiving geniposide at 60 mg/kg or greater. No deaths occurred up to the highest dose of 200 mg/kg. When a feed-admixture containing gardenia yellow powder at 3% (approx. 0.024% as geniposide) was administered to rats, only dark brown discoloration of the liver was observed in 1 of the 6 females.

Decreased food consumption (avoidance of feed) was not evident. Therefore, 60 mg/kg (as geniposide), a dose at which some toxic effects were expected, was selected as the highest dose for this study. Lower dose levels of 20 and 6 mg/kg were selected by a common ratio of approx. 3. Since dosing via feed-admixtures was selected for this study based on the actual use Ma KhangDaeng and Ma KhangKhao powder extracts, the concentration of Ma KhangDaeng and Ma KhangKhao powder extracts in the feed was set at 3% at which no feed avoidance was expected. Clinical observations were conducted twice daily (between 8:00 and 10:00 and between 15:00 and 17:00) during the dosing period. Body weights and food consumption were recorded every 3 or 4 days, and daily food consumption was calculated. Ophthalmological examinations and urinalysis were conducted during Week 13 of dosing on 6 animals/sex/group. Urinalysis included the following parameters: pH, protein, glucose, ketone bodies, bilirubin, occult blood, urobilinogen and urinary sediment using samples collected within 4 h of voiding; urinary volume, color, specific gravity, sodium, potassium and chloride using 16-h urine samples. Water consumption during 16-h urine collection was also determined. Hematology and clinical chemistry evaluations were conducted on all animals at terminal necropsy. Hematological parameters included: red blood cell counts, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, reticulocyte counts, platelet counts, prothrombin time, activated partial thromboplastine time, white blood cell counts and white blood cell differential counts. Clinical

chemistry parameters included: aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, creatine kinase, glucose, total bilirubin, urea nitrogen, creatinine, total cholesterol, triglycerides, phospholipids, inorganic phosphorus, calcium, sodium, potassium, chloride, total protein, albumin and albumin/globurin ratios [5].

One day after the last dosing, animals fasted for at least 16 h were anesthetized with ether, and blood samples were collected. Then, animals were sacrificed by exsanguination from the posterior vena cava and abdominal aorta for complete macroscopic postmortem examinations. The following organ weights were recorded: adrenals, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pituitary, prostate (ventral lobe), salivary glands (submaxillary and sublingual), seminal vesicles, spleen, testes, thymus, thyroids (with parathyroids) and uterus. Organ to terminal body weight ratios (relative organ weights) was calculated. The following organs/tissues were fixed and preserved in 10% (v/v) neutral bufferedformalin: adrenals, aorta, bone and marrow (sternum and femur), cecum, cerebellum, cerebrum, coagulating glands, colon, duodenum, epididymides, esophagus, Harderian glands, heart, ileum, jejunum, kidneys, liver, lungs with bronchi, mammary glands (females only), mandibular lymphnodes, medulla oblongata, mesenteric lymph nodes, ovaries, pancreas, parathyroids, parotid glands, pituitary, prostate, rectum, sciatic nerve, seminal vesicles, skeletal muscle (thigh), skin, spleen, spinal cords (thoracic), stomach, sublingual glands, submaxillary glands, thymus, thyroids, tongue with laryngopharynx, trachea, urinary bladder, uterus and vagina. The testes and eyes with optic nerves were fixed in Bouin's and Davidson's solutions, respectively. All organs/tissues from the control and gardenia yellow C (high dose) groups were routinely processed, stained with hematoxylin and eosin (HE) and examined microscopically.

Statistical analysis

All values are presented as a mean value (Mean±SD). The statistically significant differences between the means of the samples were calculated by one-way ANOVA. The differences were considered significant at a level of $p<0.05$ (*).

Results

The oral administration of the extracts of Ma KhangDaeng and Ma KhangKhao were not effected to rats. After 42 days of orally administrated with extracts, the weights of male and female rats were not different to the control group. Besides, the weights of male and female

rats were not different to the control group (data not shown). The hematology and clinical chemistry evaluations were shown in **Table 1-8**. The data show statistic value with mean ± SD of experiment group and control group. The differences were considered significant at the level of $p<0.05$ (*). The pathological results from the control group compared with experiment groups were normal, and there was no change in the appearance of tissue damage.

Table 1 The Hematology evaluations profiles of the male rat after oral administrated with Ma KhangDaeng extracts 500 mg/kg.

Heamatology profile	The Hematology and clinical chemistry			
	Day 28	Day 28 (control)	Day 42	Day 42 (control)
Hb	16.26±0.23	15.75±0.63	16.83±0.11	15.75±0.63
Hct	50.30±0.62	48.50±2.82	53.11±1.01	48.50±2.82
RBC	8.38±0.17	8.16±0.21	8.81±0.22	8.16±0.21
WBC	7246±1394.28	9960.00±367.69	7286.660±990.80*	9960.00±367.69
RDW	12.83±0.20	12.45±0.35	12.86±0.20	12.45±0.35
MCV	60.03±0.55	59.45±1.90	60.23±0.80	59.45±1.90
MCH	19.43±0.20	19.30±0.28	19.10±0.36	19.30±0.28
MCHC	32.33±0.15	32.50±0.56	31.70±0.40	32.50±0.56
Plt	922333.33±117627.94	739000.00±46669.05	912666.70±146473.00	739000.00±46669.05
Neutrophic	7.33±1.52	6.00±0.00	6.33±2.51	6.00±0.00
Lymphocyte	88.00±5.29	91.00±1.41	91.33±4.04	91.00±1.41
Monocyte	0.33±3.78	0.00±1.41	2.00±1.52	0.00±1.41
Eosinophil	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Basophil	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 2 The Hematology evaluations profiles of female rat after oral administrated with Ma KhangDaeng extracts 500 mg/kg.

Heamatology profile	The Hematology and clinical chemistry			
	Day 28	Day 28 (control)	Day 42	Day 42 (control)
Hb	12.62±7.00	17.90±0.00	17.18±0.76	16.85±0.49
Hct	37.98±21.56 *	56.05±1.06	44.70±15.40	52.75±1.76
RBC	6.69±3.76	9.71±0.17	8.56±0.78	8.67±0.28
WBC	4000.00±3497.79	7230.00±1258.60	7850.00±4063.28	5220.00±1909.18
RDW	12.82±0.58	12.35±0.00	12.94±0.39	12.80±0.28
MCV	56.76±1.14	57.70±0.00	61.32±0.37	60.85±0.07
MCH	19.84±2.33	18.45±0.35	20.20±1.39	19.45±0.07
MCHC	34.96±3.88	31.90±0.56	32.84±2.00	31.90±0.14
Plt	888000.00±713587.06	1156500.00±350017.90	978200.00±4624813.00	852000.00±110308.70
Neutrophic	12.60±5.02	23.00±21.21	9.00±2.34	15.00±8.48
Lymphocyte	84.40±2.96	73.50±21.92	89.80±2.77.00	83.50±7.77
Monocyte	2.00±2.91	2.50±0.70	1.00±1.00	1.50±0.70
Eosinophil	1.00±1.73	1.00±0.00	0.20±0.44	0.00±0.00
Basophil	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 3 The Hematology evaluations profiles of the male rat after oral administrated with Ma KhangKhao extract 500 mg/kg.

Heamatology profile	The Hematology and clinical chemistry			
	Day 28	Day 28 (control)	Day 42	Day 42 (control)
Hb	17.33±0.66	17.20±0.42	16.33±0.11	17.20±0.42
Hct	56.93±3.74	53.45±2.61	52.23±0.47	53.45±2.61
RBC	9.27±0.26	9.26±0.68	8.97±0.24	9.26±0.68
WBC	9140.00±667.75	9960.00±1767.76	6320.00±1135.82*	9960.00±1767.76
RDW	15.46±2.83**	13.80±0.14	14.40±0.26	13.80±0.14
MCV	61.96±5.57	57.75±1.48	58.23±1.60	57.75±1.48
MCH	18.86±0.90	18.55±0.91	18.33±0.65	18.55±0.91
MCHC	30.50±1.30	32.20±0.70	31.36±0.30	32.20±0.70
Plt	1055666.70±33857.54	938500.00±34648.23	926000.00±29546.57	938500.00±34648.23
Neutrophic	9.33±7.57	19.00±11.31	7.00±5.19	19.00±11.31
Lymphocyte	87.33±7.37	81.00±11.31	87.00±7.81	81.00±11.31
Monocyte	3.33±0.57	0.00±0.00	5.66±2.08	0.00±0.00
Eosinophil	0.00±0.00	0.00±0.00	0.33±0.57	0.00±0.00
Basophil	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 4 The Hematology evaluations profiles of female rat after oral administrated with Ma KhangKhao extracts 500 mg/kg.

Heamatology profile	The Hematology and clinical chemistry			
	Day 28	Day 28 (control)	Day 42	Day 42 (control)
Hb	17.00±0.51	17.20±0.42	16.63±0.42	17.20±0.42
Hct	52.76±1.58	53.45±2.61	53.16±0.07	53.45±2.61
RBC	9.27±0.26	9.26±0.68	9.00±0.11	9.26±0.68
WBC	7246.66±804.32	9960.00±1767.76	7286.66±2354.66	9960±1767.76
RDW	13.73±0.15	13.80±0.14	13.83±0.35	13.80±0.14
MCV	56.90±0.60	57.75±1.48	59.03±0.77	57.75±1.48
MCH	18.33±0.37	18.55±0.91	18.46±0.70	18.55±0.91
MCHC	32.23±0.30	32.20±0.70	31.30±0.77	32.20±0.70
Plt	1007000.00±160149.93	938500.00±34648.23	981333.30±55154.33	938500.00±34648.23
Neutrophic	7.33±1.52	6.00±0.00	6.33±2.51	6.00±0.00
Lymphocyte	89.66±3.51	81.00±11.31	82.33±6.36	81.00±11.31
Monocyte	4.66±0.57	3.00±0.00	2.33±0.00	3.00±0.00
Eosinophil	0.66±1.15	0.00±0.00	0.00±0.00	0.00±0.00
Basophil	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 5 The clinical blood chemistry evaluations profiles of female rat after oral administrated with Ma KhangDaeng extracts 500 mg/kg

	The clinical blood chemistry			
blood Chemistry	Day 28	Day 28 (control)	Day 42	Day 42 (control)
Glucose	107.80 ±19.53	106.50 ±28.99	270.00 ±54.01	272.00 ±26.87
BUN	16.00 ±2.54	15.00 ±0.00	18.20 ±1.92	18.00 ±0.00
Creatinine	0.44 ±0.05	0.50 ±0.00	0.86 ±0.11	0.75 ±0.07
Total Protein	6.12 ±0.40	6.15 ±0.21	6.30 ±0.56**	6.95 ±0.35
Albumin	3.30 ±0.31	3.45 ±0.07	3.72 ±0.29	3.65 ±0.07
AST	154.60 ±31.45	243.00 ±70.71	147.40 ±31.85	81.50 ±6.36
ALT	42.40 ±8.64	68.5 ±30.40	56.8 ±6.14	48.00 ±8.48
Alk	48.00 ±9.32	46.5 ±3.53	126.8 ±28.76	115.50 ±9.19
Na+	146.00 ±1.00	144.50.70	147.8 ±1.48	149.50 ±2.12
K+	6.42 ±1.32	8.80 ±0.70	9.68 ±1.04	8.85 ±2.19
cl-	106.20 ±1.30	104.50 ±0.70	102.40 ±1.14	103.00 ±0.00
CO_2	27.40 ±4.15	30.00 ±0.00	35.00 ±2.34	35.00 ±2.82

Table 6 The clinical blood chemistry evaluations profiles of male rat after oral administrated with Ma KhangDaeng extracts 500 mg/kg.

blood Chemistry	The clinical blood chemistry			
	Day 28	Day 28 (control)	Day 42	Day 42 (control)
Glucose	287.00 ±36.05	299.00 ±1.41	297.66 ±25.00	299.00 ±1.41
BUN	19.00 ±2.64	18.00 ±0.00	18.33 ±0.57	18.00 ±0.00
Creatinine	0.90 ±0.00	0.85 ±0.07	0.80 ±0.10	0.85 ±0.07
Total Protein	6.00 ±0.26	6.00 ±0.28	6.43±0.30	6.00 ±0.28
Albumin	3.30 ±0.10	3.40 ±0.00	3.30 ±0.10	3.40 ±0.00
AST	74.00 ±12.12	70.50 ±17.67	61.66 ±4.93	70.50 ±17.67
ALT	60.00 ±8.88	48.00 ±1.41	51.00 ±1.00	48.00 ±1.41
Alk	216.00 ±74.05	172.00 ±11.31	198.66 ±7.76	172.00 ±11.31
Na+	147.66±1.15	147.50 ±0.7	148.66. ±1.52	147.50 ±0.70
K+	8.13±0.51	7.65 ±0.07	7.90 ±0.62	7.65 ±0.07
cl-	99.00 ±1.00	99.00 ±0.00	99.00 ±2.64	99.00 ±0.00
CO_2	36.66 ±2.08	37.50 ±2.12	35.66±3.21	37.50 ±2.12

Table 7 The clinical blood chemistry evaluations profiles of female rat after oral administrated with Ma KhangKho extracts 500 mg/kg.

blood Chemistry	The clinical blood chemistry			
	Day 28	Day 28 (control)	Day 42	Day 42 (control)
Glucose	296.33 ±58.39	306.50 ±7.77	276.33±43.24	306.5 ±7.77
BUN	14.00 ±1.00	20.00 ±0.00	18.66 ±1.52	20.00 ±0.00
Creatinine	0.73 ±0.05	0.75 ±0.07	0.93±0.15	0.75 ±0.07
Total Protein	5.96±0.65	6.35 ±0.35	6.73 ±0.35**	6.35 ±0.28
Albumin	3.73±0.05	3.65 ±0.07	3.60 ±0.10	3.65 ±0.07
AST	96.33±32.71	103.50 ±36.06	110.66±25.89	103.53 ±6.06
ALT	66.33 ±19.00	65.50 ±14.84	68.66±11.01	65.50 ±14.84
Alk	201.66 ±29.53	459.00 ±83.43	189.00 ±35.17	459.00 ±83.43
Na+	149.00 ±2.64	151.00 ±1.41	150.33±0.57	151.00 ±1.41
K+	7.76±0.75	7.20 ±0.28	8.13 ±0.68	7.20 ±0.28
cl-	102.66 ±2.08	101.00 ±0.00	104.00 ±1.73	101.00 ±0.00
CO_2	31.33±1.52	34.00 ±0.00	33.00 ±1.73	34.00 ±0.00

Table 8 The clinical blood chemistry evaluations profiles of the female rat after oral administrated with Ma KhangKho extracts 500 mg/kg.

blood Chemistry	The clinical blood chemistry			
	Day 28	Day 28 (control)	Day 42	Day 42 (control)
Glucose	305.33 ±40.01	299.00 ±1.41	240.00 ±17.34	299.00 ±1.41
BUN	18.66 ±1.52 **	18.00 ±0.01	17.66±1.52	18.00 ±0.00
Creatinine	0.73±0.11	0.85 ±0.07	0.80 ±0.17	0.85 ±0.07
Total Protein	6.50 ±0.80	6.00 ±0.28	6.43±0.05	6.00 ±0.28
Albumin	3.46 ±0.25	3.40 ±0.00	3.26±0.11 *	3.40 ±0.00
AST	84.33±14.36	70.50 ±17.67	69.66±15.04	70.50 ±17.67
ALT	77.00 ±17.52	48.00 ±1.41	62.33 ±11.15	48.00 ±1.41
Alk	225.00 ±42.93	172.00 ±11.31	232.00 ±39.83	172.00 ±11.31
Na+	149.50 ±750.84	147.50 ±0.70	149.33 ±1.15	147.50 ±0.70
K+	8.40 ±0.10	7.65 ±0.07	7.86±0.49	7.65 ±0.07
cl-	100.00 ±1.00	99.00 ±0.00	100.00 ±0.00	99.00 ±0.00
CO2	36.00 ±2.64	37.50 ±2.12	38.00 ±0.00	37.50 ±2.12

Discussion

The results showed no signs of toxicity such as general behavior change, mortality, or change in the gross appearance of internal organs. Subacute toxicity was studied by daily oral doses 500 mg/kg (intermediate dose) orally for 28 days. The results showed no abnormalities in treated groups as compared to the controls. Although significantly different, all of the values were within normal limits. According to research in the genus, Gardenia found effective compounds such as Monoterpenoid including Iridoid glycoside, showed the effect of some anti-microbial. It also found that the composition of the substances caused by plant stress or chemical plants generated when harmed as Phytoalexins, which inhibit fungi or phenolic compounds were found in different parts of the plant, in particular, makes colorful flowers and the effect of antioxidants. In addition, they found that genocide and genipin with an effectively anti-inflammatory [6]. Monoterpenoid were toxic to nerve cells of insects and animals in Annelid but have low toxicity in mammal [7], it is possible that the substance may cause toxicity. The geniposide compound exposure to mice to test the toxicity of the liver, found that the mice died more than 50% (LD50) dose administrative is 1431 mg/kg, and the effects of acute toxicity tests indicate that the size geniposide 574 mg / kg cause liver toxicity (Hepatotoxicity) after 24 to 48 hours caused by the stress of the anti-conservative (oxydative stress) to reduce the enzyme superoxide dismutase, an enzyme system that is destroying the free radicals caused by metabolism process within cells leading to an increase in liver Malondehyde or MDA, is a toxin made by the structures of proteins and DNA. Future more, sub-chronic geniposide dose 24.3 and 72.9 mg / kg feed to the mice for 90 days, causing liver toxicity, suggest that geniposide in a 24.3 mg / kg or less will not cause hepatotoxicity [8],so it's possible that in this study the rat receiving the extract of Ma KhangDaeng and Ma KhangKhao with compounds geniposide levels less than 24.3 mg / kg.

Acknowledgements

In this project, I would like to thanks, laboratory animal research center, the University of Phayao for good care animals trough experiment, IACUC of University of Phayao for advice toxicity protocol in the rat, Department of Pathology, Faculty of Medicine, Chiangmai University for histopathology section and result, and Government budget 2015.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Effect of purple rice bran in proteomic profile of pancreas of diabetic pancreas

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Abstract

Type 2 diabetes is a metabolic abnormality of body to respond to elevated blood glucose through a different degree of insulin resistance and β-cell dysfunction. Destruction of pancreas leads to insulin insufficiency and hyperglycemia that develop to a diabetic condition. A major bioactive component in purple rice (*Oryza sativa L. indica*), Anthocyanin, have been proved to have antioxidant, anti-inflammatory and anti-diabetes activities. However, the underlying mechanism of antidiabetic activity of purple rice bran in type 2 diabetes still needs more investigation. In this study, the proteomic technique was used to identify the expression of proteins in the pancreas tissues of streptozotocin (35 mg/kg body weight) induced type 2 diabetes rats supplemented with or without purple rice bran compared with non-diabetes, non-diabetic supplement with purple rice bran. Protein expression was identified by LC-MS/MS and protein identification were searched against NCBI database. Protein functions were categorized according to Uniprot database. Protein-protein interactions were analyzed by using STITCH 4.0 to explain the anti-diabetic activity of purple rice bran**.** Approximately 1297 polypeptide was expressed in all four groups. Thirty-six unique proteins in type 2 diabetic rat supplemented with purple rice bran mostly involved in pancreatic β-cell survival, proliferation

and improved insulin signaling and glucose metabolism. From these results revealed that purple rice bran could be considered as a good therapeutic option for diabetic treatment.

Keywords: purple rice (*Oryza sativa L. indica*), type 2 diabetes, insulin resistance, pancreatic β-cell, proteomic analysis.

Introduction

Type 2 diabetes is characterized by hyperglycemia with insulin resistance. The pathogenesis of type 2 diabetes is not the only dysfunction of pancreatic beta cells but also insulin resistance in peripheral tissue including skeletal muscle, liver, and adipose tissue (1). Hyperglycemia occurs when pancreatic β cells unable to compensate insulin resistance in peripheral tissue. Insulin resistance is defined as the diminished tissue response to insulin at one or more of the signaling pathway (1, 2). To compensate insulin resistance, pancreatic β cells increase insulin secretion (3).

The destruction of the pancreas by streptozotocin leads to oxidative stress, insulin insufficiency which resulting in hyperglycemia and develops to the onset of diabetes (4).

Therefore, controlling blood glucose level is important for prevention of diabetic patients. However, the molecular mechanisms of β cell dysfunction and insulin resistance remain largely unrevealed

Purple rice (*Oryza sativa L. indica*) is cultivated in Southeast Asia, including Thailand, China, and Indonesia. Major functional component of pigmented rice is anthocyanins. They have been proved to have antioxidant, anti-inflammatory, anti-diabetes and anti-hypercholesterolemia properties(5-7). In addition to this, they prevent cardiovascular disease and obesity(7,8). In streptozotocin (STZ) induced diabetic male Wistar rat found anti-hyperglycemic and hypolipidemic effects of anthocyanin-rich bran from purple rice by improving β-cells function and insulin secretion(9). Therefore, purple rice bran may be a good candidate for diabetic treatment. However, the mechanisms of antidiabetic activity of purple rice bran in type 2 diabetes still need more investigation.

In this study proteomics analysis was applied to investigate the proteome profile of the pancreatic tissues of streptozotocin-induced type 2 diabetes rat that supplemented with or without purple rice bran to describe changes in protein expression and modification. By using shotgun proteomics and LC-MS/MS quantitative proteomic method, we identified the expression of total proteins in the pancreas. With this proteomic approach differentially expressed proteins were discovered and found the molecular mechanism of anti-diabetic effect of purple rice**.**

Material and method

1. Animal and treatment

In this study, a total of 20 rats were used and divided into four groups (n=5) including normal rats control (NDC), normal rats with purple rice bran supplement (NDR), type 2 diabetic rats control (DM2C) and type 2 diabetic rats with purple rice bran supplement (DM2R).

Pancreatic tissue samples of diabetic rats were supported by Dr.NarissaraLailerd and colleagues from Department of Physiology, Faculty of Medicine, Chiang Mai University (9).In brief, adult male Wistar rats, weighing 180-200 g, were obtained from the National Laboratory Animal Center, Mahidol University. Diabetic rats were induced with streptozotocin (STZ). Rats were injected STZ intraperitoneally at a dose of 35 mg/Kg body weight. Meanwhile the control group was injected citrate buffer (pH 4.4).

2. Sample preparation for proteomic analysis

2.1 Tissue homogenization and protein concentration determination

100 mg pancreatic tissues sample were pulverized in liquid nitrogen using mortar and pestle. Then fine powder was dissolved in 1 ml of 0.5% SDS. Proteins were precipitated with 2 volumes of cold acetone incubated overnight at -20˚C followed by centrifugation (12,000 rpm, 15 min at 4˚C). Finally, the protein pellets were resuspended in 0.5% SDS and stored at -20˚C.

2.2 Sample preparation for proteomic analysis

Protein concentration was determined by Lowry's method using bovine serum albumin (BSA) as standard (10). Then the individual sample was adjusted to an equal protein

concentration base on the least concentration and pooled together according to groups for further SDS-PAGE analysis.

3. Proteomic analysis

3.1 SDS-PAGE and in-gel digestion

In SDS-PAGE, proteins were separated by one-dimensional gel electrophoresis based on their polypeptide length and molecular weight. Then the gels were stained with silver staining. The gel pieces were in-gel digested using an in-house protocol developed in the Proteomic laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand (11). Spots were excised and destained. Proteins were reduced and alkylated by 10 mM 1,4-dithio-D-threitol and 55 mM iodoacetamide respectively. Tryptically digested peptides at 37 °C overnight were extracted with 5% formic acid in 50% acetonitrile. (12)

3.2 Protein identification

Digested peptides were analyzed by LC-MS/MS by mass-to-charge (m/z) ratios**.** DeCyder MS 2.0 Differential Analysis software (GE Healthcare, Sweden)(13,14) was used for protein quantitation with high statistical confidence at p-value < 0.05.

The analyzed data from were submitted to database search using the Mascot software version 2.2 (Matrix Science, UK) (15) and NCBI database for protein identification. As the way to demonstrate the distribution of identifying proteins with differential abundance in the different experimental groups, the number of proteins between four groups by using a Venn diagram that is a method of visualizing the amount of overlap between two or more lists of data.

3.3 Bioinformatic analysis.

The group of interested proteins from the Mascot search results were categorized based on biological processes. UniProt (http://www.uniprot.org/) was used to search for molecular weight and understanding of the molecular function and biological process. Search tool for the retrieval of interacting between genes/proteins with drugs including metformin, sulfonylurea, insulin; for the treatment of diabetes, celecoxib; for the treatment of inflammation, doxorubicin,

cisplatin; for the treatment of cancer) STITCH version 5.5 was used to propose possible mechanism(s) of diabetes and anti-diabetes effects of purple rice.

3.4 Statistical analysis

Comparison between multiple groups was analyzed using the analysis of variance (ANOVA). P value < 0.05 is regarded as significant.

Result

Identification of pancreatic proteins

2472 proteins were differentially expressed proteins in all four group as shown in **Figure 1**. The numbers of the identified proteins in NDC, NDR, DM2C, and DM2R were 2097,1985,2019 and 1996 respectively. Total of 1297 proteins was found in all four groups consist of 51, 29, and 36 unique proteins in the NDC, DM2C, and DM2R group respectively.

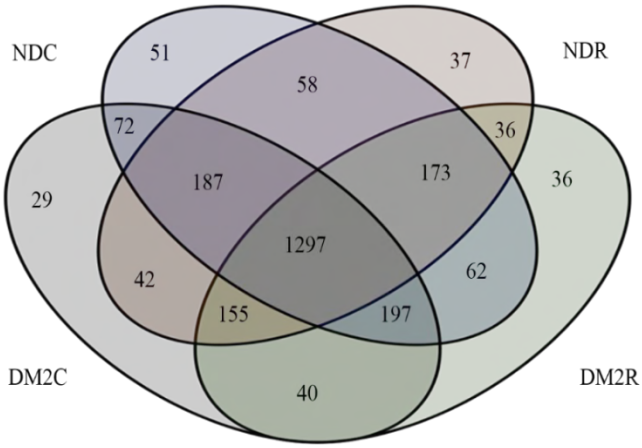

Figure 1 Venn diagram shows the total of proteins in 4 groups of pancreatic rats.
NDC: normal rats control, NDR: normal rats with purple rice bran supplement, DM2C: type 2 diabetic rats control, DM2R: diabetic rats with purple rice bran supplement

Bioinformatic analysis of unique proteins in diabetic rats with purple rice bran supplement

To get more information about the molecular mechanism of type 2 diabetes, we investigate the relationship between unique proteins of the DM2C group and drugs by using the STITCH (ver.4.0).

The results showed that the unique proteins of DM2C were linked with metformin and not with insulin, revealing that unique proteins are related to type 2 diabetes mellitus **(Figure 2)**. In addition, there are 4 unique proteins (Nae1, Atg14, Ptgr1 and Map1l3cb) were found to be associated with proteins related with oxidative stress and proteins related with autophagy pathway (Becn1, and Pik3r4). This will support the involvement of pancreatic β cell death in the development of diabetes.

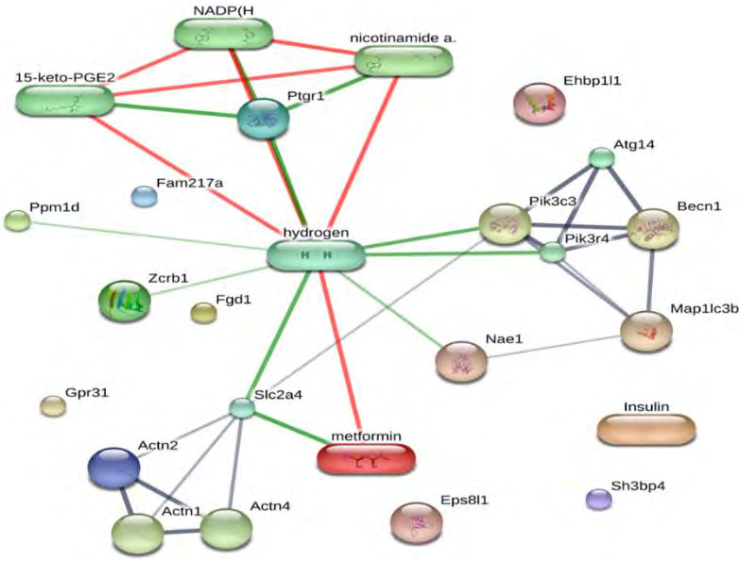

Figure 2 Protein chemical interaction network (STITCH, ver. 4) of unique proteins found in diabetic rats' pancreas. Protein-protein interactions are shown in grey, chemical-protein interactions in green and interactions between chemicals in red. Thicker lines represent the stronger association. The circular nodes indicate the different proteins.

Table 1 Identification and functional categories of 4 unique proteins found in purple rice bran supplemented diabetic rats' pancreas

Accession No.	Protein name	Gene name	Functional categories	Peptide sequence
gi50400224	NEDD8-activating enzyme E1 regulatory subunit	Nae1	Autophagy	EKEDFR
gi32264635	Zbs559	Map1lc3b	Autophagy	TLHSLY
gi157822327	Beclin 1 associated autophagy-related key regulator	Atg14	Autophagy	EVLKAMEGK
gi20302022	Prostaglandin reductase 1	Ptgr1	oxidoreductase	VVESK

Bioinformatic analysis of unique proteins in diabetic rats with purple rice bran supplement

To find out the molecular mechanism of antidiabetic activity of purple rice bran, we investigated the unique proteins in DM2R group. The STITCH chemical proteins interaction software (ver.4) confirmed that the unique proteins of the DM2R rats were linked with metformin and not linked with insulin, pointing out those unique proteins are associated with type 2 diabetes (**Figure 3**). Five unique proteins (Rasgrp3, Mfn1, Panx1, Abcc4, Lap3) were found to be linked with other protein functioning in Glut 4 translocation, myosin I heavy chain (Myo 1c) and it was upregulated in both NDR and DM2R rats' pancreatic protein of this study. According to UniProt database, these unique proteins were found their functions involved in cell signaling (Panx1, Rasgrp3), cytoskeletal protein (Mfn1), protein metabolism (Lap3), transporter proteins (Abcc4) as shown in Table 2. Based on this information, purple rice bran supplement might involve in insulin signaling and glucose metabolism.

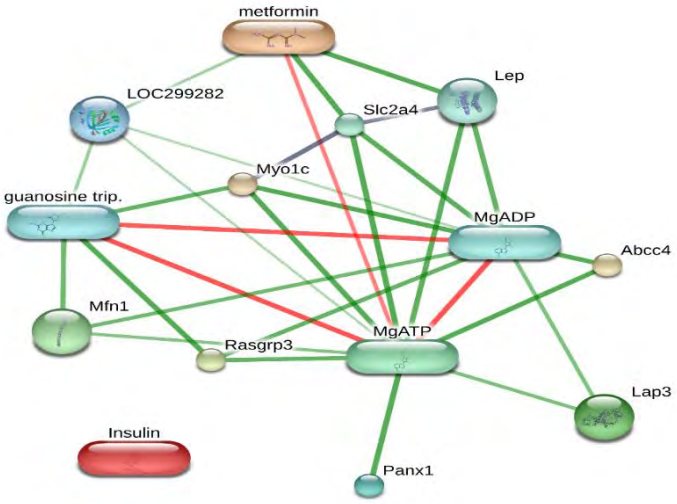

Figure 3 Protein chemical interaction network (STITCH, ver. 4) of unique proteins found in purple rice bran supplemented diabetic rats' pancreas. Protein-protein interactions are shown in grey, chemical-protein interactions in green and interactions between chemicals in red. Thicker lines represent the stronger association. The circular nodes indicate the different proteins

Table 2 Identification and functional categories of 6 unique proteins found in purple rice bran supplemented diabetic rats' pancreas

Accession No.	Protein name	Gene name	Functional categories	Peptide sequence
gi306530844	Pannexin1c	Panx1	Cell signaling	ILLNESS
gi157819575	Rasguanyl-releasing protein 3	Rasgrp3	Cell signaling	DKGFAK
gi20376820	Mitofusin-1	Mfn1	cytoskeleton	NPELDR
gi58865398	Cytosol aminopeptidase	Lap3	Protein catabolism	QVIDCQLADVNNLGK
gi1416570	Multidrug resistance protein 4	Abcc4	Transporter	NRILIIDEATANVDPR
gi58865398	Cytosol aminopeptidase	Lap3	Protein catabolism	QVIDCQLADVNNLGK

Discussion

In the present study, rats were induced by high-fat diet combination with a low dose of streptozotocin injection. The STITCH network showed the unique proteins found in the pancreatic tissue of DM2R were related to metformin, not linking to insulin (Figure 2,3). From these data confirm that animal models were typed 2 diabetes rats.

According to the chemical–-protein interaction network (STITCH 4.0) showed that the unique proteins expressed in the diabetic rat group were correlated with oxidative stress (Nae1, Atg14, Ptgr1 and Map1l3cb and linked to autophagy protein (Becn1, and Pik3r4). These data supported that pancreatic β-cells death, and oxidative stress can affect to pancreatic β-cell mass resulting in insulin resistance in peripheral tissue and impaired insulin secretion (16).

In addition, the STITCH 5.0 showed the relationship between drugs and unique proteins expressed in the type 2 diabetic rat treated with purple rice bran group. Guanyl nucleotide-releasing protein for Ras 3 (Rasgrp 3) found in type 2 diabetic rats supplemented with purple rice bran. Rasgrp 3 is a member of the RasGRP family that activated MAP kinase kinasekinase phosphorylation (Raf). (19). It has previously reported that Raf 1 is required for β-cell survival and proliferation (20). From this finding, it can be summarized that purple rice bran can affect to Rasgrp3 expression to increase pancreatic β-cell proliferation and β-cell survival. According to STITCH 4.0, we found the interaction of Rasgrp3 with Myo 1c that associated with Glut 4 translocation.

Myo 1 c, myosin I heavy chain, is an actin-based motor protein. It facilitates insulin-stimulated Glut 4 translocation in adipocyte and skeletal muscle. Previously, in 3T3-L1 adipocytes, the high expression Myo1c promoted the insulin-stimulated Glut 4 transport in adipocytes (21). Moreover, there was enhanced glucose uptake in skeletal muscles expressing wild-type Myo1c and impaired glucose uptake in skeletal muscles expressing mutant Myo1c. And they proposed that Myo1c increase glucose uptake by transporting Glut 4 to cellular membrane(22). However, Glut 4 is not a major glucose transporter in the pancreas, and Myo1 c expression in pancreas had not been reported. Interestingly, we found that Myo1c expressed in the pancreatic tissue of purple rice bran supplemented diabetic group.

Conclusion

In conclusion, purple rice bran can regulate β cell mass, β cell-survival, improve insulin signaling and glucose homeostasis. However, the mechanism of purple rice bran still needs more investigating.

Acknowledgements

This study supported by Faculty of Medicine, Chiang Mai University and the researcher groups at National Center for Genetic Engineering and Biotechnology (BIOTEC) and National Research Council of Thailand (NRCT).

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
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Fortification omega 3 in low fat set type yoghurt

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Abstract

The research was aimed to fortify omega 3 in low fat set type yoghurt (LFY) with healthy benefit, no syneresis and high acceptance. The basic recipe was done at total soluble solid (TS) 20 ºBrix and pasteurized at 90 ºC for 5 mins and cooled down to 42 ºC then 0.02 % culture was added. After that incubated at 42 ºC until the pH reduced to 4.8-4.9 finally, the fermented yoghurt was stored at 4-6 º C. TS (15, 20 and 25 ºBrix), types of cultures (Y-350, Yo-Mix R496 and Yo-Mix R05) were varied. The syneresis of the products was analyzed by centrifuged method. The sensory evaluations used 9 points hedonic scale. Finally, the products were tested for the viscosity, chemical changes (pH, acidity), microbiological changes, sensory evaluation, syneresis and shelf life. The results found that the panelists preferred LFY with TS 20 or 25 º Brix more than TS 15 º Brix. Different cultures Y-350, Yo-Mix R496 and Yo-Mix R05used different fermentation time,18, 8 and 6 hours respectively to reach pH 4.8-4.9. In comparison, the most suitable recipe of LFY was fermented by Yo-Mix R05and started with TS 20 º Brix gave the highest acceptance, 7.86 from 9 points among 50panelists and the product viscosity was 31,319 cP. Fortification of omega 3 was done by adding omega 3 in oil form and encapsulated form at 250 mg/serving 100 gin LFY base on recommended daily intake. The results found that fortification in oil form made the finished products have fishy smell and unacceptable. However, fortification with encapsulated powder form made all the characteristics of the products close to control yoghurt. The fortified product had the viscosity 29,880 cP. The lactic acid bacteria count of finished products was$7.2x10^{12}$cfu/g, no syneresis after storage at 4-6 ºC for 28 days. In conclusion omega 3 could be fortified in low fat set type which had no effect on the growth of cultures, no syneresis and enough shelf life for sale.

Key words: yoghurt, syneresis, fortification, omega 3

Introduction

Recently, the consumer's demand for foods with balanced nutritional composition that may offer additional health benefits has been increasing. Yoghurt is gathered as a healthy food due to its high protein and calcium content. There are many varieties of yoghurt such as non-fat or low-fat. Low fat yoghurt is one of the dairy products that have potential health benefit but the fat reduction in yoghurt normally significantly affects physical and textural properties (Crion et al., 2012). In addition, the roles of omega 3 which are bioactive compounds and high nutrition have benefits on health and make it more interesting for consumers (Siro et al., 2008). Omega 3 is precursor of essential fatty acids, eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) which cannot be produced by the human body. The EPA and DHA related to function of brain and eyes (Kolanowskiet al.,2006). Therefore, in this research tried to fortify omega 3 in low fat set type yoghurt in order to make a new product with high sensory evaluation and consumer acceptance similar to normal low fat set type yoghurt. The daily uptake of eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) and Alpha Lipoic Acid (ALA) are recommended at 250 mg and 2 g respectively (European Council, 2006; EFSA, 2009; European Union, 2012).

The objective of this study was aimed to produce low fat set type yoghurt with different starter cultures and produced more value added product of by fortification with omega 3 with high sensory evaluation and consumer acceptance.

Materials and Methods

Skimmed milk powder was obtained from a local producer and immediately brought to the laboratory for preparation of yoghurt samples. Freeze dried lactic cultures (Yo-Mix380, Yo-Mix496 and Yo-MixR05) of DuPont was used as a starter culture for yoghurt production. Two types of omega 3, oil form and powder encapsulated form were selected for fortification in yoghurt.

Yoghurt preparation

All ingredients were dissolved with hot water at 55 – 60 ºC by hand mixer and adjust the total solids which varied 3 levels of 15, 20 and 25 ºBrix. Then pasteurized at 90 - 95ºC for 5 mins and cooled down to 42 ºC then 0.02 % culture was added. After that incubated at 42 ºC until the pH reduced to 4.8-4.9. Finally, the sample yoghurt was stored in a refrigerator at 4-6 º C for 28 days. Physico-chemical, sensory properties and shelf-life were determined on day 7, 14, 21 and 28 days.

Fortification omega 3 in yoghurt

Two types of omega 3, oil form or powder encapsulated from were fortified in yoghurt at dissolving step after that carried out following the methods of yoghurt preparation.

Measurements of quality parameters

The pH of yoghurt samples was measured with a pH meter. The titratable acidity (as percent lactic acid) was determined according to method of AOAC (AOAC, 1995) Forsyneresis measuring, 10 g yoghurt sample was centrifuged at 3000 rpm x g for 10 min at 8-10 ºC. Syneresis is calculated as the percentage of clear supernatant per initial weight of yoghurt sample (Keogh and O'Kennedy, 1998).

Determination of Viscosity

Viscosity measurement was carried out using a rheometer (Brookfield LVDV-I+, MA, USA) equipped with a helipath stand and spindle rotating (S63) at a speed 4.0 rpm.

Sensory Evaluation

Sensory evaluation was compared with control yoghurt. Yoghurt samples were stored at 4-6 ºC and served to the panelists after taking the yoghurt samples out of the refrigerator, and immediately assessed by the panelists. The samples were served randomly with 3 digits random numbers. Sensory parameters were tested, appearance, texture, color, odor and overall preference. Scaling was done using 9 points hedonic scale.

Shelf-life Evaluation

Shelf-life of yoghurt was determined on day 1, 7, 14, 21, 28 after storage at 4-6 ^{o}C. Shelf-life parameters, pH, TA, Syneresis and Lactic acid bacteria were analyzed.

Results and discussions

1. Effect of total solids on low fat set type yoghurt

The results obtained on physico-chemical properties of yoghurt samples were presented in table 1. Yoghurt prepared with total solid of 20 ^{o}Brix and 25 ^{o}Brix reached to pH at 4.79 and 4.83 respectively and they onset to be curd at the sixth hour of fermentation. Incontrast, yoghurt with total solid of 15 ^{o}Brix dropped from 6.61 to 4.58 pH and it onset at the ninth hour of fermentation.

The total solids of yoghurt were affected on the curd formation in low fat set type yoghurt. Thus, yoghurt with total solids of 20 ^{o}Brix and 25 ^{o}Brix onset to be curd and pH reduced faster than yoghurt with total solid of 15 ^{o}Brix. So, the yoghurt with total solid of 20 ^{o} Brix or 25 ^{o} Brix should be chosen for yoghurt production because it took only 6 hours for fermentation. Moreover, the different starter cultures also affected on rate of pH reduction. The culture of Yo-Mix 496 and Yo-MixR05 had similar rate of pH reduction while YC 380 had slowly rate of pH reduction which took 18 hours for fermentation (Figure 1).

Table 1. Effect of total solids on low fat set type yoghurt

Time of fermentation (hrs.)	TS 15 °Brix			TS 20 °Brix			TS 25 °Brix		
	pH	TA	Curd	pH	TA	Curd	pH	TA	Curd
0	6.61	0.35±0.01	-	6.61	0.35±0.01	-	6.72	0.38±0.01	-
1	6.60	0.35±0.01	-	6.59	0.34±0.00	-	6.55	0.39±0.00	-
2	6.52	0.34±0.02	-	6.38	0.44±0.02	-	6.51	0.39±0.01	-
3	6.29	0.46±0.03	-	6.22	0.45±0.04	-	6.45	0.42±0.02	-
4	5.83	0.48±0.01	-	5.25	0.51±0.01	-	5.97	0.49±0.02	-
5	5.53	0.55±0.04	-	4.98	0.76±0.04	+	4.86	0.76±0.04	++
6	5.11	0.71±0.03	-	4.79	0.94±0.03	++	4.83	1.01±0.01	++
7	4.93	0.93±0.01	-	4.69	0.96±0.03	+++	4.76	1.06±0.04	+++
8	4.74	1.04±0.02	-	4.66	1.05±0.02	+++	4.67	1.11±0.01	++++
9	4.58	1.14±0.03	+	4.53	1.13±0.04	+++	4.52	1.26±0.09	++++
10	4.49	1.15±0.04	++	4.44	1.15±0.03	+++	4.50	1.33±0.01	++++
11	4.35	1.16±0.02	++	4.38	1.17±0.02	+++	4.35	1.36±0.01	++++
12	4.27	1.16±0.01	+++	4.30	1.19±0.02	+++	4.29	1.43±0.01	++++

- means no curd
+ means very little curd
++ means curd
+++ means heavy curd
++++ means very heavy curd

The result was similar to the work of (Prudencio and Petrus, 2010) who also found that the total soluble solid and total solid will be increased base on fat replacers which was added atmilk preparation such as adding inulin and sucrose.In addition, buffalo's yoghurt which adjusted fat level at 1.5 g per milk 100 g and gave the lowest fat level and total solid resulting in the lowest of pH reduction (Kaminarides et al., 2007; Shiby et al., 2008). The results were also similar the previous study, the addition of various concentrations of rehydrated carrot cell wall particles (CWP) at 0.5, 1, 1.5, 2 % to yoghurt production. The faster rate of pH reduction was observed

when the CWP concentration was increased to 1-2 %. The faster rate of pH reduction decreased the fermentation time almost 1 hour. The results were similar reported by McCann et al. (2011).

2. Effect of the different starter cultures on properties of yoghurt.

The different starter cultures affected on rate of pH reduction. The result showed that the cultures of Yo-Mix 496 and Yo-MixR05 gave the same rate of pH reduction but particularly culture of YC 380 gave slowly rate of pH reduction and it took 18 hours of fermentation time (Figure 1). Wang et al. (2002) reported that *Lactobacillus bulgaricus* and *Streptococus thermophillus* as the starter cultures and probiotic strains used for yoghurt manufacture, when grown singly or blends with yoghurt cultures affected the fermentation time at the rate of acidification step. The significant differences of fermentation time in these experiments were obtained.

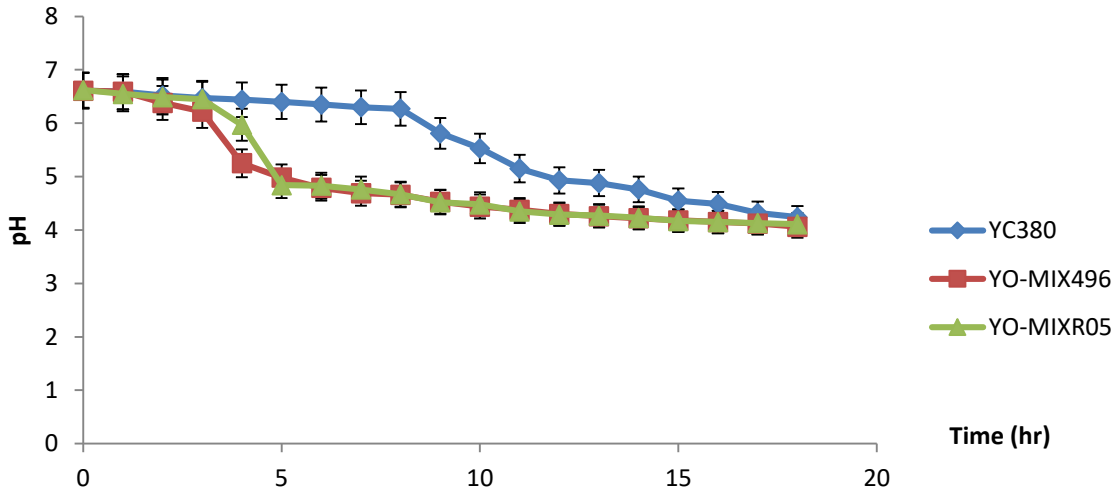


Fig. 1 Effect of the different starter cultures on the changes of pH in low fat set type yoghurt.

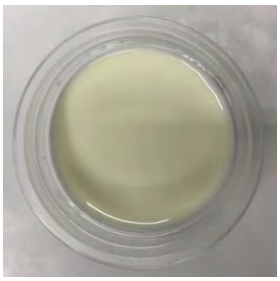
3. Fortification omega 3 in low fat set type yoghurt

The sources of omega 3 were selected from two forms, in oil and encapsulated powder form that omega 3 were fortified in low fat set type yoghurt amount 250 mg per serving size (100 ml). The result showed that the finished products gave all characteristics such as texture, sourness, odor and overall preference close to the control yoghurt except appearance as the remaining oil still existed on top (Figure 2). Moreover, the yoghurt had strong fishy smell so

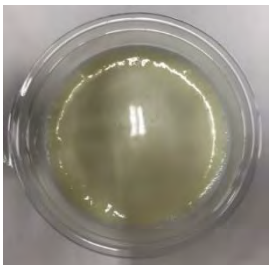
an appearance was unacceptable because the major hurdles associated with the incorporation fish oils into food of products are insolubility in water, susceptibility of PUFAs to oxidative deterioration, and development of undesirable rancid odor and taste (Augustin et al., 2006).

In addition, sensory evaluation of yoghurt fortified with omega 3 in oil form was done by 50 panelists using 9 point hedonic scale due to the finish products have strong fishy smell thus it made some panelists disgusted which omega 3 in oil form gave strong smell in both milk preparation and the finished products. Therefore, omega 3 in oil form was not suitable for yoghurt production.

(a) Control yoghurt



(b) Yoghurt fortified with omega 3 in oil



(c) Yoghurt fortified with encapsulated powder form

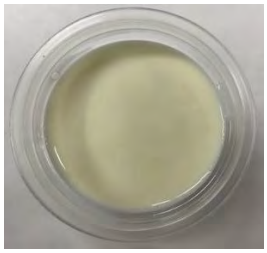


Fig. 2 Appearance of yoghurt samples between (a) control yoghurt (b) yoghurt fortified with omega 3 in oil form (c) yoghurt fortified with encapsulated omega 3 powder form

For yoghurt fortified with encapsulated omega 3 powder forms. The result was show that the finished products gave all characteristics close to control yoghurt and encapsulated omega 3 powder was produce by high technology which could entrap and release bioactive compounds so it had less smell during milk preparation and finished product. Thus, omega 3 encapsulated powder was optimized to yoghurt production. The result are similar to (Patrick et al., 2013) reported that utilization of fish oil in aqueous food systems can be improved by using encapsulated technology.

4. Shelf life of yoghurt fortified with encapsulated omega 3 powder.

Comparison shelf life parameters of yoghurt between control yoghurt and yoghurt fortified with encapsulated omega 3 powder amount 250 mg per 100 ml. The result was showed that the tendency of pH, TA, syneresis and lactic acid bacteria result was likely to be the same. Therefore, adding encapsulated omega 3 did not affect any difference to the finished products. The pH and lactic acid bacteria value dropped as time went on because acid increased. The result was the same Kehagias et al. (2006) stated that the addition of bifidobacteria to yoghurt starter increased acidity of yoghurt which attributed to the formation of both acetic acids by *B.bifidum.* In bio-yoghurt special attention should be given to avoid over acidification since this could affect the stability of starter during storage period. On the other hand, TA and syneresis seemed to increase when time went on due to the greater amount of acid which affected protein re-arrangement in the matrix. Generally, during storage titratable acidity values of all yoghurt increased due to the activity of starter culture. These results agreed with Vijayalakshmi et al. (2010) who found that a significant increase in acidity and decrease in pH were noticed in low fat yoghurt during the storage period but within the permissible levels. Regarding of syneresis, yoghurt fortified with encapsulated omega 3 powder lower syneresis values than those of control.

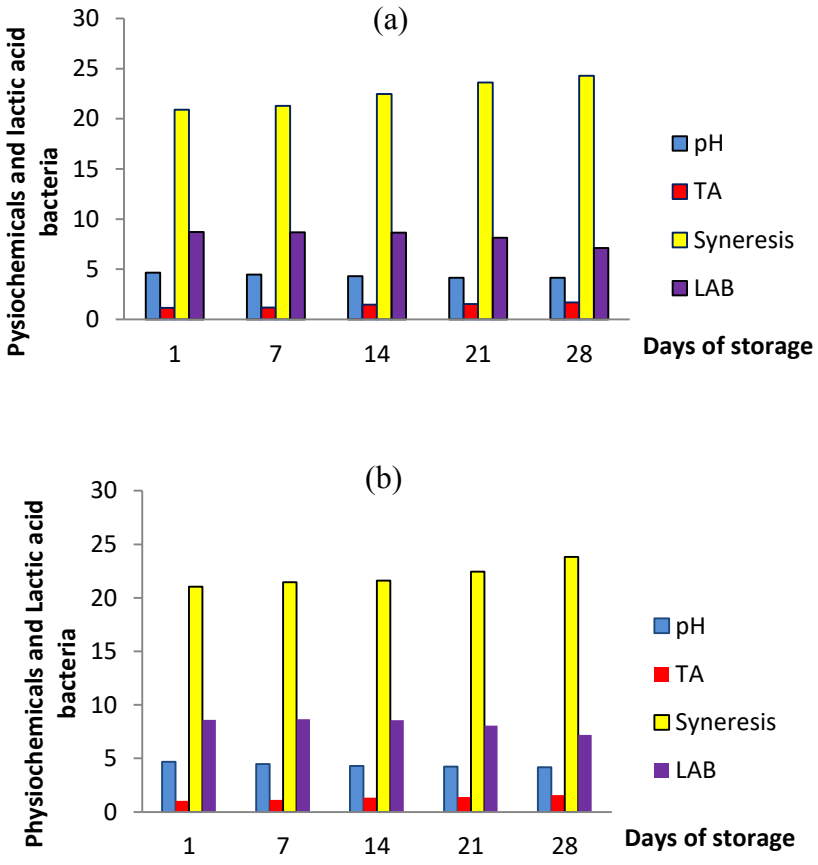


Fig. 3 Changing pH, Total acidity, Syneresis and lactic acid bacteria during yoghurt samples, storage period 28 days (a) Control yoghurt (b) yoghurt fortified with encapsulated omega 3 powder amount 250 mg per 100 ml, keep at 8-10 ^{o}C

Conclusions

Production of low fat set type yoghurt had total solid of 20 ^{o}Brix and fermented by using starter culture Yo-MixR05 which gave good all characteristics so it could improve syneresis. Moreover, yoghurt could be developed by addition of omega 3 for the amount 250 mg per 100 ml, the product has 28 days shelf life and it contains lactic acid bacteria 7.2 log cfu/g over storage period.

Acknowledgments

This study was supported by Betagen Company for scholarship and all equipments.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets ***January 22nd-24th, 2018***
University of Phayao, Phayao Province, Thailand
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Cytotoxic effects of *Pseuderanthemum palatiferum* (Nees) Radlk leaf extracts on human hepatocellular carcinoma HepG2 Cells.

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Abstract

Introduction: Liver cancer is the most highest incidence and mortality rate, which becomes a major cause of death in Thai male population. *Pseuderanthemum palatiferum* (Nees) Radlk, a medicinal plant, is commonly found in VietNam and Thailand. From previous studies, the leaf of this plant is recommended for cancer treatment. Nevertheless, the scientific evidence supporting its biological effects related to cancer is still limited.

Materials and Methods: Dry and fresh leaf of *P. palatiferum* were extracted by using water and 95% ethanol. The cytotoxic effects of water and ethanolic extracts of *P. Palatiferum* were investigated against human hepatocellular carcinoma HepG2 cell line by MTT assay.

Results: The fresh leaf ethanolic extract (FEE) exhibited the most cytotoxic effect on HepG2 cells in a dose dependent manner which fifty-percent inhibitory concentration (IC_{50}) were 446± 25.40, 277± 31.49 and 399 ± 41.69 µg/ml at 24, 48 and 72 hours, respectively. In contrast, the dry leaf ethanolic extract; dry and fresh leaf water extracts of *P. palatiferum* were nearly not effective on growth inhibition towards HepG2 cells.

Conclusions: These preliminary results showed that ethanolic extracts of fresh (FEE) of *P. palatiferum* inhibited proliferation of HepG2 cells. Therefore, this plant has a high potential for liver cancer treatment and increase value-added to the plant in ethanolic extract from. The investigation of mechanisms of death induction by *P. palatiferum*on cancer cells are further needed and *in vivo* study is also required.

Keywords: *Pseuderanthemum palatiferum,* cancer, medicinal herb, cytotoxicity, HepG2 cell

Introduction

In Thailand, cancer is the leading cause of death. The public health statistics reported that in2012 and 2016, liver cancer is the most of mortality rate among malignant neoplasm groups and likely to rise steadily (1). It indicates that mortality from liver cancer is a major problem that needs to be controlled, prevented and reduced. The current primary treatment of cancer is surgery, radiotherapy, immune therapy and chemotherapy. Chemotherapy is also toxic to normal cells (2). At present, there are many researches on cancer treatment. Traditional and alternative medicine are chosen as a potential source of new remedies for cancer. Plant extracts or natural products cause cancer cell death and reduce dose of the chemotherapy (3)**.** *Pseuderanthemum palatiferum* (Nees) Radlk is a medicinal herb commonly used in folk-medicine. Leaf of *P. palatiferum*is recommended for therapy of various diseases and symptoms, including diarrhea, stomachache, diabetes and cancer. The phytochemicals of *P. palatiferum* consist of flavonoids, β-sitosterol, stigmasterol, kaempferol, apigenin, phytol, triterpenoid, saponin and salicylic acid (4). Recent studies have reported of its anticancer propertiesagainst, *P. palatiferum*extracts were toxic tovarious human cancer cell lines vizcolon cancer (HCT15, SW48, SW480 (5) andCaco-2(6), leukemia Jurkat, hepatoma HepG2, breast cancer MCF-7 and prostate cancer PC-3cells (7). However, few studies of death-inducing effects have been reported. In this study, we investigated the cytotoxic and mechanistic death of inducing effects of *P. palatiferum* leaf extracts on liver cancer (HepG2 cells).

Materials and Methods

Plant materials and extraction

The medicinal herb is derived from garden of The Prolac (Thailand) Corp., Ltd.by organic planting method, no chemicals were used. *P. palatiferum* is identified and characterized by a botanist at the Queen Sirikit Botanical Garden with the voucher specimen number WP2615. Fresh and dry leaf of *P. palatiferum* were ground with a blender and then dried in a hot air oven at 50 °C. After that, ground leaf was mixed with distilled water for 24 hours at room temperature and filtered through Whatman filter paper No. 1. The water fractions were dried with freeze-dryer (lyophilization), collected and stored at -20°C. Ground leaf from water extracts were further extracted by fermented with 95% ethanol for 4 days at room temperature

and filtered through Whatman filter paper No.1. The ethanolic fractions were evaporated with vacuum. The extracts were also collected and stored at -20°C.

Cell culture

Hepatocellular carcinoma HepG2cell line were obtained from JCRB Cell Bank, Japan. The cells were seeded in a 96-well plate at a density of 5,000 cells/well and cultured in Dulbecco's Modified Eagle Medium (DMEM)with 25 mM $NaHCO_3$, 100 Units/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum at 37°C under 5 %CO_2 (8).

Cytotoxicity test

Cells were plated and cultured overnight in a 96-well plate and treated with each fraction of extract include fresh leaf water extract (FWE), dry leaf water extract (DWE), fresh leaf ethanolic extract (FEE) and dry leaf ethanolic extract (DEE)at various concentrations between 0-500 µg/ml for 24, 48 and 72 hours.After incubation, the cultured media were removed (100 µl)and 15 µl of 0.5 mg/ml (final concentration) MTT(3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide) were added and incubated for 4 h at 37°C. Then, the media were removed and 200 µl of DMSO were added to each well to dissolve the formazan crystal. The absorbance was measured at 540 nm with a reference wavelength of 630 nm by microplate spectrophotometer (9).

Statistical analysis

Data were an alyzedas mean ± S.D. and compared among experimental groups using One-way ANOVA (Kruskal Wallis) followed by Student's t test. The significance was considered at $P<0.05$.

Results

The water extract (DWE and FWE) and dry leaf ethanolic extract (DEE) were not significantly toxic to HepG2 cells as illustrated in Figure 1A, 1B and 1C, respectively. The fresh leaf ethanolic extract (FEE) was found to be the most effective on cell growth and proliferation inhibition of HepG2 cell in adose and time response manner at 24, 48 and 72 hours, respectively as in Figure 1D, 1E and 1F, respectively. The Inhibitory concentration (IC) values at IC_{10}, IC_{20} and IC_{50} of FEE on HepG2 cell were shown in Table 1.

Table 1. The inhibitory concentrations at 10%, 20% and 50% (IC_{10}, IC_{20} and IC_{50}) of ethanolic fresh leaf extract (FEE) from *P. Palatiferum* on HepG2 cells treated for 24 h, 48 h and 72 h. Data are presented means ± S.D. of triplicates from 3 independent experiments.

HepG2 cell	Fresh leaf ethanolic extracts (FEE)		
	24 hr	48 hr	72 hr
IC_{10} (µg/ml)	311 ± 31.39 *	146± 21.96**	267 ± 44.35*
IC_{20} (µg/ml)	352± 21.72$^{\#*}$	184± 23.28$^{*\#}$	286 ± 43.29
IC_{50} (µg/ml)	446± 25.40 $^{\#\#*}$	277± 31.49	399 ± 41.69

*****$p<0.05$, ** $p<0.01$ compared to IC_{10} at 24 h; $^{\#}p<0.05$, $^{\#\#}$ $p<0.01$ compared to the same incubation time.

Discussion

Pseuderanthemum palatiferum (Nees) Radlk. Is an intriguing medicinal herb. The previous studies reported of the antiproliferative activities on various human cancer cell line by many kinds of solvents being used. In this study the fresh and dry leaf of *P. palatiferum* were extracted by serial extraction with water and 95% ethanol. The four groups of the extracts were investigated for their cytotoxicity effects on HepG2 cells. It was found that the fresh leaf ethanolic extract (FEE)was the most effective on the growth inhibition of HepG2 cells in a dose dependent manner($P<0.05$). While other extracts were nearly not effective on growth and proliferation towards HepG2 cells ($P>0.05$).

The different antiproliferative efficiency of each extract may arise from different quantities of active compounds in the extract. Active ingredients contain in each specific solvent used in extraction depend on the polarity or non-polarity of the solvent. The solvents using for extraction of natural products are significant to clarify the kinds and amount of active ingredients from herbal extraction. The analogs or derivatives of active compounds also determine their activities, e.g. number and sites of side chains; methoxy groups (10) or hydroxyl groups at flavonoid compounds; catechin and its derivatives (11).

There are several reports exhibit natural extracts containing polyphenolic contents, which inhibit cancer cells growth and induce cancer cells to undergo apoptosis (12). *P.*

palatiferum contains several phenolic compounds as active components (13). It is probable that the effect of the FEE on the inhibition of cancer cell growth may be related to apoptosis induction. Therefore, the mechanisms of death induced by *P. palatiferum* in cancer cells are further needed for investigation.

Conclusions

*P. palatiferum*is high of potential against cancer cell inhibition. Its fresh ethanolic extract (FEE) is of highest efficiency to inhibit HepG2 cells growth and proliferation. The mechanism(s) and mode(s) of cancer cell death will be identified further, which would apply for acute and sub-acute toxicity in rodents before employing in normal human-beings and then cancer patients.

Acknowledgements

The authors are grateful to Research and Researcher Industrial Fund (RRI) for Master Degree (MSD5810017) .The RRI-MAG grant was partly shared by Prolac (Thailand) Corp., Ltd., Endowment Fund from Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand and National Research Council of Thailand (NRCT). PK appreciated of technical assistance from Mr. Patompong Khaw-on and Ms. Benjawan Wudtiwai.

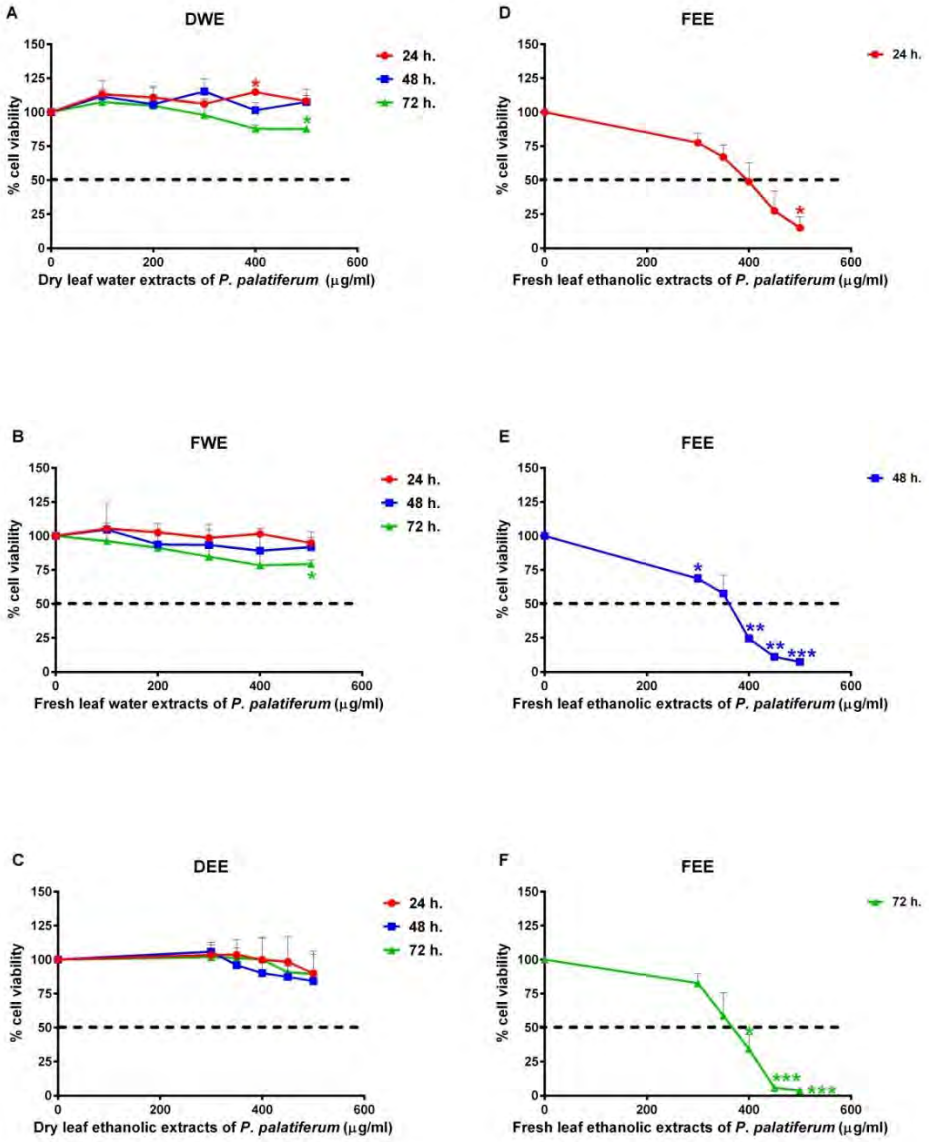

Figure 1. Cytotoxic effects of ethanolic fresh leaf extracts of *P. palatiferum*on HepG2 cells growth and proliferation by MTT assay. Cells were treated with various concentrations of each extract for 24 h, 48 h and 72 h. The percent viabilities of HepG2 cell when treated with DWE (A), FWE(B), DEE (C) and FEE at 24 h (D), 48 h (E), and 72 h (F) were illustrated and compared to control (untreated) cell. Results present as means ± S.D. of triplicates from 3 independent experiments.(*$p<0.05$, ** $p<0.01$, ***$p<0.001$)

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Preparation of pigskin collagen for an osteoblast culture scaffold

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Abstract

Collagen is the most abundant protein, which function as a structure protein, in animal including human found to be rich in bone, tendon, and skin. Collagen type – I is the first pioneer scaffold that used in tissue engineering to produced functional tissues for treatment certain abnormalities. However, pure collagen type – I is too expensive to develop a scaffold. To avoid this disadvantage our research group chose skins from pigs (*Sus domesticus*) as collagen type – I sources to extract and produce the scaffolds for osteoblast cells (MG-63). The collagen was successfully extract by simple method using NaOH with HCl neutralization. The extracted collagen was lyophilized to yield at 33.64% and stored for scaffold preparation. Two types of scaffold preparation were exploited. The collagen gel scaffold and the dried coated surface scaffold were successfully prepared. The concentration of the collagen from 50 mg/mL to 300 mg/mL could perform gel scaffold on the culture well. The dried coated scaffold at similar concentration was also prepared. Only, collagen gel scaffold could potentially support the expression of bone marker, alkaline phosphatase (ALP) activity. The effects of activation of the enzyme activity was dose dependent. However, the dried coated scaffold showed no support of bone marker cell expression. From these preliminary results, collagen gel scaffold was suitable for further investigations of osteoblast cell growth to assure that the scaffold might be potential to be developed for the use as a biomaterial to treat certain bone diseases in the future.

Keyword: Bone tissue engineering, Collagen, Pigskin

Introduction

Collagen is the most abundant protein found more than 90%in the body of mammal, including human. There are various types of collagen depending on the structure and site of synthesis. For example, collagen type – I is the main of the bone and skin, collagen type – II is the main of cartilage and collagen type – III is found in the internal organs (1).

Collagen has the same unique structure of triple helix, in each polypeptide in the structural chain of collagen might be synthesized from the same or different genes (2). Collagen type – I is mostly found as structural proteins in bones, tendons, and skins when compare with the other types (3). Interestingly, it has been used for many products such as food, supplement along with medical devices (tissue engineering and drug delivery)(4-8). Therefore, the beneficial effects of collagen type – I in tissue engineering have been applied in this experiment.

Tissue engineering is a crucial technique which required for cells, growth factors and scaffolds that used to produce functional tissue for certain diseases treatment (9). One of the most popular biological scaffolds is collagen from various sources of animals such as fish and bovines (10).

Pure collagen type – I and its product are high cost for using as a scaffold of bone tissue engineering. Recently ordered from the Sigma company, a pure collagen type – I from rat tails (C7661- 5 mg) cost us 4,500 THB. This price is too expensive to develop a scaffold for research that required 50 mg collagen type – I per one scaffold (11). To avoid this disadvantage our research group has extracted collagen from animal tissues in order supply for bone tissue engineering research.

Aforementioned, bones and skins are the organ that has many collagen type – I and collagen type – I are low antigenicity. Bones and skins of pigs are the most collagen type – I source, but collagen from bones is difficult to extract and its yield is lower than from the skins. Thus, we used pigskin as a collagen type – I source in this study.

Pigskin is easily available in local market and less expensive, and there has not been any report about collagen type – I extracted from pigskin for bone tissue engineering yet. In this study, we can add the value pigskin as a source of collagen type – I to use in the medical applications. Therefore, this study was to extract and prepare collagen type – I from pigskin for further use as a biological scaffold for bone tissue engineering.

Material and method

1. Collagen extraction from pigskins

1.1 Extraction methods

Collagen from pigskins was extracted by using the assay modified from Sato and colleagues (12). 122.6 grams of the cut skins was soaked in 480 mL of 3% (w/v) NaOH for 5 days. The mixture was adjusted to pH = 7 with 5M HCl and filtered through a filter paper (Wattman No.4) using a vacuum pump. The solution will be harvested for lyophilization.

1.2 Detection of the extracted collagen by (SDS-PAGE)

The lyophilized collagen was visualized by SDS-PAGE along with the pure standard collagen type – I (SIGMA, C7661). Coomassie blue staining was used to stained collagen bands after gel after electrophoresis.

2. Collagen scaffold preparation

The lyophilized collagen will be tested for gel forming ability and coating surface. Various amounts of the collagen will be mixed with 1 mL phosphate buffer saline (PBS), pH=7

- Gel scaffold

The mixtures were vigorously stirred at 60°C until completely resuspended and then leave at 4°C until the gel form completely. The gel forming ability was observed. Only the gel-forming amounts of collagen were chosen for further experiments.

- Dried-surface-coating scaffold

The mixtures were vigorously stirred at 60°C until completely dissolved and loaded into the 24 well-plate. The solution was left dried at 60°C for 12 hours.

2.1 Cell culture

MG-63 cells (ATCC, CRL-1427), a human osteosarcoma cell line, and/or primary human osteoblast (PromoCell, C-12720) was used to test the pigskin collagen as a scaffold for bone tissue engineering. The cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) until they reach 80-90% confluence. The cells were harvested and counted for loading into the collagen gel with loading cell density at 1×10^4 cells/scaffolds/well. The cells will be incubated at 37°C, humidify incubator with 5% CO_2 for 4 days.

Osteogenic medium (OM), the normal growth medium supplemented with osteogenic factors (10 μM dexamethasone, 1 M β-glycerophosphate, and ascorbic acid) was also used to enhance the osteoblaststo maintain mature osteoblast characteristics.

2.2 Biochemical analysis of the cells cultured with the collagen gel scaffold

- Alkaline phosphatase (ALP) activity

Alkaline phosphatase is a classical enzyme producing from osteoblast cells. The activity of this enzyme could imply osteoblastic functions upon bone formation. The conversion rate of p-nitrophenolphosphate (pNPP) substrate into p-nitrophenol (pNP) was applied for ALP activity determination.

3. Statistical analysis

ANOVA was used to compare the significant difference among groups with the p-value set at 0.05.

Result

Collagen extraction

The 41.27 g of lyophilized collagen powder (Fig. 1) was obtained from 122.66 g of wet pigskinaccounting as 33.64% yield of collagen extraction. SDS-PAGE confirmed 3 collagen bands on the gel along the standard collagen (Fig 2).

Fig. 1 Lyophilized collagen powder extracted from pigskin

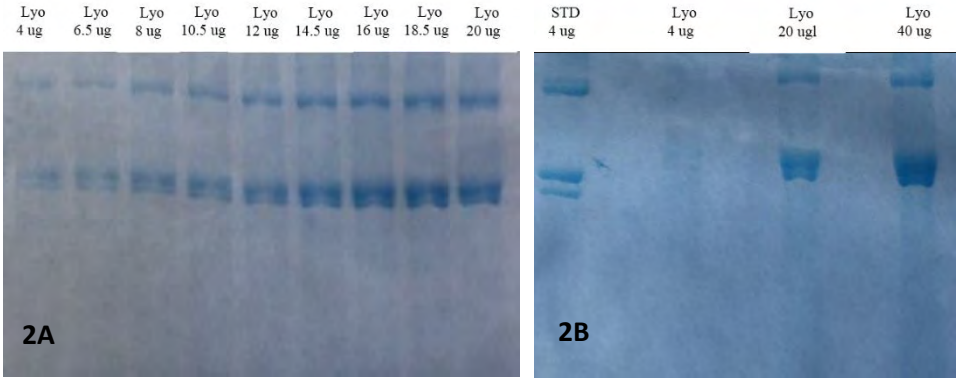


Fig. 2 SDS-PAGE analysis of extracted collagen;(A) Collagen extracted from pigskin with various amount in each lane (4-20 µg), (B) Collagen standard and the extracted collagen

Collagen scaffold preparation

The extracted collagen powder was performing gel from the concentration of 50 mg/mL to 300 mg/mL. The turbidity was increased when the concentration of the collagen was increased (Fig. 3A). The collagen could be coated dried on the surface of the culture wells (Fig. 3B).

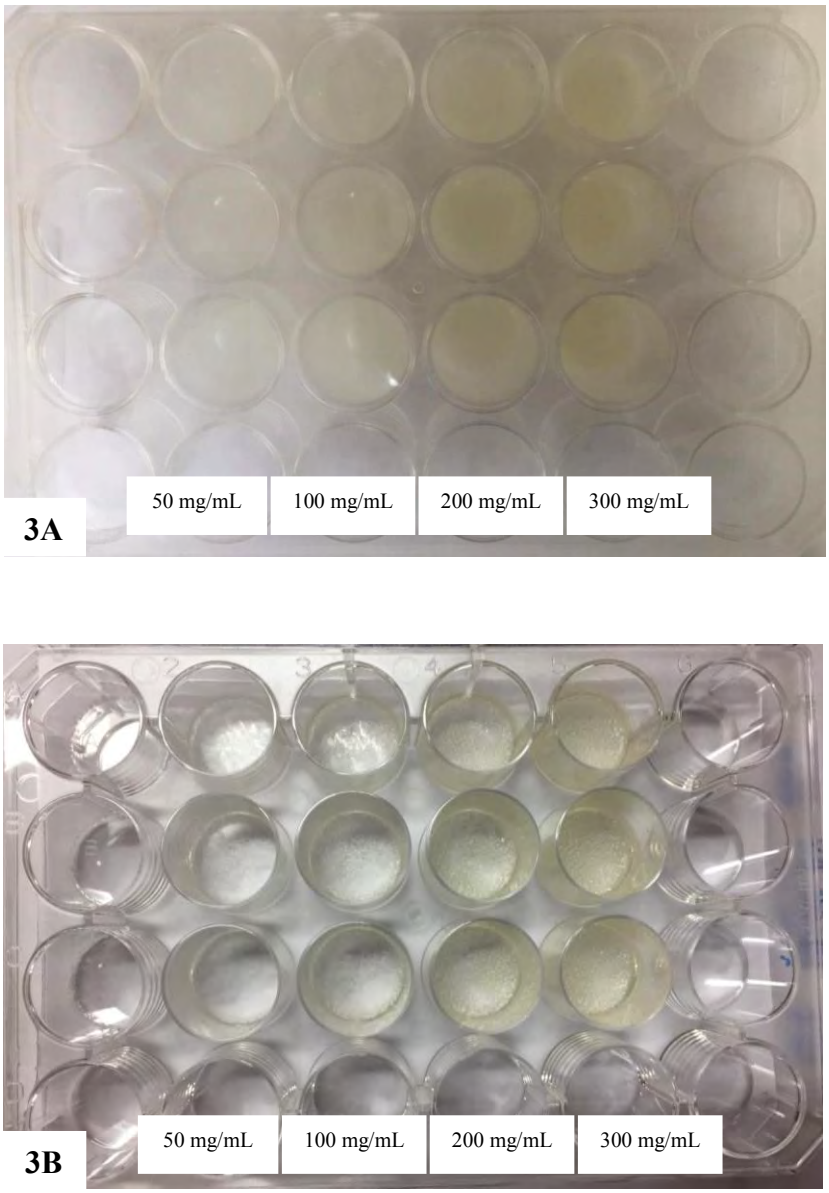
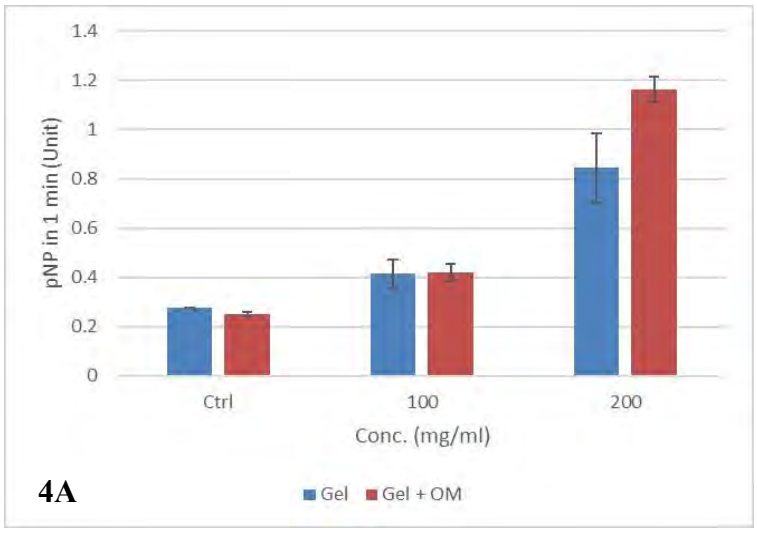


Fig. 3 The collagen scaffold preparation; (A) Collagen gel formation and (B) Dried coated preparation of the extracted collagen from the concentration of 50-300 mg/mL

Alkaline phosphatase (ALP)activity of MG-63 cells cultured on collagen scaffold

MG-63 cells cultured in the pigskin collagen gel scaffold expressed significantly higher ALP activity at both 100 and 200 mg/ml when compared with control group. The effects of osteogenic factor (OM) could only enhance the ALP activity only when the cells were in the collagen scaffold at 200 mg/mL (Fig. 4A).

In contrast, the ALP activity in the cells cultured with the collagen scaffold dried coated on the surface of the well was significantly lower than that of the control regardless of the presence of OM (Fig. 4B)



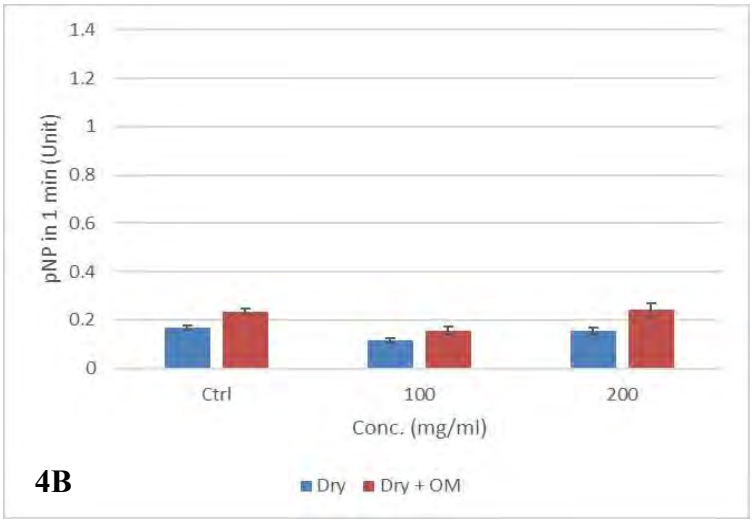


Fig. 4 Alkaline phosphatase (ALP) activity of MG-63 cells cultured on collagen scaffold (A) Collagen gel; (B) Dried coated surface

Discussion

Pigskin is the raw material and easily found in the market and with cheap to purchase. Furthermore, it is a collagen type – I abundant collagen source but its applications in bone tissue engineering is limited. Therefore, the collagen type – I in pigskin was investigated to increase value of this material in this experiment. To confirm collagen type – I by SDS-PAGE,

the result showed that the band of pigskin sample was consistent with standard band of pure collagen type – I to assure that the extracted sample was collagen type – I.

In this study, pigskin collagen was extracted to use as a scaffold for osteoblast cell culture by setting as gel and dried coated on the surface. Collagen gel was prepared to mimic microenvironment of extracellular matrix (ECM) in the human body. The results showed that MG-63 cell in normal media with 100 and 200 mg/mL of collagen gel scaffold showed the increasein ALP activity when compared with normal control. Meanwhile, only 200 mg/mL of collagen gel scaffold with osteogenic medium (OM) significantly increase ALP activity in MG-63 cell when compared with OM control. In contrast, dried coated collagen scaffold was also utilized for culture MG-63 cell according to easy preparation and the prolong storage. The present study revealed that the level of ALP activity in normal media was markedly lower than OM. This data indicated that the efficiency of dried coated collagen scaffold property was less than collagen gel scaffold. Although this technique was such a convenient preparation which was used for culture MG-63 cell, it was not able to mimic physiologically.

Besides, osteogenic medium (OM) was reported that it can induce human mesenchymal stem cells (hMSCs) to differentiate in osteoblast cells. In addition, osteogenic medium comprises of dexamethasone, β-glycerophosphate and ascorbic acid. Typically, hMSCs are difficult to be induced for differentiation into some lineages. Therefore, the culture technique with osteogenic medium was able toelevate ALP activity that affect to cell growth and bone mineralization(13, 14).

Taken together, the data indicated that pigskin was a good potential source to extract collagen type – I using in bone tissue engineering. It has possibly applied for bone tissue engineering because this material was not only non-toxic to the cells, but also promoted the cells to express bone marker significantly that confirm with the results from the experiment without prior purification. However, there are still more experiments to assure the potential of this scaffold as the good scaffold for bone tissue engineering.

Conclusion

From these results it could be summarized that pigskin collagen could be used for the scaffold of bone growth for further application in bone tissue engineering. However, the form of the scaffold must be suitable for the bone cell growth. It was suggested from this experiment

that the collagen gel scaffold rather than collagen dried coated surface was suitable for bone cell growth to express their marker, ALP.

Acknowledgement

This study was supported by Department of Biochemistry, Faculty of Medicine, Chiang Mai University.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
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A comparative study of antioxidant activity, total phenolic and total flavonoid contents in four banana (*Musa* spp) cultivars

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Abstract

Introduction: Plants of the genus *Musa* (Musaceae) or banana is one of the most important crop in Thailand. Fruits of various banana cultivars are produced for domestic consumption and export market. Since ripen bananas are eaten as dessert. The over produced bananas may go waste due to over ripen. The application of green bananas as a source for functional food can improve product value and reduce food losses in production chain. The purposes of this study was to screen for functional food properties of parts of the green bananas indicated by antioxidant activity, total flavonoid and total phenolic contents.

Materials and Methods: Four Thai banana cultivars namely *Musa*(ABB) KluaiNamwa, *Musa* (BB) cv. KluaiThani, *Musa* (AAA) cv. KluaiHomKheiw and *Musa*(AAA) cv. KluaiHom Thong were selected for investigation. Peels and pulps of the green fruits were separated and extracted with 96% EtOH. The antioxidant activity was evaluated by DPPH radical scavenging assay using vit C as a standard antioxidant. The total flavonoid contents were determined by aluminium chloride complexation method and total phenolic contents were determined by Follin-ciocalteu method.

Results: The ethanolic extracts (1 mg/ml) of banana cultivars exhibited different degree of antioxidant activity. *Musa* (BB) cv. KluaiTani pulp extracts showed the strongest antioxidant activity (94.81±0.25% inhibition equivalent to 50.12±0.13mg vitC/g extract). The highest total phenolic contents was detected in the pulp of *Musa* (BB group) cv. Kluai Tani (253.13±82.64

mgGAE/g extract) whereas the highest total flavonoid contents were identified in the peel of *Musa* (BB group) cv. KluaiTani (4.13±0.53 mgRUE/g extract).

Conclusion: This comparative study has illustrated the antioxidant activity, total flavonoid and total phenolic contents in various banana cultivars and could be used for selecting the potential banana cultivars to further development as functional food.

Keywords: *Musa* sp., antioxidant activity, total phenolic contents, total flavonoid contents

Introduction

Plants of the genus *Musa* (Musaceae) consists of banana and plantain is one of the most important fruit crops consumed worldwide as dessert as well as staple food source. They originated in South East Asia and later traveled to other tropical and temperate climate zones by traders. The edible bananas and plantains nowadays derived from the two ancestors *Musa acuminata* (genome A) and *Musa balbisiana* (genome B). The banana cultivars were classified in groups according to number of chromosome set (ploidy) and the present of characters inherited from the two ancestors. The main groups of banana cultivars are diploid and triploid for example AA, AB, AAA, ABB and *etc.* [1]. Although the world largest banana producers are in South America but the most diversity of *Musa* cultivars are in south east Asia where it originated. It has been reported that over 50 cultivars of bananas were cultivated in Thailand [2].

The banana plant is one of the most useful plants and is closely related to Thai culture. Almost all parts of it were utilized in different ways. Ripe banana fruits consumed as dessert has high nutrition value. Unripe bananas, stems and flowers were used in cooking and as side dish. Leaves were used as food container, used in handicraft for decoration in ceremony [2]. In Thai traditional medicine, unripe banana pulp is used in diarrhea and peptic ulcer; sap from pseudo stem is used in pain reduction due to snake bite; roots and stems were as wound healing agent in burn; banana flowers is used for promoting lactation and diabetic control [3].

Numbers of researches on chemical constituents of *Musa* sp. identified various groups of bioactive natural products including, triterpenoids, biogenic amines, phenolic compounds, flavonoids [4,5,6], carotenoids [4,7], phenylphenalenones[8]. Investigation of biological activities of banana extracts from parts of bananas and the isolated compounds revealed wound

healing effect, antipeptic ulcer effect [4]. hypoglycemic effect [4,9], antimicrobial activity[10,11], antidiarrheal effect [12] and antioxidant activity[12-19].

This literature review has highlighted the *Musa* plants as sources of natural antioxidants, which are valued for human health. The plants could be used as potential material for devilment of neutraceutical and functional food. Although Thailand has a big diversity of *Musa* cultivars but only some are more preferable for the consumers than the others. The most popular cultivars in Thailand are for example *Musa* (ABB) KluaiNamwa, *Musa* (AA) KluaiKhai and *Musa* (AAA) KluaiHom.Most banana fruits are produced as dessert banana for domestic consumption as well as export market. Since the ripen bananas are eaten as dessert. The over produced bananas may go waste due to over ripen. The application of green bananas as a source for functional food can improve product value and reduce food losses in production chain. The purposes of this study was to screen for functional food properties of parts of the green bananas of different cultivars indicated by antioxidant activity, total flavonoid and total phenolic contents.

Materials and Methods

Plant materials

Green fruits from four cultivars of bananas (*Musa* spp.) were purchased from central fruit and vegetable market, Warinchamrab, UbonRatchathani province, Thailand. They were *Musa* (ABB) cv.KluaiNamwa, *Musa*(BB) cvKlauiTani, *Musa*(AAA) cv. KluaiHom Thong, *Musa*(AAA) cv. KluaiHomKheiw. The fruits were cleaned with mild detergent and dried with towels. Peels and pulps were separated and cut into small pieces before oven drying at 50^{o}C for 36 h. The plant material were then grinded into powder by food processor. The plant material were kept in tight-container until extraction.

Extraction

The powdered plant materials of 5 g were extracted with 50 mL of 96% EtOH under shaking condition (150 rpm) at 27^{o}C for 30 min. The extracts were then filtered through filter paper (Whatman No.1). The filtrate was collected and the marc was re-extracted using the same procedure for 3 times. After the last extraction, the mixtures were stand for 24 h and then filtered.t The filtrates were pooled and evaporated to dryness at 50^{o}C using rotary evaporator. The EtOH extracts were kept at -20 ^{o}C until use.

Evaluation of antioxidant activity by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH radical scavenging assay was performed according to Mandal et al [19]. The EtOH extracts from pulps and peels of different Musa cultivars were dissolved in MeOH to produce a concentration of 1 mg/ml. Two hundred milliliter of the methanolic solution of the extracts (200 µl) was transferred to the test tube containing 0.1mM DPPH in MeOH (1800µl), and mixed by vortex. The mixtures were kept in the darkness at room temperature for 30 min. The absorbance was read at 520 nm against blank. The percentage of inhibition was calculated from following equation; % inhibiti= $[(Abs_{control}–Abs_{sample})/Abs_{control}]x100$, where $Abs_{control}$ is the absorbance of control DPPH and Abs_{sample} is the absorbance in the present of extracts or standard antioxidant. Ascorbic acid or vit Cwas used as a standard antioxidant. The antioxidant capacity of the extracts were calculated from the standard curve of vit C (1–1000 µg/ml) and expressed as mg vitC equivalent/ g extract.

Determination of total flavonoid contents (TFCs)

The total flavonoid contents were determined by aluminium chloride complexation method [20]. The *Musa* extracts were dissolved in MeOH and diluted to optimum concentration. One milliliter of the extract solution was transferred into test tube. One milliliter of 2% $AlCl_3$(w/v) solution was added, mixed thoroughly and allowed to react for 60 min at room temperature. After that the absorbance of the flavonoid complex was recorded at 415 nm. Rutin (1-1,000 µg/ml) was used as standard flavonoid to create a calibration curve. The TFCs were calculated and expressed as mg rutin equivalent per g extract (mg RUE/g extract).

Determination of total phenolic contents (TPCs)

The total phenolic contents were determined using FollinCiocalteu reagent as previously described [21]. Briefly, 750 µl of FollinCiocalteu reagent (1/10) was transferred into a test tube. The extract solution of 100 µl was added, mixed well and the mixture was left at room temperature for 5 min. After that 750 µl of 6% (w/v) Na_2CO_3 was added. The mixture were kept at room temperature for 90 min. The absorbance was measure at 765 nm. Gallic acid (0.03-0.3mg/ml) was used as a standard phenolic compound to create a clalibration curve. The total phenolic contents were calculated and expressed in mg gallic acid equivalent per g extract (mg GAE/g extract).

Statistical analysis

The experiments were done in six replicate. The results were expressed inmean±S.D. Statistical analysis was performed using one-way ANOVA (SPSS 13.0 for window). Statistically significant difference was assessed at P <0.05. The correlation between two variables was estimated from Pearson's correlation coefficient.

Results

Antioxidant activity

The EtOH extracts (1mg/ml) from pulps and peels of various *Musa* cultivars exhibited different degree of antioxidant activity against DPPH radical (Table 1). The DPPH scavenging activity of the extracts ranged -0.73±0.64 to 94.81±0.25 % inhibition. The antioxidant capacity equivalent to vit C ranged from 0.45±0.20 to 50.12±0.13mg vitC eq/g extracts (Table 1). The antioxidant activity in descending order were *Musa* (BB) cv KluaiTani pulp >*Musa* (AAA) cv KluaiHomKheiw peel>*Musa* (AAA) cv KluaiHom Thong peel >*Musa* (ABB)cv KluaiNamwa peel>*Musa* (ABB) cv KluaiNamwa pulp>*Musa* (AAA) cv KluaiHomKheiw pulp >*Musa* (AAA)cv KluaiHom Thong pulp.The antioxidant activity of the peel extracts were stronger than that of the pulp extract in all *Musa*cultivars except *Musa* (BB) KluaiTani. In which, the DPPH antioxidant activity of the pulp extracts was much higher that the peel extract.

Table 1. Antioxidant activity of *Musa* sp. Extracts

Plants	Parts of fruits	DPPH scavenging activity (% Inhibition)	Antioxidant capacity equivalent to Vit C (mg vitCeq/g extract)
Musa(ABB) cv KluaiNamwa	Pulp	7.00±2.96^{A}	3.90±1.56 ^{A}
	Peel	23.08±4.19^{B}	12.37±2.27 ^{B}
Musa(BB) cv KluaiTani	Pulp	94.81±0.25^{C}	50.12±0.13 ^{C}
	Peel	14.68±1.66^{D}	7.94±0.87 ^{D}
Musa(AAA) cv KluaiHomKheiw	Pulp	2.31±2.23$^{A, E}$	1.43±1.30 $^{A, E}$
	Peel	35.22±9.77^{F}	18.54±5.90 ^{F}
Musa(AAA) cv KluaiHom Thong	Pulp	-0.73±0.64^{E}	0.45±0.20 ^{E}
	Peel	27.47±1.61^{B}	14.68±0.85 ^{B}

Note: The different letters in the same column indicate the significant difference (P<0.05).

Total flavonoid contents

The total flavonoid contents of the extracts were determined by aluminium chloride complexation method. The total flavonoid contents (Table 2) of the *Musa* extracts range from 1.11±0.22 to 4.13±0.53 mg RUE/g extract. The total flavonoid content values was decreased as following; *Musa* (BB) cv KluaiTani peel>*Musa* (ABB) cv KluaiNamwa peel>*Musa* (BB) cv KluaiTani pulp >*Musa* (AAA) cv KluaiHom Thong peel>*Musa* (ABB) cv KluaiNamwa pulp>*Musa* (AAA) cv KluaiHomKheiw peel>*Musa* (AAA) cv KluaiHom Thong pulp >*Musa* (AAA) cv KluaiHomKheiw pulp. The total flavonoids contents of the peel extracts were higher than pulp extracts in all studied *Musa* cultivars.

Total phenolic contents

The total phenolic contents of the extracts were estimated by Follinciocalteu method. The total phenolic contents (Table 2) of the *Musa* extracts varied greatly from 1.68±0.22 to 253.13±82.64 mg GAE/g extract. The total phenolic contents of the extracts was decreased in following order; *Musa* (BB) cv KluaiTani pulp>*Musa* (BB) cv KluaiTani peel >*Musa* (AAA) cv KluaiHomKheiw peel>*Musa* (AAA) cv KluaiHom Thong pulpl>*Musa* (ABB) cv KluaiNamwa peel>*Musa* (AAA) cv KluaiHom Thong peel >*Musa* (ABB) cv KluaiNamwa pulp>Musa (AAA) cv KluaiHomKheiw pulp. The total phenolic contents of the peel extracts were higher than pulp extracts in *Musa* (ABB) cv KluaiNamwa and *Musa* (AAA)cv KluaiHomKheiwbut in *Musa* (AAA) cv KluaiHomKheiw and *Musa* (BB) cv KluaiTani, the pulp extracts contained higher content of total phenolic compounds.

Table 2. Total flavonoid contents (TFCs) and total phenolic contents(TPCs) of *Musa* sp. extracts

Plants	Parts of fruits	TFCs (mgRUE/g extract)	TPCs (mgGAE/g extract)
Musa (ABB) cv KluaiNamwa	Pulp	1.42±0.37^{A}	2.73±0.48^{A}
	Peel	3.81±0.39^{B}	13.19±1.40^{A}
Musa (BB) cv KluaiTani	Pulp	3.25±0.33^{C}	253.13±82.64^{B}
	Peel	4.13±0.53^{B}	17.30±0.09^{A}
Musa (AAA) cv KluaiHomKheiw	Pulp	1.01±0.16^{A,D,E}	1.68±0.22^{A}
	Peel	1.20±0.33^{D,F}	14.61±3.39^{A}
Musa (AAA) cv KluaiHom Thong	Pulp	1.11±0.22^{A,E,F}	14.53±0.22^{A}
	Peel	2.07±0.57^{G}	10.70±1.25^{A}

Note: The different letters in the same column indicate the significant difference ($P<0.05$).

Correlation analysis

The Pearson's coefficients showed the positive correlation between DPPH antioxidant activity with total flavonoid contents and total phenolic contents. The total flavonoid contents and total phenolic contents were correlated (Table 3).

Table 3. The Pearson's correlation of DPPH antioxidant activity, TFCs and TPCs

	DPPH	TFC	TPC
DPPH	1	-	-
TFC	0.378**	1	-
TPC	0.871**	0.327*	1

**Correlation is significant at the 0.01 level

*Correlation is significant at the 0.05 level

Discussion

In this study the variation of antioxidant activity, total flavonoid contents and total phenolic contents of pulps and peels from four *Musa* cultivars including *Musa* (ABB) cv. KluaiNamwa, *Musa* (BB) cv KlauiTani, *Musa* (AAA) cv. KluaiHom Thong, *Musa* (AAA) cv. KluaiHomKheiw. The antioxidant activity of the ethanolic extracts were evaluated by DPPH radical scavenging method. The DPPH radical is a stable purple free radical, which was decolorized by accepting an electron or one protron from antioxidant compound [22]. Antioxidant activity of parts of *Musa* sp. was reported [12-18]. Our results showed that in three *Musa* cultivars, *Musa* ABB cv KluaiNamwa, *Musa* AAA cv KluaiHomKheiw and *Musa* AAA cv KluaiHom Thong, the antioxidant activity of the peel extracts were higher that of the pulp extracts.This finding is in agreement with previous reports. Someya et al has reported the higher antioxidant activity of the peel than the pulp of banana (*Musa Cavendish*) attributed to the higher content of the flavonoid, gallocatechin [16]. Although numbers of researches has demonstrated the peel extracts from various banana cultivars as potential sources for antioxidant compounds [23,24]. Interestingly, in this study the pulp extracts from *Musa* (BB) KluaiTani showed the strongest antioxidant activity among the test samples. At the same concentration the *Musa* (BB) cv KluaiTanipulp extract was able to scavenge over 90% of DPPH free radical (94.81±2.25%), which was approximately three to five times more than the other extracts. *Musa* ABB KluaiNamwa, *Musa* AAA cv KluaiHomKheiw and *Musa* AAA cv KluaiHom Thong are consume when ripe as dessert bananas. They are triploid banana which seeds are not well developed. This is different from *Musa* (BB) cv KluaiTani, a seeded banana, which its green fruits are eaten fresh as salad ingredient. Imama et al has investigated antioxidant activity of seeds, pulps and peels of the seeded banana, *Musa sapientum* L. spp. *sylvestris*. They have found that the seed methanolic extract was more potent antioxidant than pulp and peel extracts and it also contained a good amount of phenol phenols (244.38 mg/g of plant extract in GAE) [11]. Jain et al also studied on antioxidant activity of the hexane, ethyl acetate and ethanol extracts from seeds, pulps and peels of *M. sapientum* L. subsp. *sylvestris* from Bangladesh. The ethyl acetate extract of banana seed showed strongest antioxidant activity (1238.33 mg ascorbic acid equivalent antioxidant capacity/100 g extract). The high antioxidant correlate to the high content of polyphenol (19.46 mg GAE/g extract) in the extract [12]. This could be estimated that the strong antioxidant of *Musa* (BB) KluaiTani pulps was due to the antioxidant activity of the seed that were parted of the pulps. Seeds are

The total flavonoid contents of the extracts were estimated by aluminium chrolide complexation. Flavonoid compounds show wide ranges of physiological effects including antioxidant, anti-inflammatory and antimicrobial activity. This investigation has disclosed that the total flavonoid contents in *Musa* peel extracts were higher than in pulp extracts. *Musa* (BB) cv KluaiTani peel extracts contained the highest level of total flavonoids contents (4.13±0.53 mgRUE/g extract) but not significantly different from that of the *Musa* (ABB) cv KluaiNamwa peel extracts(3.81±0.39 mgRUE/g extract). The *Musa*cv KluaiTani pulp extract contained slightly lower level of total flavonoid contents (3.25±0.33 mgRUE/g extract). Previous study displayed that flavonoids were predominant in *Musa* peel, while phenolic were predominate in pulps of *Musa* [5]. Taken together, *Musa* peel could be good source for antioxidant of the flavonoid group.

The total phenolic contents of the extracts were determined by colorimetric method using Follin Ciocalteua regent. The phenolic compounds are one of the major groups of natural antioxidant found in fruits and vegetables. The total phenolic contents were higher in the peel extracts than in the pulp extracts in all studied *Musa* cultivars exceptin *Musa* (BB) cv KluaiTani. The *Musa*(BB) cv KluaiTani pulp extract contained the highest total phenolic contents (253.13±82.64 mg GAE/g extract) and were approximated 10 times higher than the other extracts. The could due to the seed component which are part of the pulp of *Musa* (BB) cv KluaiTani as previously described [11-12].

The Pearson's correlation coefficient between antioxidant activity and total phenolic displayed a high positive correlation (0.871). This indicated that the antioxidant activity of the *Musa* extracts were attributed to the phenolic compounds.

In conclusion this study has revealed the potential of bananas or *Musa* plants as sources of natural antioxidants. *Musa* (BB) cv KluaiTani pulp was identified as the best source of antioxidant phenolic compounds. This provide basis in functional food development.

Acknowledgements

The authors would like to thank Ubon Ratchathani University for research funding.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Effect of *Ficusdubia* latex and root extracts in lipopolysaccharide-induced macrophages

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Abstract

Introduction: Chronic inflammation is an vital cause of various chronic diseases, including metabolic syndromes, cancers, and diabetes mellitus. Macrophage plays important roles during inflammation process by the production of several inflammatory mediators and cytokines, for instance, tumor necrotic factor (TNF)-α, nitric oxide (NO), interleukin-1β (IL-1β), and IL-6. Therefore, it would be remarkable to find the effective natural compounds, which act as alternative medicine to suppress chronic inflammation. This research focused on the anti-inflammation activity of *Ficus dubia* root and latex. This plant is a new *Ficus* species which has unique characteristic by its red latex. The latex is valued and is consumed with believe of its health benefit without any scientific data. In this study, we therefore investigated whether *Ficus dubia* latex (FDLE) and root (FDRE) extracts inhibit inflammation in lipopolysaccharide (LPS)-induced RAW 264.7 macrophage.

Materials and Methods: The root was extracted with 80% ethanol, while the latex was extracted with DI water. Total phenolic and total flavonoid contents were examined by Folin–Ciocalteu and aluminum chloride colorimetric methods, respectively. Next, the antioxidant activity of the extracts was determined by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays. For

cytotoxicity testing, RAW 264.7 macrophage was treated with various concentrations of the extracts (0-250 µg/ml) for 48 hours and subjected to MTT assay. Next, anti-inflammation property of FDLE and FDRE was determined using LPS-induced RAW 264.7 macrophage model. Production of NO, an inflammation mediator, by the cells was examined using Griess's reagent assay.

Results: The extraction provided % yield of FDLE and FDRE at47% and 4.9%, respectively. Total phenolic content of FDLE and FDRE was 248.53±1.46 mg GAE/g extract and 208.31±9.9 mg GAE/g extract, respectively. Total flavonoid content of FDLE and FDRE was 55.78±4 mg CE/g extract and 157.33±1.36 mg CE/g extract, respectively. Besides, the extracts showed $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging ability. Interestingly, non-toxic dose of the extracts was significantly reduced NO production in LPS-stimulatedRAW264.7.

Conclusions: These results suggest that FDLE and FDRE showed anti-oxidant and anti-inflammation activities. This study provides scientific evidence to develop this plant using for prevention of chronic inflammation that can be a major cause of several chronic diseases.

Keywords: Inflammation, Macrophage, *Ficus dubia*, Lipopolysaccharide (LPS)

Introduction

Chronic inflammation isacause of health problems worldwide, especially non-communicable diseases, including metabolic syndromes (1), cancers (2), and diabetes mellitus (3).Chronic inflammation is regulated by several immune cells, especially macrophages that play critical roles in several processes of inflammation. Macrophages release diverse pro-inflammatory cytokines, such as tumor necrotic factor (TNF)-α, interleukin-1β (IL-1β), and IL-6. The releasing cytokine scan stimulate inducible nitric oxide synthase (iNOS) that catalyze the changed of L-arginine to L-citrulline and lead to NO production. NO is a widespread signaling molecule which able to activate vasodilation, tissues damage, pain and expression of inflammation-related proteins (4). Free radicals, atoms or groups of atoms with an odd (unpaired) number of electrons, which are high reactivity and unstable. In normal physiology condition, free radical and antioxidant activity in the cells is balance.Imbalance of the process

results in oxidative stress and oxidative damage that cause inflammation and tissue injury (5). Prevention or inhibition of oxidative stress and inflammation-induced chronic diseases by natural product is recently attractive. Natural phenolic and flavonoid compounds are widely found in plants and possess several biological activities. These compounds have been reported their abilities to suppress oxidative stress and tissue damage, which are the promising activity to prevent diverse diseases, such as diabetes type 2 (6), and cancer (7). It was found that phenolics and flavonoids showed antioxidant t(8), antimicrobial(9), antiulcer(10) and anti-arthritic properties (11), and also can be used for regression of several inflammatory-related diseases (12). Recently, biological activities of *Ficus* specieshave been reported. For instance, *Ficus racemosa*, which contains highcontent of flavonoids showed anti-diabetes, anti-inflammatory, and anti-oxidant properties (13). The latex of *Ficus carica* showed inhibition effect of the proliferation of several cancer cells (14). Besides, leaf extract of *Ficus lutea* exerted anti-diabetic property by stimulated glucose uptake and insulin secretion (15). Furthermore, leaf extract of *Ficus amplissima* exhibited significant anti-inflammatory activity in animal models (16).

This study focused on *Ficus dubia*, which is a new *Ficus* species. Itis abundantly found in Tropical Asia, including Thailand, Malaysia and Sumatra.Its red latex is a unique characteristic, which is expensive for trading due to the believe of its health benefit without any scientific data. In this study, we aim to investigate the anti-inflammatory activities of *Ficusdubia* latex (FDLE) and root (FDRE) extracts in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages.

Materials and methods

Plant materials and plant extracts

The plant samples were collected at Baan bala, TambonBukeata, AmphoeWaeng, Narathiwat, Thailand. The Voucher specimen is Chantarasuwan 040117-1. Dried root sample was extracted with 80% ethanol at room temperature overnight. While dried latex sample was soaked in DI water at room temperature. The extracts were filtrated and freeze-dried by lyophilization. The crude extract of *Ficusdubia* latex and root (FDLE and FDRE) were kept at -20 ^{o}C for further experiment.

Phytochemical screening

The total phenolic content was determined by Folin-Ciocaiteu assay according to Song FL, et al. (2010)(17). Briefly, 20 µl of FDLE or FDRE (0-100 µg/ml) were mixed with Folin-Ciocaiteu reagent 100 µl and incubated for 3minutes in dark. Next, 80 µl of 10% sodium carbonate was added into the mixtures and incubated for 30minutes in dark. Finally, the blue-color solution was measured at absorbance 765 nm. Gallic acid was used as a standard.

The total flavonoid content was examined by aluminum chloride colorimetric method according to Subedi L, et al. (2014)(18). Briefly, 25µl of the extracts were mixed with 125 µl of distilled water, 7.5µl of sodium nitrite, and incubated at room temperature, in dark for 6 minutes. Next, 15µl of aluminum chloride hexahydrate, sodiumhydroxide was added with the mixture, and incubated at room temperature, in dark for 15minutes. The flavonoid content was determined at absorbance 532 nm. Catechin was used as a standard.

Antioxidant assays

2, 2-diphenyl-1-picrylhydrazylor DPPH is a radical used for determined anti-oxidant activity according to Wang HF, et al. (2008)(19). Briefly, 180 µl of $DPPH^{\bullet}$ radical (5.07×10^{-4} M) solution was mixed with 20 µl of FDLE or FDRE (0-500µg/ml), and incubated in dark for 30 minutes. After that, the absorbance was measured at 517 nm. The antioxidant activity was calculated as a % of radical (relative to control).

ABTS scavenging activity was determined following the method of Kumar J, et al. (2014)(20) with some modification. $ABTS^{\bullet+}$ will be produced by the reaction of ABTS stock solution and potassium persulfate for 12- 16 hours, in dark, at room temperature. The $ABTS^{\bullet+}$ solution, which contains2,29- azinobis (3- ethylbenzothiazoline- 6- sulfonic acid) diammonium salt, and potassium persulfate (di- potassium peroxdisulfate) was diluted with ethanoland adjusted anabsorbance to 0.70 (±0.02) at 734 nm. Next, 190 µl of diluted $ABTS^{\bullet+}$ solution was mixed with10 µl of FDLE or FDRE in ethanol. The percent of control was measured at absorbance 734 nm

Cell culture

RAW 264.7 macrophage-like cell line was obtained from ATCC.The cells were cultured in ultra-low attachment culture dish in DMEM with L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin solution under 5% CO_2 at 37°C. When the cells reach

70–80% confluence, they were harvested and plated either for subsequent passages or for the treatments.

Cytotoxicity testing

Cytotoxicity of FDLE and FDRE in RAW 264.7 macrophages was examined by MTT assay. The cells (25,000 cells/well) were plated into 96-well plate, and incubated for 24 hours. Next, various concentrations of FDLE or FDRE (0-250 µg/ml) were added and further incubated for 48 hours. After that, the cytotoxicity was determined by MTT assay. MTT dye solution was added into each well and incubated for 4 hours. Then, formazan product was dissolved by DMSO and measured the cell viability by spectrophotometry at 540, 630 nm. In each experiment, determinations were carried out in triplicate.

NO production assay

RAW 264.7 macrophages were plated in 96-well plate and incubated for 24 hours. After that, the cells were pretreated with non-toxic concentration of FDLE or FDRE (0-200 µg/ml) for 1 hour. Next, LPS 1 µg/ml was added into each well and further incubated for 24 hours. Then, culture supernatant was collected to examine NO production by Griess's reagent assay. Griess reagent and supernatant were mixed and incubated at room temperature for 15 minutes, in dark. The absorbance was determined at 540 nm. Besides, cell viability was determined by MTT assay.

Inhibition of inflammatory cytokine protein expression

RAW 264.7 macrophages were plated in 6-well plate, and incubated for 24 hours. Next, the cells were pretreated with non-toxic doses of the extracts as previous described. After the treatment, the culture supernatant was collected to determine the protein level of pro-inflammatory cytokines (IL-6 and TNF-α)using a sandwich Enzyme Link Immuno-Sorbent Assay (BioLegend's ELISA MAXTM Deluxe Set, CA). Dexamethasone was used as a positive control.

Statistical analysis

All values were given as mean ± standard derivation (X ± SD) from triplicate samples of two or three independent experiments. Overall differences among the treatment groups were

determined using one-way analysis of variance (ANOVA) by Prism 5.04software. *P* values<0.05 are regarded as significance.

Results

Total phenolic and flavonoid contents of the FDRE and FDLE

Percent yield of FDRE and FDLE is4.9% and 47%, respectively. It was found that total phenolic content of FDLE and FDRE was 248.53±1.46 mg GAE/g extract and 208.31±9.9 mg GAE/g extract, respectively (Table 1). While, total flavonoid content of FDLE and FDRE was 55.78±4 mg CE/g extract and 157.33±1.36 mg CE/g extract, respectively (Table 1).

Table 1: Total phenolic, total flavonoid contents and extraction yield of FDLE and FDRE

Plant extracts	Total phenolic (mg GAE/g extract)	Total flavonoid (mg CE/g extract)	Extraction yield (%)
FDLE	248.53±1.46	55.78±4.00	47
FDRE	208.31±9.90	157.33±1.36	4.9

Each value of total phenolic and flavonoid contents is represented as mean ± SD (n= 2).

DPPH and ABTS scavenging activities of FDRE and FDLE

We found that the extracts significantly scavenged DPPH and $ABTS^{\bullet+}$free radicals in a dose dependent manner. Results from DPPH assay showed that the half maximal inhibitory concentration (IC_{50}) of FDRE and FDLE was 83.12±8.86 µg/ml and 210.59±6.13 µg/ml, respectively (Figure.1A-B). While $IC_{50}$of FDRE and FDLE was 9.44±3.26 µg/ml and 33.59±6.26 µg/ml, respectively, as shown in Figure 1C-D, respectively determined by ABTS assay.

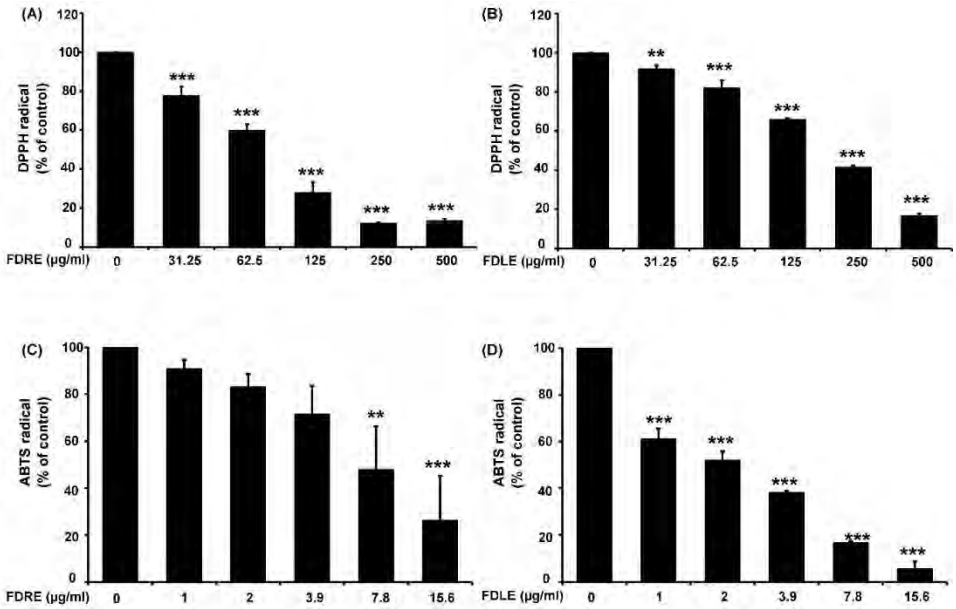

Figure 1. Antioxidant activities of FDRE (1A&C) and FDLE (1B&D) determined by DPPH and ABTS assays. Each value represents mean ± SD (n = 3), ** *p<0.01*,*** *p<0.001* vs. Control

Cytotoxicityof FDRE and FDLE on RAW 264.7 macrophages

The extracts at the dose up to 250µg/ml did not show any significant cytotoxicity to the cells. Inhibitory concentrations, IC_{20} and IC_{50} of FDRE and FDLE were more than250 µg/ml(Figure2A). Non-toxic dose or IC_{20} of FDLE and FDRE was then applied for further experiments.

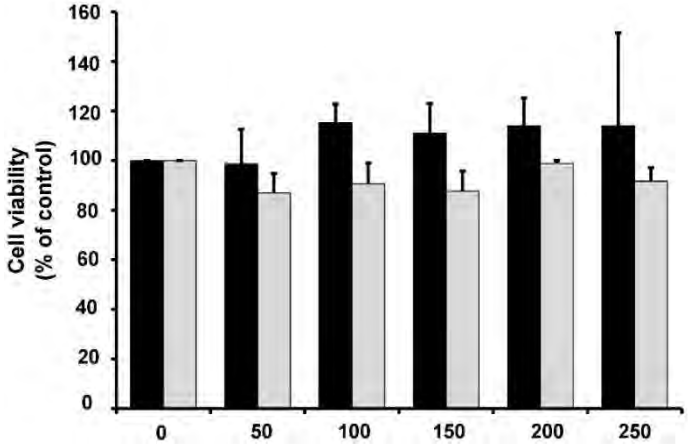

Figure 2. Cytotoxicity testing of FDRE and FDLE in RAW 264.7 macrophage cells. RAW 264.7 cells were treated with various concentrations of FDRE and FDLE (0-250 µg/ml) for 48 hours. Cell viability was examined by MTT assay. All data were performed in three replicates (n =3).

FDRE and FDLE inhibited nitric oxide (NO) production in LPS-induced RAW 264.7 macrophages

To evaluate the anti-inflammatory activity of FDLE and FDRE in LPS-induced RAW 264.7 macrophages, NO production was measured by the accumulation of nitrite level in the culture medium of LPS-treated macrophages. Certainly, LPS 1 µg/ml significantly increased NO production in RAW 264.7 macrophages. The treatment of FDRE at the dose 50, 100, and 200 µg/ml significantly inhibited the NO level by 26%, 33%, and 49%, respectively, whereas FDLE at the dose 200 µg/ml significantly decreased the NO level by 38.52% as compare with LPS-treated group (Figure3A). Co-treatment of LPS and the extract did not toxic to RAW 264.7 macrophages (Figure3B). Therefore, the decreased of NO production was not due to the reduction of cell viability.

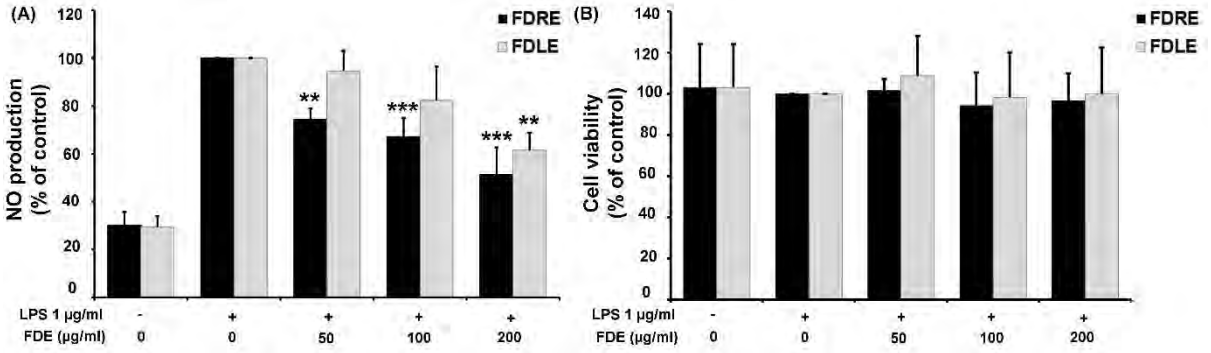

Figure 3. Anti-inflammation effect of *Ficus dubia* extracts. LPS-induced macrophages treated with or without FDRE and FDLE was subjected to Griess's reagent and MTT assays for determination of nitrite level (A) and cell viability (B), respectively. Each value represents mean ± SD (n = 3), ** $p<0.01$, *** $p<0.001$vs control.

Effect of FDRE on protein level of pro-inflammatory cytokines, IL-6 and TNF-α secreted from LPS-induced RAW 264.7 macrophages

FDRE showed the greater NO production inhibitory ability than that of FDLE. We therefore next investigated whether FDRE alter the inflammatory cytokine level in LPS-induced RAW 264.7. The results showed that FDRE significantly reduced IL-6 protein level

secreted from LPS-stimulated RAW 264.7 compared with control (Figure4A). Whereas, the treatment of FDRE slightly decreased the level of TNF-α (Figure4B).

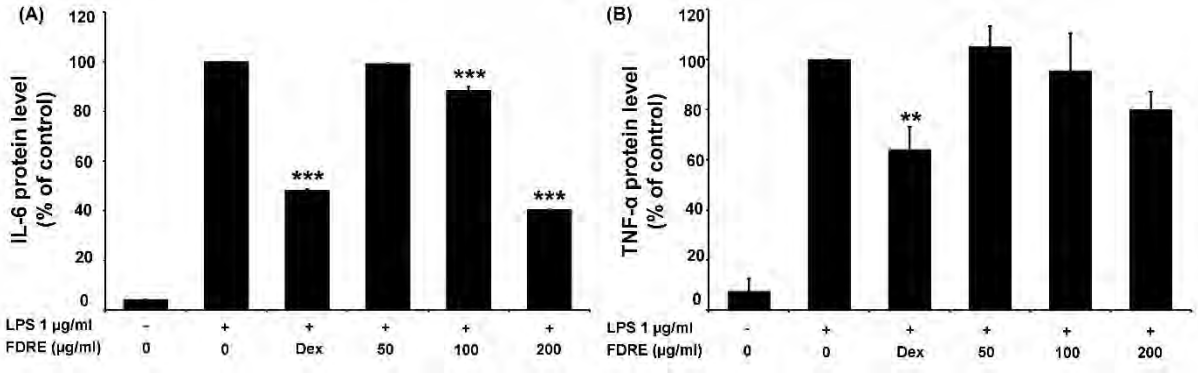

Figure 4. Effect of FDRE on LPS-stimulated IL-6 (A), and TNF-α (B) secretions from RAW 264.7 cells. The cells were pretreated with FDRE for 1 hour, and further incubated with LPS (1 µg/mL) for 24 hours. Culture medium was collected for the measurement of IL-6and TNF-α levels by ELISA, ** $p<0.01$, *** $p<0.001$vs. Control. Dexamethasone was used as a positive control

Discussion

Deregulated or imbalanced of inflammatory mediator production is a harmful factor to generate several chronic diseases such as heavy fever (21), rheumatoid arthritis (22), atherosclerosis (23), cardiovascular diseases (24), autoimmune disorders (25), cancer (26), and diabetes mellitus (27). For example, the inflammatory cytokines, IL-1β, IL-6, TNF-α, COX-2, iNOS, NO, and NF-κB are the key inflammatory mediators linking the inflammation and cancer in promotion and progression stages(28). Moreover, the inflammatory cytokines such as TNF-α can stimulate insulin resistance, which is a major cause of diabetes type2 via IKK/NF-κB and JNK signalings. Nowadays, non-steroidal anti-inflammation drugs (NSAIDs)are usually used for treatment of inflammation. Unfortunately, the side effects of the drugs in many patients who have continue treatment for long times have been reported (29). Therefore, the natural alternative medicine may bea suitable use for treatment in chronic inflammation.Recently, apigenin, a natural flavonoid from vegetables and fruits such as parsley and oranges, could inhibit LPS-induced inflammatory response in macrophages(30). Previous

study reported that fruits of *Ficusdeltoidea* showed the high quality of phenolic content that provided anti-oxidative stress ability(31). Furthermore, *Ficuspumila* leaves served as the natural source of antioxidant by contained flavonoid glycosides(32). In this study, root extract of *Ficusdubia* (FDRE) showed higher flavonoid content than latex extract (FDLE). Moreover, FDRE exhibited higher efficacy scavenging DPPH and $ABTS^{\bullet+}$radicals and inhibiting of NO production in LPS-induced macrophages than that of FDLE. The antioxidant and anti-inflammation activities of *Ficusdubia* might be correlated with its flavonoid content. We next investigated the anti-inflammatory activityof FDREby determined of inflammatory cytokines, IL-6 and TNF-α secreted from LPS-induced macrophages. It was found that the treatment of FDRE significantly decreased the protein level of IL-6, but not TNF-α. Thus, FDRE exerted its anti-inflammation ability through the reduction of oxidative stress, secretion of IL-6, and NO productions. Nevertheless, the molecular mechanism of anti-inflammation activities of FDLE and FDRE need to be further investigated.

In conclusion, we have demonstrated that FDLE and FDRE showed anti-oxidation and anti-inflammation activities. Moreover, the extracts at the dose up to 250 µg/ml were not toxic to RAW 264.7 macrophages. Finally, the knowledge from this study will provide scientific data for use and development of this plant as an alternative medicine or functional food.

Acknowledgement

This work is supported by the Faculty of Medicine Research Fund, Chiang Mai University, Chiang Mai, Thailand.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Study on chemical composition and mineral constituents of three selected myanmar functional foods

Win Win Khaing

Abstract

In this research work, some of functional foods which are manufactured in Myanmar such asThazin biscuit, Butter margarine and Penguin milk powder were selected to assess the nutriontial values and mineral compositions. The nutritional values such as total fat in each selected sample was determined by soxhlet extraction method, protein content by Kjeldahl digestion method. Ash content of each sample was determined by carbonization and incineration of sample in muffle funnace at 550˚C for six hours. The moisture content was determined by oven drying method at 105˚C . The crude fibre of each selected sample has been treated by boiling with acids and alkali. Inaddition, the mineral composition of these samples were studied by Energy Dispersive X Ray Fluorescence (EDXRF) analysis.

Key words:functional foods, mineral compositions, soxhlet extraction , Kjeldahl total nitrogen method, iodometric titration

Introduction

Functional foods are foods that provide health benefits beyond basic nutrition due to certain physiologically active components. In Myanmar, there are so many functional foods. The aim of the present research is to study the proximate mineral compositions, nutritional values of Penguin milk powder, butter margarine and Thazin biscuits which are manufactured in Myanmar. These functional foods were purchased from Ocean super market, Chanmyathazi Township,Mandalay Region, Myanmar. One of the selected samples, Penguin milk powder, is suitable not only for nutrition but also for bone strength.Butter margarine provides nutrient to consumers. Moreover Thazin biscuit is also source of carbohydrate, protein and fat together with some fibers,ash and minerals. Therefore, these three samples were chosen for analysis in this research. Penguin milk powder is designed with a combination of nutrients that work together to support strong bones, joints muscle for consumers. The ingredients of Penguin milk

powder are dried milk powder, sugar, minerals such as calcium and flavouring agent. The primary raw materials used in margarine production include vegetable oils, water,salt,skim milk and salt emulsifiers. The principle ingridents of Thazin biscuit which is used in the present study is wheat flour. Otheringredients of the considered biscuit are sugar, butter, milk powder , egg and vanillin powder.In Myanmar, these foods have been widely consumed. Therefore these foods were selected to evaluate their nutritional values and mineral compositions.

Objectives

To purchace the samples

To determine moisture, ash of each sample

To determinenutrional values such as protein, fat, carbohydrate, sugar of each sample

To examine the mineral compositions of the selected samples by EDXRF

Materials and Methods

1.Materials

Some of the foods which are manufactured in Myanmar such as Penguin milk powder, Butter margarine and Thazin biscuit were purchased from Ocean Super Market, ChanmyathaziTownship, Mandalay Region, Myanmar in July2017. These foodswere kept in the glasscontainers were kept in the glass containers till the analysis.The analytical reagents were usedin each experiment as necessary.

2. Preparation of each sample

Some chips of Thazin biscuits wasground and was used throughout the research. The samples Penguin milk powder and margarine were directly used for my research.

3.Methods

3.1 Physical Valuation

The physical properties of the chip of Thazin biscuit, the sachets of Penguin milk powder and butter margarine were studied firstly.

3.2 Determination of gross chemical composition

The moisture content, protein, fat, crude fibre and ash of each sample were determined.

3.2 (a) Determination of moisture

The moisture content of each sample was determined by oven drying method at 105˚C.

3.2 (b) Determination of proteinby Kjeldahl digestion method

About 5g of Thazin biscuit sample (finelyd till the mixture became colourless ground powder) was weighed and placed in the Kjeldahl digesting flask. About 5g of annular sodium sulpouhate, 0.25g of anhydrous copper II sulphate and 12.5 ml 98% sulphuric acid were added into it in order to wash down any solid adhering to the nesk.The flask was shaken until the contents were throughly mixed and it was heated till the mixture became colorless. The digestion was continued for half an hour to make sure that all nitrogen in the sample was converted to ammonium sulphate. It was allowed to cool and 10ml of distilled water was carefully added while shaking. The Kjeldah's distillation apparatus was setup,taking come that the tip of the condenser extended below the surface of the 50ml standard sulphuric acid solution in the receiver. The digested solution was poured into the flask together with 100ml of 40% sodium hydroxide to make mixture strongly alkaline. The evolved ammonia was distilled off.The distillate was titrated with standard sodium hydroxide solution, using methyl orange as an indicator. A blank determination was carried out without sample using all the reagents as in the case of sample. The results were described in results and dissucion.

3.2 (c) Determination of fat content by Soxhlet method

About 50 g of sample was placed in a thimble and the bag was then placed in a Soxhlet extractor. Petroleum ether(300cm^3)was poured into the extractor until some of it overflowed into the flask. The flask was heated on a water bath until the was previously removed from the sample. A duration of about 8 hours was required for complete extracrion. After the extraction, the oil dissolved in the solvent was removed by distillation. The last trace of the solvent was then removed by placing the content in an oven at about 100˚C until the constant weight was obtained. The results were expressed in results and dissucion.

3.2(d)Determination of crude fiber content

About 5 g of sample was put into a 500ml flask and then 200ml of 1.25% sulphuric acid solution was added. The flask was connected with reflux condenser and digested for 30 minutes. The flask was rotated every five minutes in order to mix the contents and then the particles were removed from the side of the flask. After digestion, the boiling solution with insoluble materials was filtered using Buchner funnel. The residue was washed again with hotdistilled water washed down into the flask. And then 200ml of 1.25% sodium hydroxide solution was added in to the flask. The flask was connected with refux condenser and boiled for 30 minutes. After boiling, the residue was filtered again and washed with hot distilled water until free from alkali. Then it was washed with 15 ml of 95% ethanol. After washing, the residue was transferred into a crucible and it was heated in an ovan at 100˚C until the constant weight was obtained. Then the crucible was cooled in a desiccator before weighing. The contents in the crucible were ignited in an electric furnace at dull red heat for about 20 minutes until all the organic matter had been removed. It was cooled and weighed. Heating, cooling and weighing were repeated until the constant weight was obtained. The loss in weight represents the weight of crude fiber.

3.2 (e) Determination of ash of each sample

About 5g of sample was put in a previously weighed procelain crucible and dried in an ovenat ±5˚C for four hours. This was done to remove moisture that would cause foaming of the sample drying the early stages of ashing. After removing the crucibles from the oven, they were cooled in a desiccators for about four hours and weighed with the evaporared sample. The materials were then ashed in an electrical furnace at 600˚C for six hours, followed by cooling in a desiccator and then weighed.

4. Determination of mineral contents in each sample

The mineral contents of samples were investigated by applying EDXRF (Energy DispersiveX-ray Fluorescence) at University of research Center, Yangon, Myanmar. The results weredescribed in results and discussion.

5. Determination of water content in butter margarine

The water content of butter margarine was detwrmined by using Dean and Stark apparatus. About 3.5 g of flesh sample was subjected to reflux distillation in the apparatus using 200ml toluene as solvent. The volume of water collected in the gadulated collecting tube gives the water content of the sample.

Results and Discussion

According to experimental data,the nutritional values of each selected sample such as ash, moisture content and fat content were summarized in table 3.1

Table3.1Nutritional values of three selected samples

Samples	Properties		
	Ash (%)	Moisture (%)	Fat (%)
Penguin milk powders	-	-	41.1
Butter margarine	0.1	-	25.8
Thazin biscuit	0.95	3.05	9.75

It can be seen that Butter margarine and Penguin milk powder are moderately rich in fat but Thazin biscuit contains a little amount of fat. Margarine and Penguin milk powder have no moisture. Therefore these foods are suitable for consumers. By studying the experimental results from Kjeldahl digestion method, the protein content ofeach sample was described in table 3.2

Table 3.2 Protein contents of three selected samples

Samples	Nitrogen (%)	Protein (%)
Penguin milk powder	2.4	15.00
Butter margarine	0.08	0.50
Thazin biscuit	1.40	9.11

By observing these datas, it can be known that the selected food sample, Penguin milk powder, is more rich in protein than margarine and Thazin biscuit. In addition,the results of the mineral contents of each selected sample examined by EDXRF spectrometry were expressed in table 3.3. (a).3.3 (b) and 3.3 (c).

Table 3.3 (a) Mineral contents of Penguin milk powder

No	Symbol	Element	Amountof Concentration (%)
1	Ca	Calcium	75.12
2	K	Potassium	21.90
3	Fe	Iron	1.70
4	Zn	Zinc	0.43
5	Rb	Rubdium	0.24

Table 3.3 (b) Mineral contents of butter margarine

No	Symbol	Element	Concentration mg/ cm $^{-2}$
1	K	potassium	54.90
2	S	sulphur	42.92
3	Ca	calcium	34.55
4	Fe	iron	20.82
5	Cu	copper	9.24
6	Mn	manganese	4.03

Table 3.3 (c) Mineral contents of Thazin biscuit

No	Symbol	Element	Amountof Concentration (%)
1	Ca	Calcium	45.47
2	K	Potassium	39.45
3	Fe	Iron	15.07

By studying the EDXRF report, it can be observed that the selected food samples are morerich in calcium and potassium than the remaining minerals such as iron, copper, sulphur andmanganese. Moreover it can also be clearly seen that there is no toxic minerals in eachsample. Therefore the selected functional foods are safe for concumers. According to the experimental data, it can be known that Thazin biscuit is the most rich in carbohydrate than the other two. Moveover, suger is present significant amount in Thazinbiscuit but low contents in the Peguin milkpowder.

Conclusion

The experimental data revealed that the selected Thazin biscuit was found to be nutritious functional healthful food. It improved both physical, sensory characteristics for the consumers. From the analysis, Thazin biscuit containscarbohydrate (72.69%), fat (9.75%), protein (9.11%), moisture (3.05%), sugar (2.5%), fiber (1%), ash (0.95%) and minerals such as calcium (45.47%), potassium (39.45%), iron (15.07%) and energy value (436.31 Kcal/100 g). However the selected margarine sample was composed of (0.5 %) protein, (52.8%) total fat, (0.1%) carbohydrate, (0.1%) total ash, (15.4%), water content, minerals. In addition, penguin milk powder could be determined as a healthy functional food from experimental information. This selected sample was composed of (41.1 %) fat, (33.9%) carbohydrate, (15%) protein which is composed of 82% casein and 18% whey protein and minerals such as (75.12%), potassium(21.91%), iron(1.70%) and energy value (430calorie per 100g). It can be concluded that the selected functional food are not only rich in protein, carbohydrate, and minerals required for health but also safe for consumers. Therefore, it could be recommended that these selected functional foods are more suitable for malnourished children to strengthen their bodies as well as patientsto recover and toresupply the nutrients and minerals. We will

attempt to assess the quality and safty of myanmar functional foods and then will promote them to the other countries.

Acknowledgement

We wish to express our gratitude to Prof. Dr. Mondhon sanguansermsri, President, University of phayao, Prof. Emetitus Dr. maitree Suttajit , Chair of Organizing Committee and Prof. Dr. Nison sattayasai, Dean, School of Medical Sciences for invitation to IFFA conference , 2018. We also wish to express our gratitude to Dr Thida Win, Rector ,University of Mandalay for permission to submit this paper. I am extremely grateful to Dr Yi Yi Myint, Professor and Head, Department of Chemistry and Dr Khaing Khaing Kyu Professor, Department of Chemistry, University of Mandalay for their kind permission,suggestions for this research work.

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Table : The Elemental Composition of Crude Sample *Cassia nodosa* Ham. by EDXRF

Symbol	Element	Concentration (%)
Ca	Calcium	1.7250
Si	Silicon	0.4404
K	Potassium	0.2818
Al	Aluminium	0.1393
Fe	Iron	0.1153
P	Phosphorus	0.0983
Cl	Chlorine	0.0809
Sr	Strontium	0.0296
S	Sulfur	0.0246
Ti	Titanium	0.0090
Zn	Zinc	0.0039
Mn	Manganese	0.0038
V	Vanadium	0.0025

Innovation of Functional Foods in Asia (IFFA)
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Investigations of various nutritive values from milk powders and energy drinks

Dr. Thida Kyaw, Dr. Yi Yi Myint, Dr. Thida Tin, Dr. Myint Myint Khaing

Abstract

The present study reports relationship between foods and food components that provide a health benefit. Although several articles have been reported for the functional foods, we have developed investigations of various nutritive values from milk powders and energy drinks which are produced in Myanmar. Mineral composition and nutritive values are important because it determines their absorption in the intestine and their biological utilization. Nutritive values of selected functional foods were investigated by using AOAC (Association of Official Analytical Chemists) method, acid precipitation method, Soxhlet extraction method, Somogyi's reagent using iodomertric technique, Kjeldahl method, EDXRF (Energy Dispersive X-Ray Fluorescence) and redox titration method. Moreover, caffeine from energy drink samples was extracted using dichloromethane. The extracted caffeine was confirmed by measuring the melting point. The functional groups in the extracted caffeine were identified by FT-IR spectrum.

Keywords: nutritional values, milk powder, energy drinks, protein, vitamin C

Introduction

Milk powders and energy drinks are important source of functional foods in most of the country in the world. Milk powder is a manufactured dairy product made by evaporating milk to dryness. One purpose of drying milk is to preserve it; milk powder has a far longer shell life than liquid milk and does not need to be refrigerated, due to its low moisture content. Another purpose is to reduce its bulk for economy of transportation.[1] Milk powders contain all twenty-one standard amino acids, the building blocks of proteins, and are high in soluble vitamins and minerals.[2] Powdered milk is used for food and health and also in biotechnology.[3] Milk is the most nutritionally complete food found in nature.[4] All kinds of milk, human or animal, contain vitamins (principally thiamine-vitamin B_1, riboflavin-vitamin B_2, pantothenic acid-vitamin B_5

and vitamins A, B_{12}, D), minerals (calcium, potassium, sodium, phosphorous and trace minerals), proteins (most casein), and carbohydrates (principally lactose), and lipids (fats).[3,4] Moreover, milk is a rich source of high quality protein which can support muscle growth and repair.[5] There are three kinds of proteins in milk: casein, lactalbumins and lactoglobulins. The main protein found in the milk casein.[6] Nowaday, many of us are not getting enough calcium from our diets. Calcium deficiency resulting either from inadequate intake or poor intestinal absorption is an important cause of reduced bone mass and osteoporosis. To maintain good health at elderly age, an optimal balance of the intestinal tract and its microbiota is especially important.[7] The only important elements in which milk is seriously deficient are iron and vitamin C.[8] An energy drink is a type of beverage containing stimulant drugs, usually including caffeine, which is energy as providing mental and physical stimulation. They contain sugar, herbal extracts and amino acids. Energy drinks have the effect caffeine and sugar provide, but there is little or no evidence that the wide variety of other ingredients have any effect.[9] Most of the effects of energy drinks on cognitive performance, such as increased attention and reaction speed, are primarily due to the presence of caffeine.[10]Energy drinks are energy to provide the benefits among health effects of caffeine along with benefits form the other ingredients they contain.[11] The dietary supplements in energy drinks may be purported to provide detoxification, sustain mental process, protect heart health, and reduce muscle fatigue.[11,12] Excessive or repeated consumption of energy drinks can lead to cardiac problems such as arrhythmias and heart attack.[13,14]Globally, energy drinks are typically attractive to young people.[15]

Materials and Methods

Milk powder-1 | Milk powder-1 | Energy drink-1 | Energy drink-2

Figure (1) Selected Milk Powders and Energy Drinks

General experimental techniques

Milk powders and energy drinks were purchased from Zaycho Market, (Figure 1), Chanayetharzan Township, Mandalay Region, Myanmar. The main idea of this research is to analyze the measurable amount of mineral composition and nutritional values of selected functional foods. Nutritional values were studied by AOAC method. The fat contents of selected samples were determined by Soxhlet extraction method. The protein contents were also determined by Kjeldahl's method. Somogyi's reagent using iodomertric technique was used for the determination of sugar contents in selected samples. Mineral contents of these samples were studied by Energy Dispersive X-Ray Fluorescence (EDXRF) spectroscopy. Moreover, casein proteins were isolated from selected milk powder by acid precipitation method. Caffeine was extracted using dichloromethane from selected energy drink samples. The extracted caffeine was confirmed by measuring the melting point. The functional groups inthe extracted caffeine were identified by FT-IR spectrum. The vitamin C contents of selected energy drink samples were also analyzed by using iodometric titration method.

Determination of nutritional values by AOAC method

The moisture content was determined by the oven drying method. The protein content was investigated by Kjeldahal digestion method. The fat content was determined by Soxhlet extraction method using petroleum ether (b-p 60-80℃) run for 8h. The ash content was determined by placing sample in preweighted crucible and placed in muffle furnace at 500℃ for 6hr.[16,19,20]

Determination of Mineral compositions by EDXRF Spectroscopic method

Mineral compositions of selected milk powder were determined by using EDXRF spectrophotometer.

Isolation of Casein Proteins from Selected Milk Powder

Casein is a mixed complex of phosphoproteins existing in milk. It can be separated by acid precipitation method.[18]

Determination of Sugar Content

Sugar contents of selected samples were investigated by Lane and Eynon's Method. Sugar content was calculated by titrating of Fehling solution (A and B) with sample solution.

Determination of Caffeine content

Caffeine was extracted from selected Energy drink samples by using dichloromethane. The melting point of extracted caffeine crystal was measured by Electric Melting Point Determination Apparatus (SMP-30). The infrared spectrum of the caffeine was carried out by FT-IR instrument at the Department of Chemistry, University of Mandalay.

Determination of vitamin C content

The vitamin C content was determined by iodometric titration method. Each of the standard vitamin C solution and fresh juice sample solution (10mL) was pipetted into each conical flask. Then, 3 drops of 1% starch indicator solution was added to each conical flask and then, titrated with iodine solution. The end point of the titration was identified as the first permanent trace of a dark blue color due to the formation of starch-iodine complex. The final volume of iodine solution as recorded. Titration was repeated three times.[17]

Results and Discussion

AOAC method was used to determine nutritional values in selected samples. The nutritional values of moisture, ash, protein, fat and sugar content were found are recorded in Table 1 and 2.

Table (1) Nutritional Values of Two Selected Milk Powder

Sample	Moisture Content (%)	Ash Content (%)	Fat Content (%)	Protein Content (%)	Sugar Content (%)
PEP 1	1.92	4.23	18.92	17.12	56.01
Polar 2	2.13	3.95	17.86	16.78	58.23

Table (2) Sugar Content of Two Selected Milk Powder

Sample	Sugar Content (%)
1	56.01
2	58.23

According to experimental result, the ash content was more abundant in milk powder sample (1). The ash content of a sample is an inorganic residue remaining after organic matten has been burnt away. The moisture content in milk powder (2) was higher than in milk powder (1). The larger moisture content is the shorten shelf-life of food sample. Moreover, the fat content in milk powder (1) was higher than milk powder in (2). Fat and oil function as a major store house of energy. In addition, sample (1) gives more protein content than sample (2). Protein is one of the essential components of human body. Milk proteins contain all essential amino acids required by humans. By the comparison of sugar contents, milk powder (2) gave more sugar content than the other one. Lactose is the major carbohydrate fraction in milk. It is made up of two sugar, glucose and galactose.

Mineral Contents of selected milk powder sample were measured by energy Dispersive X-Ray Fluorescence Spectrometer. The results are shown in Table (3).

Table (3) Mineral Compositions of two selected milk powder

No.	Elemental Composition (%)	Sample (1)	Sample (2)
1.	Cl	1.15200	1.16810
2.	K	0.94780	0.89060
3.	Ca	0.73620	0.62110
4.	P	0.48100	0.47970
5.	Si	0.16790	0.04860
6.	S	0.00370	0.04400
7.	Al	0.04310	0.06560
8.	Mn	0.00098	0.00092
9.	Fe	0.00010	0.00010

From these results, calcium, phosphorous, silicon contents in the milk powder (1) were presented at the higher composition. It is often important to know the mineral contents of foods during processing because this affects the physicochemical properties of foods. Some minerals are essential for a healthy diet. Calcium plays an important role in the bone, teeth and other soft tissues. Potassium can act as electrolyte in the body tissues and it is also an essential mineral for maintaining normal blood pressure and heart function. Phosphorous is an important component of energy intermediates. Phosphate serves as blood buffers. Hence, it was found that the selected milk powder samples contain essential minerals for human health.

In this experiment, only glacial acetic acid was used to isolate the casein proteins from milk powders. Furthermore, the fat that precipitates along with casein can be removed by dissolving it in ethanol and pet-ether. The fat was highly soluble in organic solvent. The results indicated that milk powder (1) contains larger amount of casein protein than milk powder (2). The results are tabulated in Table (4).

Table (4) Percentage if Isolated Casein Proteins from selected Milk Powder

No	Sample	Casein Protein (%)
1	Milk powder (1)	40.25
2	Milk powder (2)	37.03

Casein protein is the majority of muscle repairing and rebuilding take place during sleep. Hence, milk powder (1) and (2) are suitable for the best muscle growth and basic building achievement.

Caffeine was extracted from energy drink samples by using sodium sulphate and dichloromethane. The results were shown in Table (5).

Table (5) The Content of Caffeine Extracted from Energy Drinks Samples

No.	Sample	Caffeine Content mg/mL
1.	Energy Drink (1)	1.5012-1.5120
2.	Energy Drink (2)	0.5371-0.5520

From this table, it was found that energy drink sample (1) contains more caffeine amount than sample (2). Moreover, comparison between melting points of extracted caffeine and reference caffeine was tabulated in Table (6).

Table (6) Comparison between melting points of extracted caffeine and reference caffeine

No.	Sample	Melting Point (℃)
1.	Extracted Caffeine	235-238
2.	Reference Caffeine	238

According to this result, melting point of the extracted caffeine was almost similar to that of the reference caffeine.

The infrared spectrum of caffeine was identified by FT-IR instrument at the Department of Chemistry, University of Mandalay. The results were shown in Table (7).

Table (7) Characteristic Absorption of RT-IR and their Assignments

No.	$\lambda_{max}(cm^{-1})$	Assignments (Functional group)
1.	3109.35	-CH stretching vibration of sp^2 hydrocarbon
2.	2928.04,2877.89	-CH stretching vibration of sp^3 hydrocarbon
3.	1703.20	C=N stretching vibration
4.	1656.91	C=O stretching vibration of carbonyl group
5.	1546.96	C=C stretching vibration of carbonyl group
6.	1485.24,1357.93	C-H bending vibration of methylene group and methyl groups
7.	1234.48,1024.24	C-N stretching vibration
8.	864.14,746.78	=C-H out of Plane bending vibration

From FT-IR spectrum, it was found that extracted Caffeine contains C=N, C-N, C=O, C=C functional group.

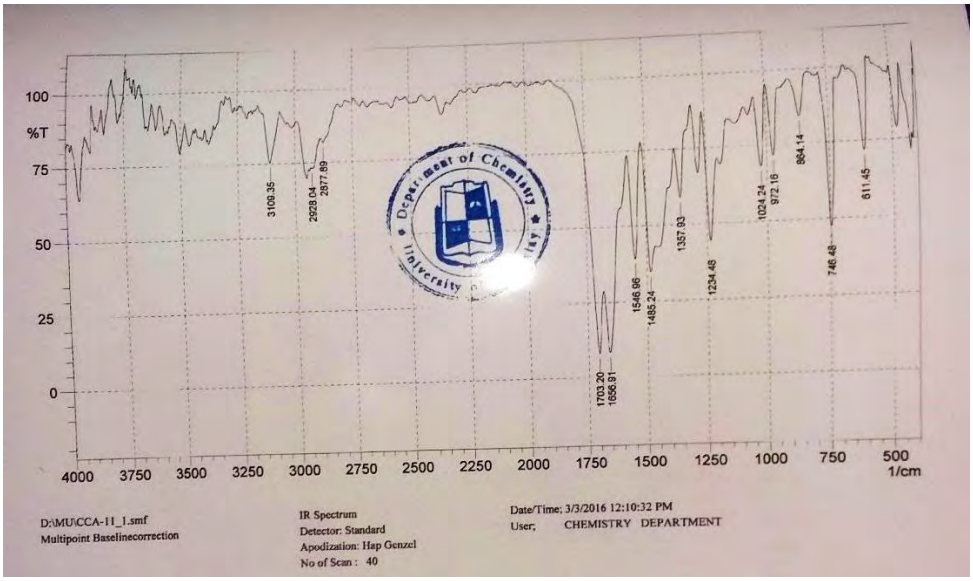

Figure (2) FT-IR spectrum Extracted Caffeine from Energy Drinks

Vitamin C contents in energy drink sample were determined by iodometric titration method. The results were shown in Table (8).

Table (8) Ascorbic acid (Vitamin C) contents of Energy Drink Samples

No.	Sample	Vitamin C content (mg/mL)
1	Sample (1)	0.0957
2.	Sample (2)	0.0876

According to Table (6), the vitamin C contents of these samples are nearly the same. Vitamin C is one of the most important vitamins for humans. Vitamin C is needed to help keeping the immune system up and going strong. It can chelate metals that produce free radicals in the body.

Glucose contents in energy drink samples were by Somogyi's regent using iodomertric technique. The results were shown in Table (9).

Table (9) Glucose Contents of Energy Drink Samples

No.	Sample	Sugar Content (mg/mL)
1.	Sample (1)	0.1295
2.	Sample (2)	0.1470

Conclusion

In the present research work, nutrition values of selected milk powder samples were investigated by AOAC method. According to the experimental results, the ash contents of selected samples were found to be 4.23 and 3.95%, moisture contents were 1.92% and 2.13%, fat contents were 18.92% and 17.86%. In addition, the protein contents of selected samples were measured by Kjeldahl's method. It was found that protein contents were 17.12% and 16.78%. Sugar content was 56.01% and 58.23%. Casein content of two milk powder samples was found to be 40.25% and 37.03%. From EDXRF spectroscopic data, two selected milk powder samples showed major constituents of calcium, potassium and phosphorous. Hence the two milk powder samples contains essential mineral for human. Furthermore, caffeine was investigated from selected energy drink sample by using sodium, sulphate and

dichloromethane. The caffeine contents were found to be 1.5012-1.5120 mg/mL and 0.5371-0.5520 mg/mL. It was found that energy drink sample contains the highest caffeine was measured and compared with that of reference caffeine. It can be seen that melting point of the extracted caffeine was almost similar to that of the reference caffeine. Then, the extracted caffeine was also identified by FI-FT spectrum. The vitamin C and sugar contents in energy drink samples were determined by Somogyi's reagent using iodometric technique. The vitamin C contents of samples were 0.0957mg/mL and 0.0846 mg/mL. It was known that the vitamin C contents in samples were nearly the same. The sugar contents were 0.1295 mg/mL and 0.1470 mg/mL. According to these data, all milk powder samples and energy drink samples are good sources of energy for human nutrition.

Acknowledgements

The author would like to thank Professor and chairman of conference committee, University of Phayao, Phayao Province, Thailand. I would like to thank the Department of Higher Education, Ministry of Education, Nay Pyi Taw, Myanmar, for the permission of presenting this research paper. Moreover, I am especially grateful to DrMaungMaungNaing, Reactor of Yadanabon University encourages me to attend "Trends in Research and Markets of Functional Food". I would like to extend my gratitude to Professor Dr Haling HlaingMyat , Head of Chemistry Department, Yadanabon University for allowing to attend this conference.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Investigation of nutritional values in cereal based foods in Myanmar

Khin Myo Myint

Abstract

Our country, Myanmar is one of the developing countries. The nutritious readymade foods such as cereal and milk products, chocolate-dipped foods were exported from different countries especially Asia and Europe since many years ago. Therefore, the investigations of nutritional composition in commercial cereal products were carried out in my research work. For the determination of chemical composition, five brands of cereal products including "Super quaker", "Calsomequaker", "Sunday quaker", "Happy quaker" and "All time quaker" were chosen for the study. The constituents of crude protein, crude fiber, crude fat, ash, moisture, total carbohydrate and total sugar were analyzed for 100 grams of each sample. The protein content in the samples was determined by Micro Kjeldahl method. Crude fiber, moisture and ash were estimated by applying the standard method of analysis. Crude fat content was measured by the soxhlet extraction method. Total carbohydrate determination in samples was performed by using phenol-sulphuric acid method. Total sugar contents in these samples were measured by employing Somogyi-Nelson method. According to the resulting data, the amounts of protein in all samples were found fewer than 5%. The total carbohydrates were also found as the range of 5% to 7%. The constituents of crude fiber, crude fat, ash and moisture have been observed as no significant differences. Among the all samples, the highest amount of total sugar was found in "Super quaker". Ash contents in these samples were also found nearly 1%. Moreover, Energy Dispersive X-Ray Fluorescence results showed that all of samples consist of Calcium, Zinc, Copper, Phosphorous, Magnesium, Iron, Potassium, Manganese, Selenium and Sodium. The experimental results of my research indicated that the samples in Myanmar local market consist of the suitable nutritional composition. But the exact amount of ingredients in these samples should describe such as fat and sugar contents for consumers.

Keywords: nutritional values, cereal products, Myanmar Market

Introduction

Consumers can be classified as uniformed consumers, consumers concerned about their health, conscious consumers and non-health conscious consumers. (Vicentini A., *et al*., 2016) Consumers were demanding better foods to provide energy and nutrient for health as the statement that food is medicine and medicine is also food. (Food Technological Magazine.1998) The foods primarily provide energy and nutrients. Furthermore, nutrients obtained from also provide sensory satisfaction and well physiological activity. (Plami S.P. *et al*., 2011)

The institute of Medicine's Food and Nutrition Board defined functional foods as any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains. (Plaami S.P., *et al*., 2001) Plants and animals are two main sources of functional foods such as many kinds of vegetables, fruits, meat and fish respectively. (Food Technological Magazine, 1998) The types of functional foods are found as fortified foods, enriched foods, altered products, non-altered products and enhanced commodities. (Joseph T. Spence, 2006; Consumer trends: functional foods, ISSN, 2009; Siro I., 2008)

We have got nutritious and fresh foods as gifts from nature. However, the people who have no enough time to choose such foods because most are struggling every day for livelihoods. Milk has huge variety of nutrients and also grain has huge variety of minerals. There are supplementary foods mainly made of milk and grain (cereal) (Kowieska A.,2011) since they have the required nutrition content for the consumers. Therefore, they are used to produce different types of supplementary foods.

Many kinds of cereal powder or flake from corn, rice, oat and wheat are mixed with malt extract, non-dairy creamer and sugar. The nutritious readymade foods were found as malted milk, coffee mate, cereal powder, biscuits, fruit juices, cheese, chocolate-dipped foods and many kinds of snack.

The nutritious readymade foods are exported to Myanmar. The most exported foods to our country markets come from Thailand, China, Singapore, Malaysia and India. Today, various kinds of nutritious readymade foods are manufactured and distributed in local like other countries. In Myanmar, nutrient contents in some products are shown on sachet but some products do not label the gradients.

Food labels can be helpful for individuals to monitor caloric intake and ensure successful weight loss or maintenance. (Jumpertz R., *et al*., 2014, Wing RR, Hill JO. 2001)

Nutrition labels have been developed specifically to help consumers identify foods that will comprise a healthful diet. (Taylar Cl., WilkeningVL., 2008; Jumpetz R. *et al*., 2013)

Many functional foods are found in local market. Among them the cereal quakers were selected for the analysis. The objectives are to investigate the nutritional composition and to compare the contents of nutrient in commercial cereal products.

Materials and Methods

Sample collection and preparation

Five brands of cereal quaker were chosen for the study. These are namely Super, Calsome, Sunday, Happy and All time. The first two brands are popular among consumers. These samples were collected from local market, Mandalay, Myanmar. The dried samples were ground into fine powder and they were stored in air tight containers.

Analysis of nutritional composition

The moisture contents were determined bydrying until constant weight in oven at 105°C for 2 hours. (AOAC. 1990; 2000) To analyze ash content, 5g of sample were accurately weighted in crucible which is placed in a muffle and incinerated at a temperature of 550 ± 10 °C for sample. The residue is cooled in a desiccator and weighted. (ISO/DIS 936: 1978; ISO 2171:1993; AACC. 1995)

The amount of protein was determined from the organic nitrogen content by micro Kjeldahl method. (AOAC. 2005) The various nitrogenous compounds are converted into ammonium sulphate by boiling with concentrated sulphuric acid. The ammonium sulphate formed is decomposed with an alkali (NaOH) and the ammonia liberated is absorbed in excess of standard solution of acid and then back titrated with standard alkali.

One g of fat free dried sample was weighed and put in one liter tall beaker; 200 ml of 1.25% H_2SO_4 was added. The solution was kept boiling for 30 minutes under bulb condensers. Beaker was rotated occasionally to mix the content and removed the particles from the sides. Content of the beaker was filtered through funnel. Sample was washed back into tall beaker with 200 ml, 1.25 per cent sodium hydroxide, brought to boiling point and boiled exactly for 30 minutes. All insoluble matter was transferred to the sintered crucible by means of boiling water until it became acid free, washed twice with alcohol, three times with acetone, dried at 100°C to constant weight, reweighed and ashed in a muffle furnace at 550°C for 1 h. Crucible

was cooled in a desiccator, reweighed and percentage of crude Fiber in the samples was calculated. (AOAC. 2008)

A weighed amount (2g) of sample was transferred to an extraction thimble dried overnight at 60°C temperature. The thimble was placed in a Soxhlet extractor fitted with a condenser and flask containing sufficient petroleum ether. After 6 h extraction, thimble was removed from the extraction apparatus and dried in the hot air oven to a constant weight, cooled in a desiccator to room temperature and weighed. Loss of weight of thimble indicated the amount of fat in the sample. (AOAC. 2006)

Total carbohydrate determination in samples was performed by using phenol-sulphuric acid method. (Menezes.E.W.,*et al*., 2004; Barikmoa I., *et al*., 2004) And total sugar contents in these samples were measured by employing Somogyi-Nelson method. (Buzarbarua. A.2000)

Results

The moisture and ash contents in the samples were shown in Table 1. While the highest amount of moisture contents were found in Sunday and Happy, the remaining three brands are nearly same. It was found that all the samples equally contain the ash composition.

Table 1 Moisture content

No.	Name of Sample Brand (Cereal Quaker)	% Moisture	%Ash
1	Super	2.127	1.99
2	Calsome	2.058	1.95
3	Sunday	2.714	1.97
4	Happy	2.603	1.96
5	All time	2.026	1.98

Table 2 represents the results of protein content in the samples. The values of protein are 3.10%i in Super, 0.01% in Calsome, 2.80 % in Sunday, 2.54 % in Happy and 2.30% in All time. Moreover, the labeled contents on sachet were described in this Table.

Table 2 Protein Content

No.	Name of Sample Brand (Cereal Quaker)	% Protein	
		Observed value	Labeled content
1	Super	5.3	6.67
2	Calsome	0.01	0
3	Sunday	2.80	-
4	Happy	2.54	-
5	All time	2.30	-

Table 4 Carbohydrate Content

No.	Name of Sample Brand (Cereal Quaker)	% Carbohydrate	
		Observed value	Labeled content
1	Super	75.95	73.90
2	Calsome	83.69	80.00
3	Sunday	83.92	-
4	Happy	87.37	-
5	All time	77.29	-

Table 5 Sugar Content

No.	Name of Sample Brand (Cereal Quaker)	% Sugar	
		Observed value	Labeled content
1	Super	45	50
2	Calsome	32	36
3	Sunday	37	-
4	Happy	38	-
5	All Time	42	-

Table 6 Crude fiber Content

No.	Name of Sample Brand (Cereal Quaker)	% Crude fiber	
		Observed value	Labeled content
1	Super	2.8	3.33
2	Calsome	3.1	4.00
3	Sunday	3.0	-
4	Happy	2.9	-
5	All Times	3.4	-

Table 7 Crude fat Content

No.	Name of Sample Brand (Cereal Quaker)	% Crude fat	
		Observed value	Labeled content
1	Super	12.0	13.3
2	Calsome	9.2	12.0
3	Sunday	5.6	-
4	Happy	12.6	-
5	All Times	13	-

Table 8 Mineral Contents

No.	Element	% Composition				
		Super	Calsome	Sunday	Happy	All Times
1	Ca	0.920	16.880	0.803	0.781	0.693
2	Na	0.220	0.251	0.190	0.244	0.181
3	Fe	4.20	0.020	0.141	0.081	0.201
4	K	0.001	0.001	0.002	0.001	0.001

According to the resulting data, the amounts The total carbohydrates were also found as the range of 70 to 90%. The constituents of crude fiber, crude fat, ash and moisture have

been observed as no significant differences. The constituents of crude fiber in samples were observed as 2.8% in Super, 3.1% in Calsome, 3.0% Sunday, 2.9% in Happy and 3.4% in All times. Crude fat contents were also found as 12.0% in Super, 9.2% in Calsome, 5.6% in Sunday, 12.6% in Happy and 13.0% in All times. The highest amount of total sugar content was found in "Super quaker". Moisture contents were found as 2.127% in Super, 2.058% in Calsome, 2.714% in Sunday, 2.603% in Happy and 2.026% in All times. Ash contents in these samples were also found as 1.99% in Super, 1.95% in Calsome, 1.97% in Sunday, 1.96% in Happy and 1.98% in All times. Moreover, elemental compositions were examined by Energy Dispersive X-Ray Fluorescence (EDXRF) method. The results showed that the significant content of calcium, was found in Calsome and iron in Super.

Discussion

The nutrients provided foods affect for consumers' health. Some products of cereal based foods are suitable for nutrient deficiency. The experimental results of the research work indicate that significant differences were not found in observed nutritional value of samples and labeled claim on these samples. But the nutritious foods like these samples in local products should label more exactly ingredients that the material is creditable or not such as fat and sugar contents for consumers.

Acknowledgements

First of all, I would like to many thanks to all participant in "The First International Conference on Innovation of Functional Foods in Asia (IFFA)" for permission to present my research paper. I would like to express my deepest thanks to DrThidar Win, Ractor of Mandalay University andDr Yi YiMyint, Professor, Head of Department of Chemistry, Mandalay University, for their authorization. I am also thankful to Lei Lei Win and Nu Yi Thin for their helpfulness and cooperation on my research work.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets ***January 22nd-24th, 2018***
University of Phayao, Phayao Province, Thailand
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Functional food: productivity of pectinase enzyme from *Asergillus niger* used in wine making

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Abstract

Aspergillus niger obtained from National Health Laboratory has been chosen for isolation of pectinase enzyme and it was used in damson wine making. Three types of wines were prepared and their nutritional values were determined. Antimicrobial activities and antioxidant activities of wines were monitored. Three different media such as Sabouraud dextrose agar, Potato dextrose agar and Starch agar were used to incubate at room temperature. The strains obtained were identified by using morphological and microscopic features. The Natham's Agar Well Diffusion Technique was used to monitor the antimicrobial activities of those wines and an antibiotic drug, streptomycin. The antioxidant activities of prepared wines were determined by using 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay. The nature of the absorbance *Vs.* concentration of pectin was a straight line passing through the origin showing that Beer's law was obeyed between 0.1 to 1.5 (g/mL) concentrations. Thus 0.5 g/mL concentration and optimum pH (4.8) were chosen for this research. The optimum temperature for the pectinase enzyme was found to be 50˚C. Average sensory evaluation test was observed that 15 % sugar of damson wine with yeast is the best flavor, odour, color, taste and overall acceptance. The antimicrobial activities tests point out that commercial wine is higher than prepared wines because of the additive such as alcohol and some other ingredients. The radical scavenging activity of wine with isolated pectinase (IC_{50}- 0.72 µL/mL) was found to be highest; it was higher than that of ascorbic acid (IC_{50}- 1.04 µg/mL) standard. It is concluded that, these wines fermented by pectinase are suitable to drink for good health due to their high antioxidant activities and these wines can reduce the risk of cardiovascular disease and some forms of cancer and anti-aging.

Keywords: Functional Food, *Aspergillus niger*, pectinase, Antimicrobial, Antioxidant

Introduction

"Functional Foods" are foods or dietary components that may provide a health benefit beyond basic nutrition. Biologically active components functional food may impart health desirable physiological effects (Cencic A., 2010). Functional food attributes of many traditional foods are being discovered. Functional foods may be "designed to have physiological benefits and reduce the risk of chronic disease beyond basic nutritional functions, and may be similar in appearance to conventional food and consumed as part of a regular diet" (Basics, 2010). Hippocrates nearly 2,500 years ago espoused that the tenet "let food be thy medicine and medicine be thy food", and it is receiving renewed interest. Hasler proposed that there has been an explosion of consumer interest in the health enhancing role of specific foods or physiologically-active food components, so called functional foods (Hasler, 1998).

There are many functional foods all over the world. Among them, wines are the health benefits functional food. Various kinds of wine are rich in micronutrients, and they also contain variety of biologically active compounds. Some non-nutritive compounds contribute color, flavor and characteristics. All of these phytochemical especially polyphenol have been reduced to chronic diseases such as cancer, osteoporosis and coronary heart disease (Johnson, 2003). Wine, red wine, is a rich source of a variety of phenolic compounds. It is estimated that a 4 oz. glass of wine contains about 200 different types of polyphenols. The most abundant antioxidants in our diet are polyphenols (Waterhouse, 2002). Antioxidant can protect against cellular damage caused by free radicals in the body. Cellular damage can lead to development of diseases like heart disease and cancer (Valko, 2004).

When an antioxidant destroys a free radical, this antioxidant itself becomes oxidized. Therefore, the antioxidant resources must be constantly restored in the body. Thus, while in one particular system an antioxidant is effective against free radicals; in other systems the same antioxidant could become ineffective (Young I, 2001). The antioxidant process can function in one of two ways; there are chain-breaking and prevention. In the chain-breaking process, when a radical releases or steals an electron, a second radical is formed. The last one exerts the same action on another molecule and continues until either the free radical formed is stabilized by a chain-breaking antioxidant or it simply disintegrates into an inoffensive product. For the preventive way, an antioxidant enzyme like superoxide dismutase, catalase and glutathione peroxidase can prevent oxidation by reducing the rate of chain initiation, e.g., either by scavenging initiating free radicals or by stabilizing transition metal radicals such as copper and

iron (Young I, 2001). Persons who frequently consume fruits, red wine, tea, chocolate or beer will have higher intake of polyphenols.

In the preparation of wine, one of the major problems is cloudiness due primarily to the presence of pectin. The cloudiness that they cause is difficult to remove except by enzymatic hydrolysis. Such enzymatic treatment also has the additional benefits of reducing the solution viscosity, increasing the volume of the wine produced, subtle but generally beneficial changes in the flavor and , in case of wine making, shorter fermentation times (Frazier, 1988; Voragen A., 1999).

The pectinase enzyme in *Aspergillusniger* plays as a major role in processing of wine since it gives rise extracellular pectinase enzyme. Pectinase can degrade plant cell wall polysaccharide (Aonymous, 2000). The function of pectinase in brewing is to fold, first it helps break down the plant typically fruit material and so helps the extraction of flavors form the mash. Secondly the presence of pectin in finished wine causes a haze or slight cloudiness, pectinase is used to break this down and so clear the wine (Anisa S.K, 2013).

For this research work, the industrial important enzyme, pectinase have been isolated for the *Aspergillusniger* and the isolated enzyme was used in wine processing. Four types of wines were prepared and the quality of wines was examined. For health safely, the microbial safety test and antimicrobial activity were determined. Antioxidant activities of the prepared wines and a commercial wine found in Myanmar market were determined.

Materials and Methods

Subculture Transfer of Isolated *A.niger* Strain

The *Aspergillus niger* (onion source) was obtained from National Health Laboratory (NHL), Yangon. The received isolated *A. niger* (NHL strain) was also transferred to SDA (Sabouraud Dextrose Agar), PDA(Potato Dextrose Agar) and SA(Starch Agar) media, and incubated at 25ºC for 6 to 7 days (Sidana A., 2014). Their growth rate, morphological changing and microscopically characteristics of *A.niger* on three different media were examined during these days.

Pectinase Enzyme Production from Isolated *A. Niger*

Sabouraud Dextrose Broth (SDB) was used as enzyme producing medium (Carlier G., 1984). Prepared SDB and distilled water were placed into the conical flask. The flask was boiled on a magnetic stirrer for 30 minutes with 1300 rpm at 200ºC. It was sterilized in microwave oven for 15 minutes at 130ºC and allowed to cool for 30 minutes. The pH of broth culture was adjusted to 4.5 (Murray P. R., 2003). Prepared enzyme producing medium, Sabouraud Dextrose Broth was incubated with pure *A. niger* strain, and incubated in water bath shaker for 72 hours at 32ºC and 120 rpm. After incubation time, mycelium suspending in liquid broth culture was removed by centrifugation with 6000 rpm for 30 minutes and filtered the solution. The clarified enzyme solution was obtained. About 75 mL of clarified enzyme solutions were taken and placed into the beakers. These breakers were placed in the ice bath in order to get cooling conditions. Acetone was slowly added drop by drop and gently stirred with glass rod. Amorphous form of pectinase enzymes was precipitated. Cold centrifuge was used to remove unwanted supernatant with 6000rpm for 20 minutes. The precipitated pectinase was dried in air.

Identification of Pectinase Enzyme by Using FTIR

The standard pectinase and isolated pectinase enzyme were subjected to analyze by Infrared spectrophotometer (SHIMAZU). Functional groups and their vibrational mode were studied.

Determination of Isolated Enzyme Activities

0.1 ml enzyme solution and 3.0 ml pectin solution were used in determination of enzyme activities. The maximum wavelength of pectinase enzyme was determined by using UV spectrophotometer between 200 to 300 nm. In optimum pH determination, phosphate buffer was used. Enzyme and pectin solution were prepared in various pH of 4.0 to 6.0. Enzyme and pectin solutions were thoroughly mixed for 5 minutes at various temperatures between 30 to 60ºC. The absorbance of these two solutions was measured during various reaction times 0 to 22 hours.

Inoculation of Native Yeast

Damson fruits were washed, sliced, deseeded and then blended using a blender for 2-3 minutes until homogeneous solution was obtained. 12% of sugar was added to the juice extract. 200 ml of the solution were put into three sterilized bottles each and stored to ferment at room temperature for 10-15 days.

Fermentation of Wine with Different Sugar Contents

About 125 g of damson fruits were thoroughly washed to remove adhering dust and dirt. Then, they were sliced and crushed and the resultant "must" was placed in each bottle containing 10%, 15% and 20% sugar syrup based on yeast (1:10) and control wine containing 20% sugar syrup with yeast and thoroughly stirred. After well stirring, the bottle was sealed carefully. The fermented bottles were stored at room temperature for 15 days. Later carbon dioxide gas was evolved from the fermentation bottle and allowed it to release. When fermentation was slowed down, the liquid was siphoned out the bottle and placed into another bottle which had been sterilized and stored for one month or three months. After three months, fermented wine bottles were filtered and pasteurized for 30 minutes at 80ºC. And then the pasteurized wines were tested for sensory evaluation test.

Determination of Sensory Evaluation upon Wines with Different Sugar Contents

For sensory test of wines with different sugar contents, four kinds of wines were prepared. The first type was controlled wine, contains 20% sugar but without yeast. Second wine contains 20% sugar with native yeast with native yeast, and the third wine contains 15% sugar with native yeast. The last one was fermented with 10% sugar and native yeast. The appearance of color, odor, taste and overall acceptance were determined by ten wine drinkers. These wines level (not acceptance, acceptance, good, very good and the best) were assigned as increasing order 1 to 5. Their remarks were carefully collected. The same procedure was done for damson fruit sample without enzyme.

Fermentation of Wine with Different Enzymes

Ripe damsons were chosen to the point of growing mold. They were washed and placed in a 1 L beaker. The ripe damson samples are blended using a food blender for (2-3) minutes until homogenous solution was obtained. Boiling water was added to the juice extract until the total volume reach 1 L and stirred, covered and allowed to settle until a thick mold crust had grown on the top. The flavor of wine will be better if there is a good thick mould. When the mould was thicker enough, the sugar (15%), yeast (mother culture) and pectinase enzyme were added and well stirred until all constituents were dissolved. The beaker was recovered again and left for 24 hours stirred by magnetic stirrer. When the preparation of wine was finished, they were transferred into sterilized bottles. The fermentation bottles were sealed with soft elastic rubber ring when bubbles form; it means that fermentation is going on. During the fermentation, CO_2 gas was evolved from the fermentation bottles and was allowed to release in water. The rate at which gas bubbles through the bung indicated the rate of fermentation. When the specific gravity of fermentation liquid became one, it indicated the end point of fermentation.

Pasteurization and Aging

After three months, fermented wine bottles were filtered by a fabric filter and pasteurized for 30 minutes at 80ºC. And then those bottles were put into other sub-bottles which had been sterilized. These bottles were stored or aged for six months or more after fermentation. During aging, slow oxidation which would alter the characteristics of the wine progressively took place (Ugliano M., 2013). The alcohol and acid present in the wine reacted to form esters that provided the delicate aroma, flavor and bouquet of well-aged wine.

Chemical Profile

Some wine parameters such as pH (Schott Gerate CG 712 pH meter, Germany), alcohol percent (Alcohol meter), sugar content (Refractometer), turbidity (HACH meter), protein content (Kjeldahl's method) (Julius B., 1910), and vitamin-C (spectrophoto meter), were examined.

Microbial Profile

The Nathan's Agar Well Diffusion Technique was used to test the antimicrobial activity of the wine (Holder I.A., 1994). This test was carried out two times, after three months and six months. After respective incubation time for tested micro bacteria, the plates were examined and the diameter of zones of complete inhibition was measured to the nearest whole millimeter with a ruler. The microbial safety for each pasteurized wine samples were carried out by serial dilution method in Biotechnology Lab.

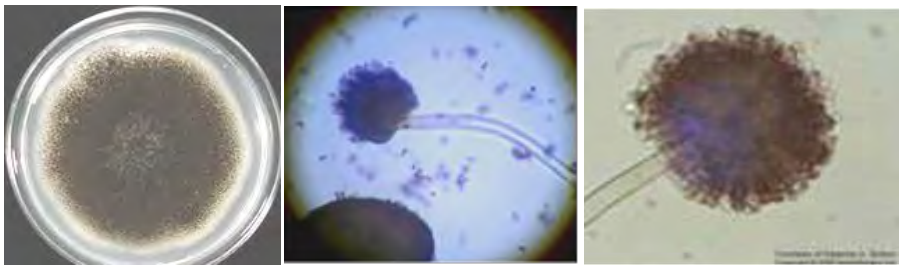
Measurement of the Free Radical-Scavenging Activity in Wine

The free radical scavenging activity was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. 2 µL of tested damson wine was dissolved in 50% ethanol and made the volume 10 cm^3. The solution was then filtered, stock solution was obtained. The desired concentration of test sample (0.625, 1.25, 2.5, 5 and 10 µL/mL) were prepared by serial dilution method by dilution the stock solution with 50% ethanol. Three solutions such as control, blank and sample solutions were prepared. Control solution was prepared by mixing 60 µM DPPH solutions and ethanol. The bland solution was prepared by mixing test solution and ethanol and the sample solutions were prepared by mixing the test solution with 60 µM DPPH solution.

Results

Isolated *Aspergillus niger* Strain

The isolated *A. niger* inoculated on three different media were identified by microscopically characteristics. Culture plates of received *A. niger* were described in Figure (1) and microscopic results of its strains was mentioned in Figure (2). For the comparison of the microscopic feature of isolated *A. niger* and of *A. niger* that found in literature, it was found that the isolated *A. niger* is in pure state. The spore bearing heads are large, tightly packed and globular and black.



(a) (b) (c)

Figure (1,a) Cultural feature of isolated *A. niger*
(1,b) Microscopic feature of isolated *A. niger*
(1,c) Microscpic feature of *A. niger* in Literature

Isolated *A. niger* strain were detected on three different cultural media (SDA, PDA and SA). Cultural media test determined the remarkable features of the strain such as mycelium formation and presence or absence of suitable pigment. Colony morphology of *A. niger* is woody, at first white to yellow, then turning black. From Morphological results, *A. niger*strain on three different media are not quite different however the strain on SDA media is more thicker than others. So SDA media is chosen for the remaining part of experiment.

Table (1) Morphological results of isolated *A. niger*strain on three different media

	SDA	PDA with 7.5 % NaCl	SA
Completion of growth	6 -7 days	6 -7 days	6 -7 days
Color of colonies on plate	White (during 2 days) Pale yellow (2-3 days) Yellow (3-4 days) Brownish black (after 4 days)	White (during 2 days) Pale yellow (2-3 days) Yellow (3-4 days) Carbon black (after 4 days)	White (during 2 days) Milky white (2-3 days) Yellow (3-4 days) black (after 4 days)
Reverse color of plate	Yellow	Cream	Pale black
Reverse Morphology	Produce radial fissures on agar	Produce radial fissures on agar	Produce radial fissures on agar
Color of conidia	Brownish black	Black	Pale Black

Isolated Pectinase Enzyme

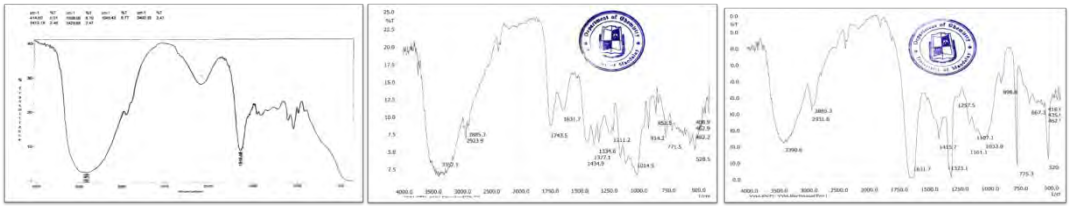
SDA media was used for incubation more *A. niger* strain to isolate pectinase enzyme. After incubation time 72 hours, mycelium was suspending in broth culture and centrifuged and filtered. The clarified enzyme solution was obtained. Acetone was used to precipitate the pectinase enzyme. This enzyme was dried in air.

Interpretation of Three Pectinase Enzymes

The infrared spectra of reference, standard pectinase and isolated pectinase were respectively shown in Figure (2), (3) and (4). The vibrational modes of standard pectinase enzyme and the isolated pectinase enzyme were compared with reference data. The band assignments and interpretation from FT-IR spectra were also shown in Table (2).

Table (2) Infrared spectral data of three enzymes

No.	Standard pectinase	Isolated pectinase	Reference	Vibrational Mode
1	3352.1	3390	3400	O-H stretching vibration
2	2923.9	2931.6	2955	CH stretching vibration
3	1631.7	1631.7	1638	C=O stretching vibration
4	1434.9, 1377.1	- 1415	1466, 1410	CH bending vibration
5	1211.2	1257.5, 1161.1	1232, 1112	C-O, C-O-C stretching vibration



(a) (b) (c)

Figure (2,a) FT-IR spectrum of reference
(2,b) FT-IR spectrum of standard pectinase enzyme
(2,c) FT-IR spectrum of isolated pectinase enzyme

Enzyme Activities

The construction of calibration curve of pectin solution obeyed Beer's Law between 0.1 to 1.5 g/mL. Thus, 0.5 g/mL concentration was chosen for this research work. The nature of the activity vs. pH curve of the enzyme from *A. niger* was found to be bell shape and the optimum pH was found to be 4.8. Enzyme-catalyzed reactions are influenced by temperature. As temperature increases, enzyme becomes more active until finally rapid thermal denaturing takes place. Enzyme also requires optimum temperature for their maximum activity. The optimum temperature for the pectinase enzyme was found to be 50ºC in citrate buffer pH 4.8. The longer the reaction time, the greater the decomposition of pectin substrate and the higher is the enzyme activity. In this research work, it can be seen that the product concentration is increased till 22 hour. It means that the enzyme reaction was carried until the reaction time 22 hour.

Native Yeast

After 10 – 15 days, mould layer had grown on the top of the fermented juice. If the mould layer is absence, the bottle was opened and this smell was tested. The smell is sweet that the fermented juice can be used as native yeast.

Wines with Different Sugar Contents

Four types of wines were prepared with different sugar contents. In these wines, control wine contained 20% sugar but yeast was absence. Others were produced with different sugar contents (20 %, 15 % and 10 %) and yeast was added and stored in cool and dark condition. During this time, external contaminant was prevented by using siphoned method. After three months, sensory tests were done by ten wine drinkers. Pasteurization was done before sensory evaluation test. The purpose is to chosen the best sugar content for wine processing.

Sensory Evaluation Test

Sensory Evaluation Test was determined upon prepared four different types of wine. The best wine was chosen by 5 male and 5 female wine drinkers. Their age is between 25-35 years. In these tests, the wine containing 15 % sugar with native yeast was chosen as the best sample because of its highest overall acceptance.

Table (3) Average sensory evaluation test for wines (female and male)

Products	Appearance (Attractive color)		Odour		Taste		Over all acceptance	
	Female	Male	Female	Male	Female	Male	Female	Male
Control	4.4	4.6	2.8	3.6	3	3	3.8	3
10% sugar	3.6	3.8	4	4	3.8	3.2	4.2	3.4
15% sugar	4	4	4.2	3.8	4.4	3.8	4.6	3.8
20% sugar	3.8	4	3.6	3.2	4	3.4	4.4	3.2

According to sensory evaluation test's results, it was observed that 15% sugar of damson wine with yeast is the best flavor, odour, colour, taste and overall acceptance.

Results from Chemical Analysis of Damson Wine

The parameters (pH, Alcohol content, sugar content, turbidity, protein and vitamin-C) were determined after one month, three months and six months, respectively. And then arsenic and lead were also determined. Experimental results were described in Table (4).

Table (4) Analysis of damson wine after one, two and six months

Parameters	Measuring Values								
	Without enzyme			With isolated pectinase			With standard pectinase		
	One month later	Three month later	Six month later	One month later	Three month later	Six month later	One month later	Three month later	Six month later
pH	3.19	3.08	2.76	3.23	3.17	2.51	3.10	3.03	2.65
Alcohol (%)	Nil	5	13	Nil	8	14	Nil	8	13
Sugar content (%)	17.50	17.5	15.8	16.50	13.5	13.2	13.50	13.5	12.2
Turbidity (FTU)	2322.0	1999.5	1715.0	882.00	793.00	537.00	746.00	625.00	549.00
Protein (%)	0.26	0.18	0.17	0.24	0.19	0.15	0.24	0.19	0.18
Vitamin-C (%)	0.15	0.18	0.21	0.19	0.23	0.30	0.21	0.24	0.30
Toxic metal – As (ppm)	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Toxic metal – Pb (ppm)	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

The pH values of all of wines became more acidic as long as their fermentation time. In wine processing, the longer the fermentation time the lower the sugar content and the higher is the alcohol content. Turbidity of wine with isolated enzyme is the lowest and wine without enzyme is the highest. This fact supported that pectinase enzyme break down the pectin in wine. Vitamin-C content of these three wines is larger with longer fermentation time. In contract, protein of these three wines is lower with longer fermentation time. The contents of toxic metal, arsenic and lead were found to be absence. The data were collected after one

month, three months and six months and there results are reliable because they are coincident with each other.

Antimicrobial Activity of Wine

To detect the antimicrobial activity of wine, Agar Well Diffusion Method was used. Four selected microbe, *E. coli, Shigellaboydii, Salmonella typhi*and *Staphlococcusaureus* were used. These tested wines have high activity on the pathogen, *S. boydii*. And they have moderate activity on *S. typhi* which causes Typhoid. Pectinase wine is in good agreement with the standard pectinase wine. Most of the data of pure wine without enzyme were lower than that of standard pectinase wine. The antimicrobial activity of commercial wine is higher than prepared wines because of the additive such as alcohol and some other ingredients.

Microbial Safety after Pasteurization

Before and after pasteurization, microbial safety test was determined by using serial dilution method. The pasteurization conditions were 30 min and 80°C. Viable cell population was carried out by serial dilution method. It was found that there was no viable cell at pasteurized wine after three months shelf-life.

Antioxidant Activities of Wines

The antioxidant activity of four type of wines such as wine with 1)isolate enzyme, 2)with standard enzyme, 3) without enzyme and 4)commercial wine. Ascorbic acid was used as a standard. DPPH assay is based on the capability of test sample to inhibit stable free radical (DPPH) that was followed by decrease in absorbance at λ_{max} 517 nm. Five concentrations (0.625, 1.25, 2.5, 5 and 10 µL/mL) for each wine were prepared and determined their radical scavenging activity. IC_{50} values of wines were found to be 0.72 µL/mL, 1.06 µL/mL, 1.54 µL/mL and 2.08 µL/mL respectively. IC_{50} value of ascorbic acid was 1.04 µg/mL Wine with isolated enzyme is highest antioxidant activity than others and standard ascorbic acid.

Discussion

Natural resources used in this research give high quality of wine and higher antioxidant activity. Higher antioxidant activity of red wine especially damson wine provides significant protection against the development and progression of many chronic pathological conditions

including cancer, diabetes, cardio-vascular problems and aging. The results of studies expressed a current understanding on the biological effects of red wine and their relevance to human health.

Acknowledgements

Firstly, I want to say many thanks to the responsible persons for your kind permission to present my paper in "The First International Conference on Innovation of Functional Foods in Asia (IFFA)". I would like to express our profound gratitude to DrThida Win (Rector, Mandalay University), Dr Yi YiMyint (Professor and Head, Department of Chemistry, Mandalay University) and Dr. KhaingKhaingKyu (Professor, Department of Chemistry, Mandalay University) for their suggestion and permission.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Determination of nutritional values of supplement foods for baby

Toe ToeKhaing

Abstract

Functional foods are thought to provide benefits beyond basic nutrition and may play a role in reducing or minimizing the risk of certain diseases and other health conditions. Examples of these foods include fruits and vegetables, whole grains, fortified foods and beverages and some dietary supplements. Foods can provide specific health benefits. Foods for health are an important part of an overall healthful lifestyle that includes a balanced diet and physical activity. In this research, supplement food samples, Myanmar made - Baby meal, stage 1 and stage 2 and Malaysia made- Nestle were chosen to analyze. They were purchased from local super market at Mandalay, Myanmar. The fat, carbohydrate, protein, fiber and elemental composition of these supplement foods were determined by some sophisticated methods. The ash and moisture contents were also determined. The crude fat content of samples was examined with soxhlet extraction method. The total carbohydrate content was determined by phenol- sulphuric acid method. The protein content was determined using Kjeldahl method. The ash, moisture and crude fiber were estimated by applying the standard method of analysis. Total sugar contents in the samples were measured by using Somogyi-Nelson method. The elemental compositions of samples were also determined by EDXRF (Energy Dispersive X-ray Fluorescence) spectroscopy. From experimental results, the fat content of samples, stage 1, 2 were found to be 2.5-3%. The protein contents were 9-14%. The carbohydrate content was 75- 80%. From elemental analysis of samples, the amount of iron and zinc were higher than other metals. The results of Baby meal, stage 1 and stage 2 were compared with that of Nestle. The experimental data from this research indicated that supplement food for baby in Myanmar local market contain suitable nutritional composition.

Keywords: Supplement Foods, baby meal, local market, Nutritional values

Introduction

The nutritional value of food refers to its capacity to nourish the body with the substance needed to live and grow. The body relies on food for fuel and to obtain the chemical compounds it needs to function. The seven major types of nutrients are carbohydrates, fats, proteins, water, fiber, vitamins and minerals. The first five nutrients are considered macronutrients, which are the nutrients the body requires in relatively large quantities. The last two nutrients vitamins and minerals are considered micronutrients, which the body only needs in relatively small amounts. The body primarily uses carbohydrates and fats as fuel to supply the body with the energy, or calories, it needs for activity. The so called "good fats" also play a role in promoting healthy cholesterol level; they assist in the regulation of some hormones. Fat actually comprises 60 percent of your brain's tissue. Proteins are important because they are the body's only source of essential amino acids. Amino acids are the "building blocks of life." They are vital to nearly every function of the body. Fiber is a significant part of the diet because it helps to facilitate digestion and works to maintain good cholesterol. Water is fundamental to all life; the human body is 60 percent to 70 percent water. The chemical compounds of vitamins and minerals contribute greatly to the formation, growth, regulation, protection, repair and conversion activities the body must carry out to maintain health. The foods with the greatest nutrient density offer the greatest nutritional value. Foods that are relatively low in calories but rich in any combination of micronutrients, fiber, essential amino acids and polyunsaturated fats are considered nutrient dense and of great nutritional value. [12]

Food, whether for human or animal consumption, are supposed to provide nutrients for maintenance of health; however, situations arise where the food may be source of the problem. Food poisoning is caused by the consumption of food or water contaminated with bacteria or their toxins, or with parasites, viruses or chemicals. The symptoms of food poisoning vary in degree and combination. They may include the following; abdominal pain, vomiting, diarrhea, headache, fever, stool changes. More serious cases of food poisoning can result in life threatening neurologic, hepatic and renal syndromes leading to permanent disability or death. The common environmental contaminants of greatest concern in food are the so-called 'heavy metals', most notably (cadmium, lead and mercury). Contamination of rice, soya bean and seafood with cadmium from local industrial and mining operations has caused cadmium poisoning. Lead occurs widely in the environment and it can enter our bodies through drinking water (either from source or via lead piping) and the air we breathe, as well as through food.

Children are the group at greatest risk, because even at levels below those that produce the usual signs of poisoning, lead can cause behavioral abnormalities. Almost all of the mercury found in food occurs in seafood. Fish and shellfish that were heavily contaminated by industrial waste caused poisoning in many of the people who ate them, resulting in damage to the central nervous system and in some instances death.[11]

In Myanmar, there are no many brand of food supplement for baby. Some people in Myanmar consume foreign made food supplement such as Dumex, Nestle for their babies. Since they are expensive, Myanmar people who have low income can't use foreign made. Baby meal stage 1 and stage 2 are made in Myanmar and they have suitable costs. Many people consume local made. Therefore, Myanmar made samples were selected to determine the nutrient compositions and Malaysia made sample was selected to compare the results.

Materials and Methods: Sampling

Supplement food samples, Myanmar made - Baby meal, stage 1 and stage 2 and Malasia made- Nestle were chosen to analyze. They were purchased from local super market at Mandalay, Myanmar.

Moisture Contents

1g of sample was accurately weighed and then dried in an oven for about 2 hr at 101C. It was then removed from the oven and cooled in a desiccator at room temperature and weight. The procedure was repeated until the constant weight was obtained[4][6]

Ash Contents

The sample 1g was weight and placed in a preheated, cooled and weighed the crucible. The crucible was heated carefully in the furnace at 550°C for 2 hours burned off without flaming or until all the carbon was eliminated. When the materials are converted to white ash powder, the crucible was cooled at room temperature in a desiccator and weighed again. To obtain a constant weight, the heating, cooling and weighing were repeated.[1][2][3][5]

Mineral Contents

Mineral contents were measured by applying EDXRF (Energy Dispersive X-Ray Fluorescence) spectroscopy.

Fat Contents

10g of sample accurately weighed was introduced into a thimble and a piece of cotton wool was placed the open end of the thimble. The thimble containing sample was then placed in a soxhlet apparatus. Then the apparatus was fixed with 500ml round- bottomed flask containing 350ml petroleum ether (b.p 40-60C). The extraction flask was heated on the water bath for 8 hours at the boiling point of petroleum ether. After the extraction was completed, most of the ether extract was distilled off. The content in the flask were carefully transferred to a weighed specimen tube. The remaining ether in the specimen tube was vapourized until constant weight was obtained.[8]

Protein Content

(a)Digestion

About 5g of sample was weighed and placed in the Kjeldahl's digesting flask. About 5g of annular sodium sulphate, 0.25 g of anhydrous copper II sulphate and 12.5 ml of 98% sulphuric acid were added into it in such a way as to wash down any solid adhering to the neck. The flask was shaken until the contents were thoroughly mixed and it was heated till the mixture became colourless. The digestion was continued for half an hour to make sure that all the nitrogen in the sample was converted to ammonium sulphate. It was allowed to cool and 5ml of distilled water was carefully added with frequent shaking.[7]

(b) Distillation

The Kjeldahl'sdistillating apparatus was set up, taking care that the tip of the condenser extended below the surface of the standard sulphuric acid solution (50ml) in the receiver. The digested solution was poured into the flask together with 100ml of 40% sodium hydroxide to make mixture strongly alkaline. The evolved ammonia was distilled off.

(c) Titration

The distillate was titrated with standard sodium hydroxide solution using methyl orange as an indicator. A blank determination was carried out without sample using all the reagents as in the case of sample. The nitrogen content of sample can be calculated by using following formula.

$$\%\text{Nitrogen} = \frac{(V_2-V_1)xN_Ax0.01401x100}{W}$$

Where, V_2 = the volume of acid used in the test (in millimeter)

V_1 = the volume of acid used in the blank (in millimeter)

N_A = the concentration of acid used (in Normality)

W = the weight of sample (in gram)

Protein (%) = Nitrogen content × 6.25

Where, 6.25 = a factor of protein - nitrogen conversion

Crude Fibre Contents

About 2g of the sample was placed into a 500ml flask and then 200ml of 1.25% sulphuric acid solution was added. The flask was connected with reflux condenser and digested for about 30 minutes. The flask was rotated every few minutes in order to mix the contents and to remove particles from the side of flask. After 30 minutes the boiling solution with insoluble materials was filtered. The insoluble residue was washed with the hot water in order to free from acid. Then the residue was washed down into the flask with 200ml of 1.25% sodium hydroxide solution and boiled for 30 minutes. After boiling, the residue was filtered again and washed with 15ml of 95% ethanol. After washing the residue was introduced into a crucible and it was heated in an oven at 100° C until the constant weighed was obtained. Finally, the substance in the crucible was incinerated in a muffle furnace dull red until the all carbonaceous matter had been removed. The contents with the crucible were cooled and weighed.

This procedure, such as heating, cooling and weighing were made until a constant weight was obtained. The loss in weight during the incineration was referred to as crude fiber.[9]

Water-Soluble Carbohydrate

The water soluble carbohydrate content was also determined by phenol-sulphuric acid colourimetic method in terms of glucose.[4]

Preparation of Sample Solution

0.1g of sample powder was dissolved in 100ml of hot water and shaken for ten minutes. 1ml of this solution was then dilute to 10ml with water and this solution was taken as the sample extract.

Preparation of Standard Sugar Solution

100mg (0.1g) of hydrated glucose was exactly weighed and dissolved in 100ml of distilled water. 1,2,4,6,8 and 10ml of these solution were drawn out and put in each 100ml volumetric flask and diluted to the mark with distilled water. These solutions contained 10, 20, 40, 60, 80 and 100µg of glucose per ml respectively.

Procedure

1ml of sample solution and six standard sugar solutions containing 10, 20, 40, 60, 80 and 100 µg of glucose per ml were put in each test tube. 1ml of 5% phenol solution was also added to each test tube and mixed. A blank also prepared with 1ml of distilled water instead of sugar solution. 5ml of 96% sulphuric acid, was again added to each tube so that the stream hit the liquid surface directly to produce good mixing. Each test tube was agitated during the addition of acid. After ten minutes, the tube were reshaken and placed in water bath at 25- 30 C for twenty minutes. The yellow orange colour was stable for several hours. Absorbance were measured at 490 nm using UV- visible spectrophotometer.

A standard curve was plotted by the absorbance of the standard solution against the concentration in ug per ml. [Using this standard curve, the concentration of glucose in the sample was calculated.

Sugar Contents in Sample

Determination of Concentration of Iodine Solution

Procedure

10 mL of glucose solution was taken in conical flask and 20 mL of 0.05 M iodine solution and 45 ml of 0.1 M sodium hydroxide solution were added into the flask. The flask was closed and left in the dark place for 15 minutes. Then, 6 mL of 1 M hydrochloric acid was added and titrated with 0.05 M sodium thiosulphate solution. When the liquid became straw color, 1 mL of starch solution was added. The liquid became dark blue color again and titrated until the

colorless content was obtained. From the experimental data, the concentration of iodine solution can be calculated.[11]

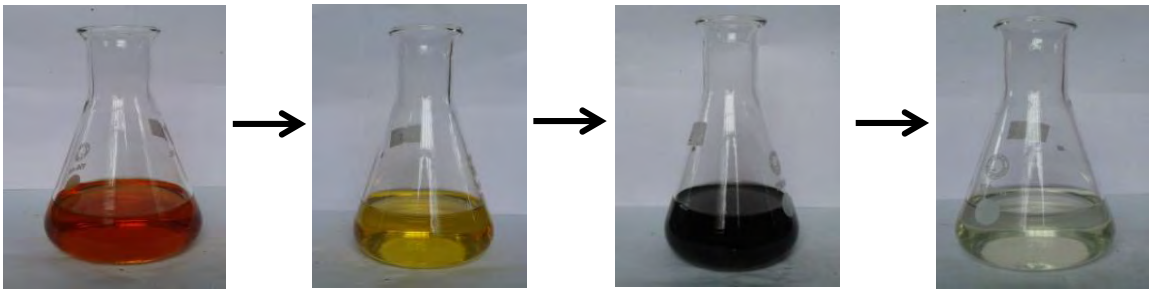

Figure (1) Iodometric Titration

Determination of Sugar Content in Samples

Procedure

10 mL of sample solution was taken in conical flask and 20 mL of 0.05 M iodine solution and 45 mL of 0.1 M sodium hydroxide solution were added into the flask. The flask was closed and left in the dark place for 15 minutes. Then, 6 mL of 1 M hydrochloric acid was added and titrated with 0.05 M sodium thiosulphate solution. When the liquid became straw color, 1 mL of starch solution was added. The liquid became dark blue color again and titrated until the colorless content was obtained. From the experimental data, sugar content in sample can be calculated.

Results

The results of nutritional compositions of supplement food samples were shown in Table 1, 2, 3, 4, 5 and 6.

Table 1 Results of moisture and ash content of samples

No.	Sample	Moisture Content (%)	Ash Content (%)
1	Baby meal, stage 1	1.12	2.25
2	Baby meal, stage 2	1.22	2.45
3	Malaysia made- Nestle	1.02	2.85

Table 2 Results of carbohydrate content of samples

No.	Sample	Carbohydrate content (%)
1	Baby meal, stage 1	80.02
2	Baby meal, stage 2	75.20
3	Malaysia made- Nestle	64.50

Table 3 Results of protein content of samples

No.	Sample	Protein content (%
1	Baby meal, stage 1	8.72
2	Baby meal, stage 2	14.10
3	Malasia made- Nestle	14.55

Table 4 Results of fat content of samples

No.	Sample	Fat content (%)
1	Baby meal, stage 1	2.45
2	Baby meal, stage 2	2.45
3	Malasia made- Nestle	8.75

Table 5 Results of crude fiber content of samples

No.	Sample	Crude fiber content (%)
1	Baby meal, stage 1	1.05
2	Baby meal, stage 2	1.25
3	Malasia made- Nestle	1.01

Table 6 Results of sugar content of samples

No.	Sample	sugar content (%)
1	Baby meal, stage 1	0.03
2	Baby meal, stage 2	0.04
3	Malasia made- Nestle	0.02

Moisture content in baby meal stage 1 and stage 2 were found to be 1.12% and 1.22%. Nestle was 1.02%. Baby meal stage 1 and stage 2 contain 2.25% and 2.45% of ash and Nestle contains 2.85% of ash. Baby meal stage 1 and stage 2 have higher content in carbohydrate and lower content in protein than Nestle. Nestle contains higher amount of fat content. From elemental analysis of samples, the amount of iron and zinc were higher than other metals.

Discussion

In this paper, supplement food samples, Myanmar made - Baby meal, stage 1 and stage 2 and Malasia made- Nestle were chosen to analyze. They were purchased from local super market at Mandalay, Myanmar.

The nutritional composition of the samples were determined. As described in experimental data, Baby meal stage 1 and stage 2 have higher content in carbohydrate and lower content in protein than Nestle. Nestle contains higher amount of fat content than Baby meal stage 1 and stage 2. From elemental analysis, the amount of iron and zinc were higher than other metals in all samples.

It can be concluded that Myanmar made - Baby meal, stage 1 and stage 2 contain valuable nutrients for baby. Therefore, Myanmar made samples should be comsumed for the nutrients.

Acknowledgements

I would like to show my sincere thanks to all of you for the permission to present my research paper. I also would like to express my deepest gratitude to Dr.Thida Win, Rector, Dr. Tin TunAung, pro-rector, University of Mandalay for their interest and encouragement on my research paper. I also wish to express many thanks to Dr. Yi YiMyint, Professor and Head, and

Dr. KhaingKhaingKyu, Professor, Department of Chemistry, University of Mandalay for their kind help and invaluable guidance for this research work.

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Innovation of Functional Foods in Asia (IFFA)
Functional Foods: Trends in Research and Markets *January 22nd-24th, 2018*
University of Phayao, Phayao Province, Thailand
Society for Free Radical Research Thailand

Study on some selected Myanmar functional foods

Thida Tin, Yi YiMyint

Abstract

In this research work, I have selected three different kinds of functional foods- premier milk powder, strawberry margarine and myintmyintkhin biscuit which are produced in Myanmar. They were purchased from ocean super market, Chanmyathazi Township, Mandalay region, Myanmar. The aim of this study is to analyze the approximate mineral composition, nutritional values of selected functional foods. Firstly, the moisture and ash contents in three selected samples were determined. Secondly, the mineral contents in these three samples were determined by using EDXRF method. Then, the fat contents in samples were determined by Soxhlet extraction method and protein contents in samples were determined by using Kjeldahl's Analyzer. Finally, carbohydrate contents in samples were determined by phenol-sulphuric acid method.

Keywords: Myanmar Functional Foods, mineral composition, EDXRF, Kjeldahl's, phenol-sulphuric acid

Introduction

The primary role of diet is to provide sufficient nutrients to meet the nutritional requirements of an individual. There is now increasing scientific evidence to support the hypothesis that some food and food components have beneficial physiological and psychological effects over above the provision of the basic nutrients. Today, nutrition science has moved on the classical concepts of avoiding nutrient deficiencies and basic nutritional adequacy to the concept of positive or optimal nutrition. Functional foods can be considered to be fortified, enriched or enhanced foods that provide health benefits beyond the provision of essential nutrients such as vitamins and minerals. The research focus has shifted more the identification of biologically active components in foods that have the potential to optimize physical and mental well-being and which may also reduce the risk of diseases. Many traditional food products including fruits, vegetables, soya, whole grains and milk have been found to contain components with potential health benefits. In addition to these foods, new

foods are being developed to enhance or incorporate these beneficial components for their health benefits or desirable physiological effects. The ingredients of premier milk powder are sodium and calcium caseinates, sucrose, sunflower oil, soy oil, milk, minerals and vitamins. Margarine is produced by combining several different types of vegetables oils. Two of the most common oils used for margarine are corn and soybean oil. And then animals fat, skim milk and salt emulsifiers. The main ingredients of myint myint khin biscuit which is used in the present study is wheat flour. Other ingredients of the considered biscuit are sugar, butter, milk powder, egg and vanillin powder for flavor.

Nutritional Components in Myanmar Traditional Food

Protein

Proteins are the fundamental building blocks of muscles, skin, hair and cellular components. Proteins are needed to help muscles contract and relax, and help repair damaged tissues. They play a critical role in many body functions as enzymes, hormones and antibodies. Protein may also be used as an energy source by the body. Milk protein consists of approximately 82% casein and 18% whey protein. Both casein and whey proteins are present in milk in milk, yogurt and ice cream. In most cheeses the casein is coagulated to form the curd, and the whey is drained leaving only a small amount of whey proteins in the cheese.

Carbohydrate

Carbohydrates are the primary source of energy for activity. Glucose is the only form of energy that can be used by the brain. Excess glucose is stored in the form of glycogen in the muscles and liver for later use. Carbohydrates are important in hormonal regulation in the body. Lack of adequate levels of glucose in the blood and carbohydrate stores leads to muscle fatigue and lack of concentration.

Fat

Fats are structural components of cell membranes and hormones. Fats are concentrated energy source and the main energy source used by the body during low intensity activities and prolonged exercise over 90 minutes. Fat is the main storage form of excess energy in the body. Fats cushion organs during movement.

Vitamins

Vitamins have many roles in the body including metabolism co-factors, oxygen transport and antioxidants. They have the body use carbohydrates, proteins and fat.

Minerals

Minerals have many roles in the body including enzyme functions, bone formation, water balance maintenance, and oxygen transport. They help body use carbohydrates, protein and fat. The content of calcium in milk is shown in the Mineral content table. Calcium plays an essential role in bone formation and metabolism, muscle contraction, nerve transmission and blood clotting. Dairy products are significant source of calcium in the diet.

Energy

Food provides energy to the body in the form of calories. There are many components in food that provide nutritional benefits, but only the macronutrients protein, carbohydrate and fat provide energy.

Materials and Methods

Sampling

The samples of three different kinds of functional foods such as premier milk powder, strawberry margarine and myintmyintkhin biscuit were chosen for analyzed which are produced in Myanmar. They were purchased from ocean super market, Chanmyathazi Township, Mandalay region, Myanmar. They were chopped into small pieces. These pieces were ground to the powder and then store in a well- stopped bottle.

Determination of nutritive value in functional foods

The nutritional values such as total fat in each selected sample were determined by the soxhlet extraction method, protein content by kjeldahl digestion method. Ash content of each sample was determined by using muffle furnance at 550˚c for 6 hours. The moisture content was determined by oven drying method at 105˚C.The crude fiber was determined by treating with acids and alkali. The mineral contents of samples were also examined by Energy Dispersive X-Ray Fluorescence (EDXRF) analysis.

Results

The ash, moisture and total fat contents of samples were measured by using muffle furnance, oven drying method and soxhlet apparatus and these data were shown in table 1.

Table 1 . Nutritive values of three selected samples

Samples	Properties		
	Ash (%)	Moisture (%)	Fat (%)
premier milk powder	5.70	2.50	13.90
strawberry margarine	0.60	3.50	17.50
myintmyintkhin biscuit	2.27	2.50	10.96

The ash contents of three selected samples were found to be 5.7% premier milk powder, 0.6% strawberry margarine and 2.27%myintmyintkhin biscuit. The moisture contents of these samples were found to be 2.5%, 3.5% and 2.5%. The fat contents of three samples were found to be 13.9%, 17.50% and 10.96%.

Table 2. Mineral content of premier milk powder

No	Symbol	Element	Amount of Concentration (%)
1	Ca	Calcium	61.158
2	K	Potassium	32.114
3	Fe	Iron	3.987
4	Zn	Zinc	2.062
5	Cu	Copper	0.679

The mineral content ofpremier milk powder contains the highest value of calcium.

The mineral content of strawberry margarine and myintmyintkhin biscuit were determined and the results were shown in table 3 and table 4.

Table 3. Mineral content of strawberry margarine

No	Symbol	Element	Amount of Concentration (%)
1	S	Sulphur	79.978
2	Ca	Calcium	69.176
3	Fe	Iron	23.058
4	Cu	Copper	9.795

The mineral content ofstrawberry margarine contained the highest value of calcium.

Table 4. Mineral content of myintmyintkhin biscuit

No	Symbol	Element	Amount of Concentration (%)
1	Ca	Calcium	55.963
2	K	Potassium	31.954
3	Fe	Iron	12.083

The mineral content ofmyintmyintkhin biscuit contained the highest value of calcium.

The protein contents of samples were measured by using Kjeldahl's method. The resulting data were shown in table 5.

Table 5. Protein Contents of Three Selected Samples

Samples	Nitrogen (%)	Protein (%)
premier milk powder	2.08	12.90
strawberry margarine	0.11	0.70
myintmyintkhin biscuit	1.48	6.24

The protein contents of premier milk powder sample was found to be 12.90%, strawberry margarine sample contain 0.70% and myintmyintkhin biscuit was found to be 6.24%.Therefore the amount of protein for premier milk powder greater than that of two other.

Table 6. Crude Fiber and Carbohydrate Contents of Three Selected Samples

Samples	Crude fiber(%)	Carbohydrade(%)
premier milk powder	-	47.40
strawberry margarine	1.06	1.40
myintmyintkhin biscuit	1.26	57.77

According to this table, the crude fiber content of strawberry margarine and myintmyintkhin biscuit were contained 1.06% and 1.26%, premier milk powder sample was no crude fiber content. The carbohydrate content of three selected samples were present in 47.40% of premier milk powder, 1.40% of strawberry margarine and 57.77% of myintmyintkhin biscuit. The present of increase in amount of carbohydrate formyintmyintkhin biscuit was found to be greater than that of two others.

Discussion

The samples of three different kinds of functional foods were purchased from ocean super market, Chanmyathazi Township, Mandalay region, Myanmar. The physico chemical parameter and some nutritional values of these samples were determined. The ash contents of three selected samples were found to be 5.7% premier milk powder, 0.6% strawberry margarine and 2.27%myintmyintkhin biscuit. The moisture contents of these samples were found to be 2.5%, 3.5% and 2.5%. The fat contents of three samples were found to be 13.9%, 17.50% and 10.96%. According to the results of table 2,3 and 4, the mineral contents of calcium, potassium, iron, zinc, copper and sulphur were observed in the sample. The higher values of calcium in premier milk powder and myint myint khin biscuit could be observed. They were important factor in maintaining physiological process. In addition, the amount of protein in premier milk powder sample was found to be 12.90%, strawberry margarine sample contain 0.70% and myint myint khin biscuit was found to be 6.24%. The crude fiber content of strawberry margarine and myint myint khin biscuit were contained 1.06% and 1.26%, premier milk

powder sample was no crude fiber content. The carbohydrate content of three selected samples were present in 47.40% of premier milk powder, 1.40% of strawberry margarine and 57.77% of myint myint khin biscuit. The experimental research shows that these three selected samples provide our daily nutrients needed for human beings, especially for child life and gieatrics as a food supplement. These practical figures inform that these food supplements have a suitable amount of protein contents and fat requirements. Our body needs different kinds of mineral contents according to our body requirements and age conditions. From my current research work, I can examine our routine that have suitable amount of protein, carbohydrate, fiber, sugar, fat. This information can provide human health and can inform future researchers who may continue similar determination concerned with functional foods and our staple foods. These practical figures inform that these food supplements, the premier milk powder and the biscuit are suitable for babies, but magarine has not a suitable amount of protein contents and fat requirements that indicate it is unfit for unhealthy person.

Acknowledgements

I would like to express our profound gratitude to Dr Thida Win (Rector, University of Mandalay), Dr Tin TunAung (Pro-rector, University of Mandalay), Dr MyintZu Min (Pro-rector, University of Mandalay), Dr Yi YiMyint (Professor and Head, Department of Chemistry, University of Mandalay) and Dr Khaing Khaing Kyu (Professor, Department of Chemistry, University of Mandalay) for their suggestion and permission.We also convey special gratitude to chairperson and committee members of IFFA 2018, Thailand.

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