Chiang Mai J. Sci. 2011; 38(3) : 430-438
http://it.science.cmu.ac.th/ejournal/
Contributed Paper

Rapid Screening of Antioxidant Compounds in Homemade Fruit Fermented Juice Using an On Line LC-ESI-MS/MS and DPPH Assay

Nitra Nuengchamnong*, Saowanit Boonpathanasak, and Plubplueng Tepwitukkij
Regional Medical Sciences Center 9, Department of Medical Sciences, Ministry of Public Health, Mueang, Phitsanulok, 65000, Thailand.
*Author for correspondence; e-mail: nitra_nuengchamnong@yahoo.com

Received: 27 October 2010
Accepted: 29 November 2010

ABSTRACT

Fruit fermented juice used for health promotion was investigated for antioxidant compound using an on line LC-ESI-MS/MS coupled to DPPH assay. The structural elucidation of the active compounds was achieved by negative ionization LC-ESI-MS/MS. Based on their mass spectra and fragmentation patterns related to antioxidant activity trace, nine compounds showing strong DPPH scavenging were identified as; HHDP-glucoside, Mucic acid, Monogalloyl glucoside, Monogalloyl diglucoside, Gallic acid, Galloyl quinic acid, Digalloyl glucoside, Gallic derivative, Ellagic rhamnoside . In addition, the confirmation of the active compounds was performed using LC-MS/MS in multiple reaction monitoring (MRM) mode. Hydrolysable tannins were found as a major component responsible for the antioxidant activity of this fruit juice.

Keywords: fruit fermented juice, hydrolysable tannin, LC-MS/MS, DPPH assay.

1. INTRODUCTION

Nowadays people concerned about their health. They need food as functional foods for health promotion and disease preventions. Fruits and their products such as wine and fermented fruit as juice are the popular form of food supplement. The process of fermenting is basically feeding sugars and nutrients in solution to yeast, which return the favor by producing carbon dioxide gas and alcohol. Raw fruit juice naturally contains many yeasts, moulds, and bacteria, derived from the surface of the fruit. Substances in fermented foods have been found to have a protective effect against the development of cancer [1]. Epidemiological evidence has been provided showing that constituents in fruits are beneficial to human health and contribute to the prevention of degenerative processes caused by oxidative stress [2,3]. Dietary intake of plant phenolics are inversely related to coronary heart disease [4] and act as anti-ulcer, antispasmodic, antisecretory, or antidiarrhoeal agents in the gastrointestinal tract [5]. Consumers need to be made aware of the numerous benefits of fermented foods, especially those traditionally produced at the home scale.

The popularity of Maha Bambad (Bio-fermented tonic) which is promoted via Pa

Cheng's cable TV channel "Super Cheng", the juice, claimed as could cure many illnesses ranging from skin diseases like psoriasis to joint pains and pain caused by cervical cancer. Meanwhile, Thai Public Health Minister Jurin Laksanawisit told a press conference on 28 January 2010 that Auntie Cheng's Nam Maha Bambad was highly acidic, had no medical value and was contaminated with *Clostridium perfringens* bacteria that can cause nausea, vomiting, diarrhea as well as gangrene. The food and drug administration (FDA) also charges her for exaggerating the properties of the liquid. The doubtful of healing power of her fermented fruit was challenged the expert. The word no medical value is used for the adulterant of modern medicine. They did not mention about the phytochemical compounds. As we know that fruits and their products contained many natural compounds that benefit for health.

That doubtful comes to the purpose of the present study. The aim of this study is to characterize the antioxidant compounds from fruit fermented juice as formula from the cable TV using an on-line LC-ESI-MS/MS coupled to DPPH assay.

2. MATERIALS AND METHODS

2.1 Materials

Gallic acid was obtained from Sigma (St. Louis, MO, USA). Methanol (LC/MS reagent) was purchased from JT Baker (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA) Formic acid (analytical grade) was purchased for Merck (Darmstadt, Germany). Water was purified using Elga USF system (Bucks, England).

2.2 Fruit Fermented Juice Sample

Fruit fermented juice sample made from the fruit, of *Morinda citrfolia* Linn, *Phyllanthus emblica* Linn. *Averrhoa carambola* L. *Sandoricum koetjape* (Burm. f.) Merr., Rhizome of *Zingiber cassumunar* Roxb. *Zingiber officinale* Roscoe and stem of *Tinospora crispa* (L.) Miers ex Hook.f. & Thoms. The ratio of the fermented formula was sugar: fruit: water 1:3:5 w/w/w and fermented for at least 4 month before use. The sample was collected from the home of Mr. Suthep Srikreuw, Wangpikul sub district,Wangthong, Phitsanulok, Thailand. The formula was from the cable TV.

2.3 Sample Preparation

The fruit fermented sample was filtered through a 0.2 μm Nylon syringe filter (Chrom Tech, Inc.MN, USA) and then direct injected into the on-line antioxidant system and the filtered samples were used for the determination of drug adulterant by LC-MS/MS. The liquid samples also used directly for testing the acidity and food poisoning bacteria.

2.4 Fruit acidity, Drug adulterant and Food Poisonous Bacteria

The sample was tested for the acidity, (pH meter, Mettler Toledo Multiseven, Switzerland), drug adulterant as medical value [6] and food poisoning bacteria; *Bacillus cereus, Staphylococcus aureus, Clostridium perfringens* and Salmonella [7].

2.5 LC-ESI-MS and Radical Scavenging Detection Analysis

The HPLC was coupled on-line to MS and a continuous flow DPPH assay as mentioned before where the effluent of the analytical column was splitted to the MS/MS and the reactor cell for the DPPH reaction[8]. The delay time between MS trace and visible trace was ca 0.6 min. The Agilent 1,100 series HPLC system (Agilent Technologies, Palo Alto, CA) was coupled to a PE SCIEX API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem, Foster city, CA) equipped with an electrospray ionization interface. The

chromatographic separation was achieved with a phenomenex Gemini column (5 µm, 250 × 4.6 mm i.d.) (Phenomenex, Torrance, CA) protected with an ODS C18 guard column, operated at 25°C. The mobile phase consisted of solvent A (1 ml formic acid in 1 L of deionized water) and solvent B (methanol). The elution program started from 90:10 solvent A:solvent B for 4 min, then changed to 80:20 solvent A:solvent B in 6 min, linearly increased to 10:90 solvent A:solvent B in 30 min. Then, the ratio of solvent A:solvent B was constant at 10:90 for 5 min, then changed to 90:10 solvent A:solvent B in 5 min and kept constant at 90:10 solvent A:solvent B for 5 min for reconditioning of the column.

Mass spectra were recorded within 55 min. The injection volume was 5 µl. The flow rate was set to 600 µl/min. The Analyst 1.3.2 software was used for data acquisition and processing. The full scan mass spectra from m/z 100-1,000 amu were acquired in negative ion modes. The optimum conditions of the interface were as follows: ESI-negative; ion spray voltage of - 4500 V, curtain gas (N_2) of 69 Kpa (10 psi), ion source gas 1 (air, for nebulizing) of 450 Kpa (65 psi), ion source gas 2 (air, for drying solvent) of 380 Kpa (55 psi). The interface temperature was set to 400°C. The entrance potential (EP) and declustering potential (DP) were -10V and -80V, respectively. The continuous flow system for antioxidant activity detection, consisted of an HPLC pump, LC20AD prominence (Shimadzu, Kyoto, Japan), home-made knitted reaction coil PEEK tubing with an inner diameter of 180 µm and a total reaction coil volume of 100 µL. The flow of 0.1mM DPPH was set to 200 µl/min and induced bleaching was detected as a negative peak at 515 nm using the UV-VIS detector (SPD 20AV, Shimadzu, Kyoto, Japan). The LC solution software was used for data acquisition and processing. The polarity of the signal output was reversed in order to obtain positive signals. The system was operated at 25°C. For the characterization of antioxidant peaks, the fragment ions from their corresponding parent ions in negative mode were induced with collision gas (CAD) of 41 Kpa (6 psi), collision energy (CE) between-5 to-50V and collision cell exit potential (CXP) of -6 V, DP in the range of -20 to -110 V.

2.6 Peak Identification

Peak identification was performed by comparison of the retention time and mass spectrum with reference compounds. Tentative identification of peaks of which standard compounds were not available was obtained by comparing their elution order and their molecular ion with the data from the literature and confirmation by multiple reaction monitoring (MRM).

3. Results and Discussion

The fruit fermented juice used as food supplement for anti-diabetic anticancer and health promotion of people in Wangthong district, Phitsanulok. This sample showed high acidity with pH 2.72. The food poisoning bacteria; *B.cereus*/1ml, *S.aureus*/0.1ml, *C. perfingens*/1ml and Salmonella/25ml did not found might be the high acidity of the juice. There are no adulterant of drug as medical value in the group of Tranquilizer (alprazolam, diazepam, nitrazepam, triazolam), Steroids (dexamethasone, prednisone, prednisolone), Antihypertensive (nifedepine), Anti-diabetic (gliclazide, glimepiride, glipizide, pioglitazone), and Antihistamines (epidine, phenylpropanolamine, psuedoepedine).

An HPLC was coupled to a triple quadrupole mass spectrometer and a DPPH assay for the rapid identification of antioxidant compound of the fruit fermented juice sample as shown in the Figure 1. DPPH assay is

based on the reduction of the stable radical, DPPH, to the formation a non radical form in the presence of hydrogen donating antioxidant. The fruit fermented juice showed an antioxidant activity by reducing DPPH to the yellow coloured diphenylpicrylhydrazine derivatives. The remaining of DPPH was measured at wavelength 515 nm. In the on-line DPPH assay monitors the activity of the antioxidant compounds that separated from the gradient reversed phase HPLC system. The mass spectrometry was used to elucidate the structures based on their molecular weights and fragmentation patterns of the active compounds.

There were tentative nine active anti-

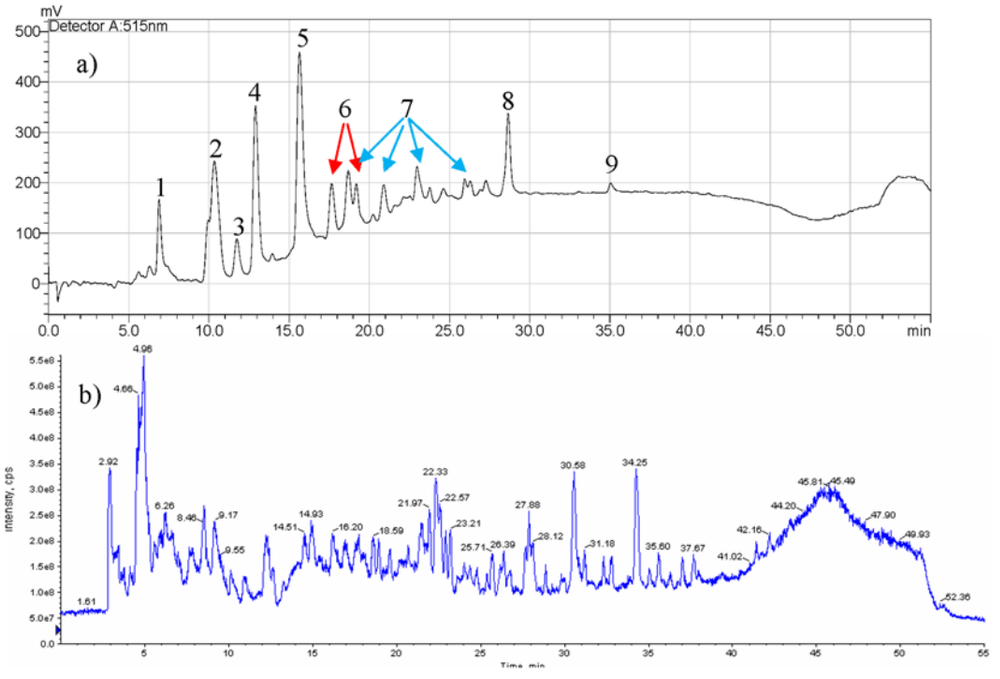

Figure 1. HPLC separation of fruit fermented compounds with simultaneous antioxidant activity assay and MS detection. a) The chromatogram from the antioxidant activity assay detection at 515 nm. b) The total ion current (TIC) output from the ESI-MS in negative mode. For peak assignments, see Table 1.

oxidant compounds in fruit fermented juice (Figure 1a). The antioxidant compounds were identified as; Peak 1 (t_R= 6.9 min) showed peak at m/z 481.4[M-H]$^-$, and the MS/MS analysis showed that fragmentation of m/z 481.4 yield ellagic acid (m/z 300.9) by comparison of the data with reported in the literature [9], it is suggested that this compound was hexahydroxyphenyl (HHDP) glucoside. Peak 2 (t_R= 10.3 min) with [M-H]- at m/z 209.1, ([mucic acid-H]$^-$) and 191.0 ([mucic acid –H_2O]$^-$) that are proposed as mucic acid. This compound was found in the fruit juice of Emblica (*Phyllanthus emblica* Linn) [10]. Peak 3 (t_R= 11.7 min) with a [M-H]$^-$ at m/z 493.0 and fragment ion at m/z 330.0, and 313.0. It was proposed as monogalloyl diglucoside. Peak 4 (t_R= 12.9

min) with a $[M-H]^-$ at m/z 331.0 and fragmentation gave ion at m/z 270.7 and 211.0, it was identified as monogalloyl glucoside [11]. The ion m/z 270.7 and 211.0 formed during the fragmentation of mono galloyl glucose. Peak 5 (t_R= 15.6 min) with a $[M-H]^-$ at m/z 169.2 and a fragment ion at m/z 125.0 $[M-H-CO_2]^-$, this compound was identification as gallic acid which the retention time and its mass data fit with authenticated compound. Peak 6 (t_R= 17.6, 19.1 min) was tentatively identified as galloylquinic acid since it had a $[M–H]^-$ at m/z 343.1 and fragmentation gave ions at m/z 190.8 and 168.9, corresponding to quinic acid and gallic acid moieties [12,13]. Peak 7 (t_R=18.6, 20.5, and 23.8 min) with $[M-H]^-$ at m/z 483.0 and fragmentation gave ion at m/z 331.3 (monogalloyl glucose), 312.9 (dehydrated monogalloyl glucose), 271.0 digalloyl quinic acid. Elimination of a galloyl ester group (m/z 331), a subsequent loss of water (m/z 313) and the occurrence of gallic acid (m/z 169) are also observed for the digalloyl-glucose anion (m/z 483) [14]. Peak 8 (t_R= 26.3, 28.7 min) with $[M-H]^-$ at m/z 241.3 and fragmentation gave ion at m/z 169.0 [gallic acid-H]$^-$ 125.2 [gallic acid-H-CO_2]$^-$, and 197.2 indicating that they were gallic acid derivatives. These two compounds need more data to identify. Peak 9 (t_R= 35.0 min) with $[M-H]^-$ at m/z 447.3 and fragmentation gave ion at m/z 301.0 as ellagic rhamnoside. The results were shown in Table 1 and confirmation of the identification using daughter ion spectra in MRM mode were show in Figure 2.

The component responsible for the antioxidant activity of this fruit juice was hydrolysable tannins. There are reported about the tannins in good and bad way. The intake of a small quantity of some tannin promotes beneficial health effects. They are beneficial to health due to their chemo-

Table 1. Identification of antioxidant compounds in fruit fermented juice using an LC-ESI-MS-DPPH assay data in negative ionization, t_R is the retention time of the peaks from the antioxidant activity trace.

Peak no.	t_R(min)	ESI-MS (m/z)		Tentative Id
		MS	MS/MS	
1	6.9	481.4	300.9, 292.7, 190.8	HHDP-glucoside
2	10.3	209.0	191.0	Mucic acid
3	11.7	493.0	331.0, 313.0	Monogalloyl diglucoside
4	12.9	331.0	270.7, 211.0	Monogalloyl glucoside
5	15.6	169.0	125.0	Gallic acid[a]
6	17.6	343.0	191.0, 168.9	Galloyl quinic acid
	19.1	343.0	191.0, 168.9	Galloyl quinic acid
7	18.6	483.0	331.3, 312.9, 271.0	Digalloyl glucoside
	20.5	483.0	331.3, 312.9, 271.0	Digalloyl glucoside
	23.8	483.0	331.3, 312.9, 271.0	Digalloyl glucoside
8	26.3	241.3	168.9, 125.2, 197.3	Gallic derivative
	28.7	241.3	168.9, 125.2, 197.3	Gallic derivative
9	35.0	447.3	301.0	Ellagic rhamnoside

[a] compared with the standard compounds

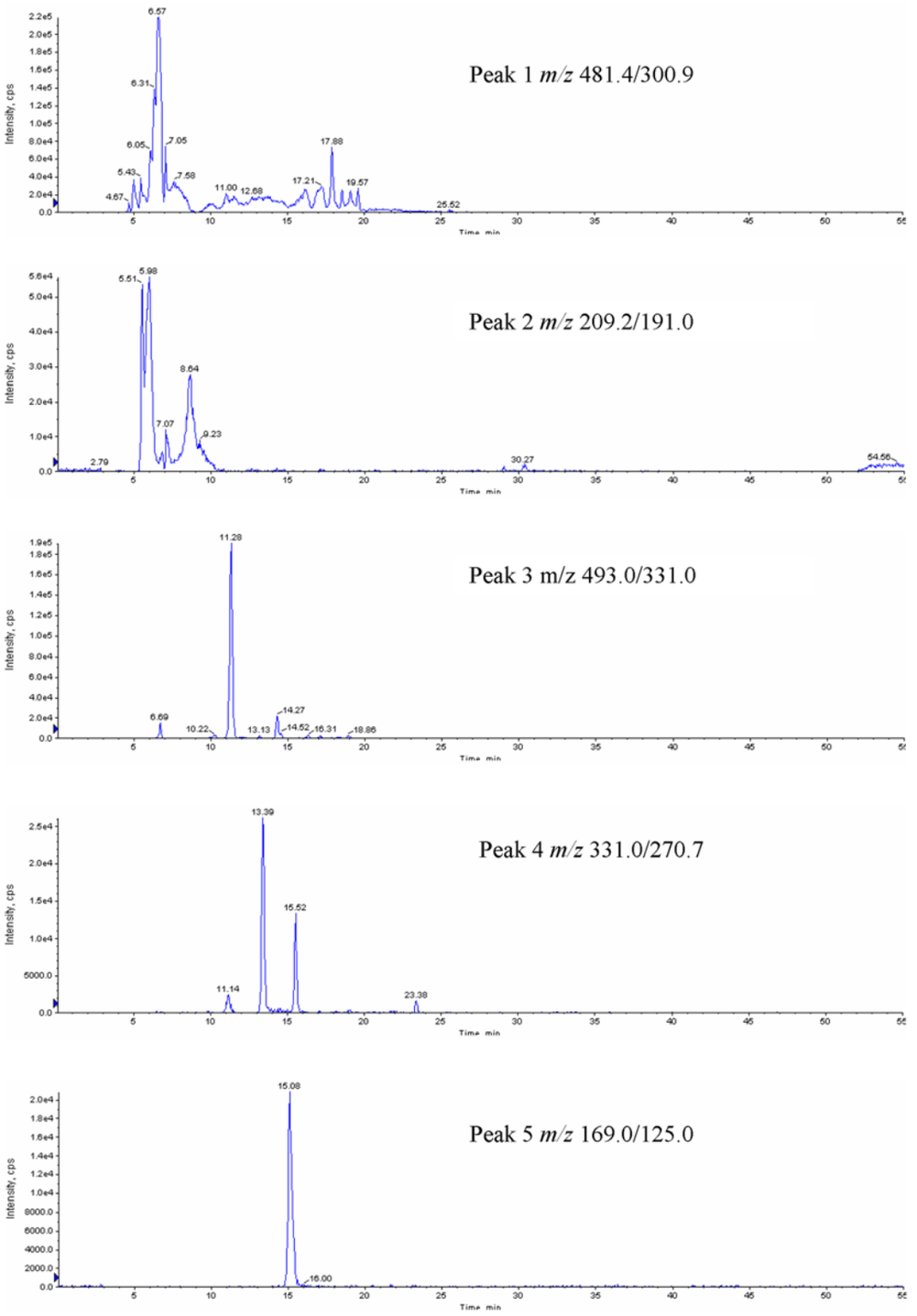

Figure 2. Confirmation of the identification using daughter ion spectra in MRM mode.

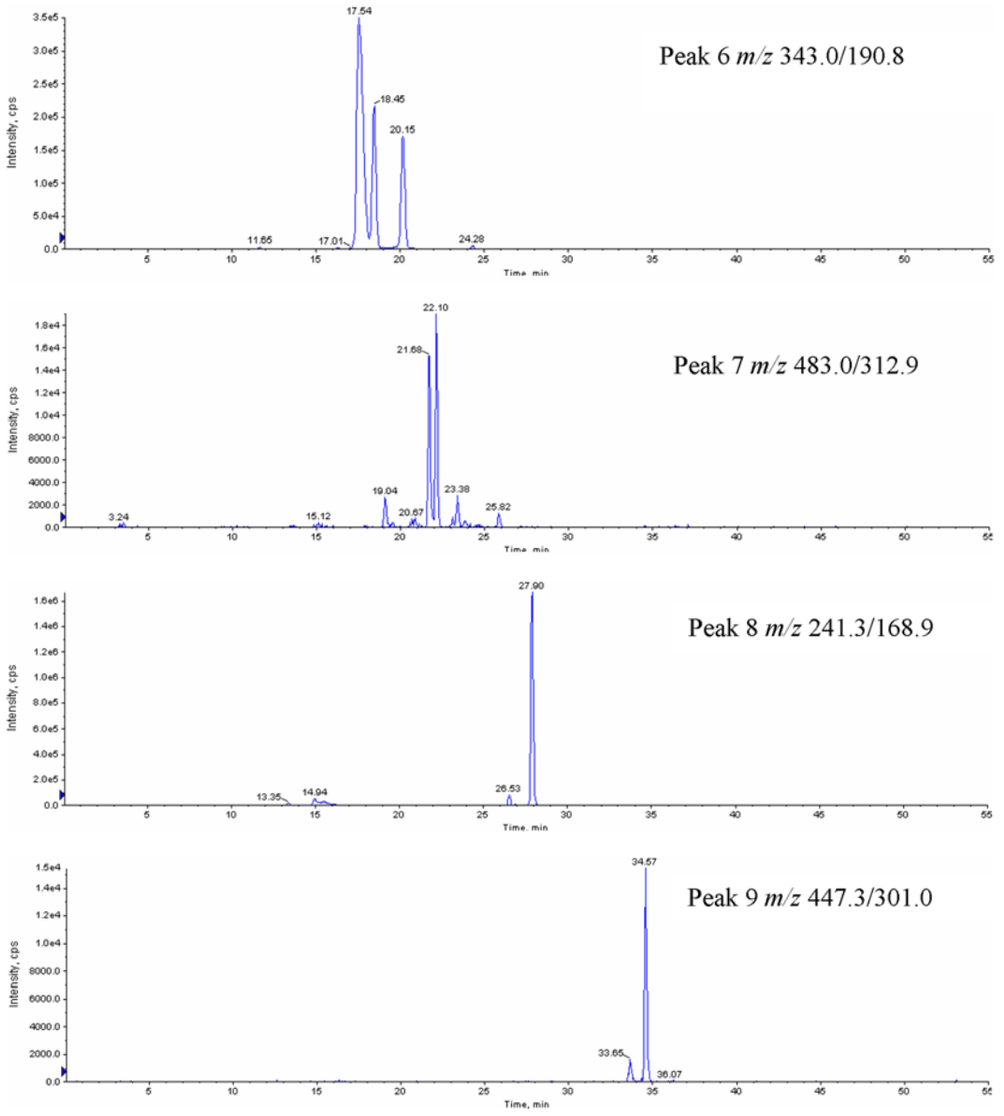

Figure 2. Confirmation of the identification using daughter ion spectra in MRM mode. (continued)

preventive activities against carcinogenesis and mutagenesis [15] but on the other hand, they may be involved in cancer formation, hepatotoxicity or antinutritional activity [16]. It is not advisable to ingest large quantities of tannins, since they may possess carcinogenic and anti-nutritional activities [17]. Thus, it is important to determine the right dose of the right kind of tannins to promote optimal health.

4. CONCLUSIONS

A method for the analysis of antioxidant compounds by RP-HPLC coupled to electrospray ionization–tandem mass spectrometry and DPPH assay was applied to the fruit

fermented juice. The results implied that this on line technique was rapid and effective for selectively analysis of the antioxidants. A wide variety of hydrolysable tannin components were detected and their structure was ascribed by daughter ion spectra obtained after collision-induced dissociation. The finding showed that these hydrolysable tannin could be utilized as food supplement if moderate intake.

REFERENCES

[1] Frohlich R.H., Kunze M. and Kiefer I., Cancer Preventive Impact of Naturally Occurring, Non-nutritive Constituents in Food, *Acta Medica Austriaca*, 1997; **24(3)**: 108-113.

[2] Kaur C. and Kapoor H.C., Antioxidants in fruits and vegetables—the millennium's health, *International Journal of Food Science and Technology*, 2001; **36**: 703-725.

[3] Vinson J.A., Xuehui S., Ligia Z. and Bose P., Phenol antioxidant quantity and quality in foods: fruits, *Journal of Agricultural and Food Chemistry*, 2001; **49**: 5315-5321.

[4] Hertog M.G., Feskens E.J.M. and Kromhout D., Antioxidant flavonols and coronary heart disease risk, *The Lancet*, 1997; 349:699.

[5] Carlo G.D., Mascolo N., Izzo A.A. and Capasso F., Flavonoids: old and new aspects of a class of natural therapeutic drugs, *Life Science*, 1999; **65**: 337-353.

[6] Nuengchamnong N, Suwannakij N. and Phrompittayarat W. Determination of Illegal Adulterant in Herbal Medicines and Dietary Supplements by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS), *Isan Journal*, 2008; **4(2)**:93-103.

[7] Bacteriological Analytical Manual (BAM). FDA US Food and Drug administration online chapter 5,12,14,16. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManual BAM/ucm071363.htm.

[8] Nuengchamnong N. and Ingkaninan K. On-line characterization of phenolic antioxidants in fruit wines from family Myrtaceae by liquid chromatography combined with electrospray ionization tandem mass spectrometry and radical scavenging detection, *LWT - Food Science and Technology*, 2009; **42**: 297-302.

[9] Soong Y.Y. and Barlow P.J. Isolation and structure elucidation of phenolic compounds from longan (Dimocarpus longan Lour.) seed by high-performance liquid chromatography-electrospray ionization mass spectrometry, *Journal of Chromatography A*, 2005; **1085(2)**: 270-277.

[10] Zhang Y.J., Tanaka T., Yang C.R. and Kouno I., New Phenolic Constituents from the Fruit Juice of Phyllanthus emblica, *Chemical & Pharmaceutical Bulletin*, 2001; **49**: 537-540.

[11] Mämmelä P., Savolainen H. Lindroos L., Kangas J., and Vartiainen T. Analysis of oak tannins by liquid chromatography-electrospray ionization mass spectrometry, *Journal of Chromatography A*, 2000; **891**: 75-83.

[12] Ku K.M., Choi J.N., Kim J., Kim J.K.,Yoo L.G., Lee S.J., Hong Y.S. and Lee C.H. Metabolomics analysis analysis reveals the compositional differences of shade grown tea (Camellia sinensis L.), *Journal of Agricultural and Food Chemistry*, 2010; **58**: 418-426.

[13] Dou J., Lee V.S.Y., Tzen J.T.C., and Lee M.R., Identification and Comparison of Phenolic Compounds in the Preparation

of Oolong Tea Manufactured by Semifermentation and Drying Processes, *Journal of Agricultural and Food Chemistry*, 2007; **55(18)**: 7462-7468.

[14] Zywicki B., Reemtsma T. and Jekel M., Analysis of commercial vegetable tanning agents by reversed-phase liquid chromatography–electrospray ionization–tandem mass spectrometry and its application to wastewater, *Journal of Chromatography A*, 2002; **970**: 191-200.

[15] Horikawa K., Mohri T., Tanaka Y. and Tokiwa H., Moderate inhibition of mutagenicity and carcinogenicity of benzo[α]pyrene, 1,6-dinitropyrene and 3,9-dinitrofluorantene by Chinesse medicinal herbs, *Mutagenesis*, 1994; **9**: 523-526.

[16] Ramanathan R., Tan C.H. and Das N.P. Tannic acid promotes benzo[α] pyrene-induced mouse skin carcinogenesis at low concentrations, *Medical Science Research*, 1992; **20**: 711-712.

[17] Chung K.T., Wei C.I. and Johnson M.G., Are tannins a double-edged sword in biology and health?, *Trend in Food Science & Technology*, 1998; **9**: 168-175.