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Antioxidant capacity and Hepatoprotective effect on Ethanol-injured Liver cell of Lemon Juice concentrates and its comparison with commercial Japanese Apricot Juice concentrates

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Abstract

Lemon is an important crop in Southern Taiwan, and its price sometimes decreases during harvest period. So, it is necessary to develop diversified products of lemon, such as lemon juice concentrate to utilize the overproduced lemons. To obtain lemon juice concentrate, lemon was processed by squeezing, filtering and evaporating, to obtain the sticky dark black lemon juice concentrate. Lemon concentrate was obtained by 10 hours evaporation followed by separation through solid phase extraction column. Obtained lemon concentrate extract was compared with commercial Japanese apricot juice concentrate extract for its antioxidant capacity and hepatoprotective activity on ethanol injured mouse hepatocytes FL83B. Antioxidant properties observed from lemon juice concentrate, which were DPPH radical scavenging activity, trolox equivalent antioxidant capacity, reducing power, ferric reducing ability of plasma and thiobarbituric acid reactive substances assay, was higher than Japanese apricot juice concentrate. Moreover, this finding was also supported by higher total phenolic and flavonoid content found in lemon juice concentrate. Mumefural, compound reported to have bioactivity and found in Japanese apricot concentrate, was also found in lemon juice concentrate by using liquid chromatography-mass spectrometry determination. In the cell culture test, 600 µg/mL of lemon concentrate extract showed significant protective ability on ethanol injured cells, which was a little bit higher compared to Japanese apricot juice concentrate. This ability may be related with antioxidant properties of lemon juice concentrate contributed by its compounds, which are phenolic, flavonoid and mumefural.

Keywords: lemon juice concentrate, Japanese apricot juice concentrate, mumefural, antioxidant, hepatoprotective activity.

Introduction

Lemon is one of Taiwan's major beverage crops and 18,105 tones of Eureka variety lemon is produced in the cultivation area of about 1,699 hectares. Lemon in Taiwan is a pale-yellow elliptically shaped berry and is mainly cultivated in southern Taiwan, which accounts for 9% of the world production[1]. During the harvesting season, price of lemon is very less due to oversupply. Therefore, processing the fresh lemon into products which have higher self life is required. One of feasible value added product of lemon is lemon juice concentrate.

Lemon juice concentrate contains many compounds that have potential health benefits. Previous study has found that mumefural, compound reported to have bioactivity in Japanese apricot concentrate, also can be found in lemon juice concentrate[2]. Mumefural, 1-[5-(2-formylfuryl)methyl]-dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate, is a compound with molecular weight 301.2 g/mol. This mumefural compound has been used as a traditional non-official medicine in Japan and has been proved to improve the blood fluidity[3] and also inhibit influenza A virus[4]. Japanese apricot juice concentrate is found to have antioxidant property and can prevent various cardiovascular diseases such as inhibition of vascular hypertrophy induced by angiotensin II[5]. It was also found to suppress the *Helicobacter pylori* induced glandular stomach lesions in Mongolian gerbils[6]. Apart from mumefural, Japanese apricot juice concentrate also contains compounds such as citric acid and malic acid that have roles in blood fluidity improvement[3].

The World Health Organization (WHO) estimates that 140 million people worldwide suffer from alcohol dependency, causing burden to their health. Harmful drinking is a major determinant for neuropsychiatric disorders, such as alcohol use disorders and epilepsy and other non-communicable diseases such as cardiovascular diseases, cirrhosis of the liver and various cancers. Alcoholic hepatitis and other forms of alcoholic liver disease are major complications of chronic excessive ethanol intake[7].

Functional properties from Japanese apricot juice concentrate have been studied by some researchers, but not for lemon juice concentrate. Since lemon juice concentrate also contains the same bioactive compounds[2] which play roles in inhibition of cancer cell proliferation, it may also have some functional properties related to oxidative stress such as ethanol injury in liver.

Material and Methods

Materials: Eureka variety lemon was purchased from market in Long-chuan, Pingtung. Commercial Japanese apricot juice concentrate was purchased from Nanako BC, Japan which

contained 500 mg mumefural per 100 g concentate. Reagent used for antioxidant capacity measurement, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and Folin-Ciocalteu's reagents, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

FL83B (ATCC: CRL-2390) was obtained from the Food Industry Research and Development Institute, Hsin Chu, Taiwan. Fetal bovine serum (FBS), 10× trypsin (5 g/L trypsin in 2 g/L EDTA-Na_4), 10,000 unit/mL penicillin-10 mg/mL streptomycin and aquaguard were purchased from Biological industries (Kibbutz Beit Haemek, Israel). Kaighn's modification of Ham's F12 medium with L-glutamine (F-12K) medium and MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (95%) was purchased from Echo Chemical Co., LTD. (Taiwan). Other chemical compounds used were of analytical grade.

Sample preparation: Lemons were washed, squeezed and filtered into lemon juice. Afterwards, lemon juice was heated for 10 hours at 97-100°C resulting in sticky dark black lemon concentrate. Freeze-dried lemon and Japanese apricot concentrates were dissolved in water by sonification for 10-15 minutes. Sonicated solution was then centrifuged at 6000 rpm 10 min, filtered through solid phase extraction (SPE) column and freeze dried. For analysis, the freeze dried extract was re-dissolved into preferred concentrations.

Antioxidant capacity: DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay: DPPH radical scavenging activity of lemon and Japanese apricot juice concentrate were determined according to Luo et al.[8]. Briefly, 1 mL of 1000 µg/mL lemon and Japanese apricot juice concentrate were added with 0.25 mL of 1 mM DPPH in methanol solution. After incubation for 30 min, generated color was measured by spectrophotometer (Shimadzu UV2550, Kyoto, Japan) at 517 nm. Radical scavenging activity was calculated as the inhibition percentage using the following formula:
(absorbance control-absorbance sample)/absorbance control × 100%.

TEAC (Trolox Equivalent Antioxidant Capacity) assay: Trolox equivalent antioxidant capacity was measured based on the isolated compounds ability against $ABTS^{\bullet+}$ by using the methods of Han et al.[9]. ABTS was dissolved in PBS (0.01 M, pH 7.4) to a 7 mM concentration. $ABTS^{\bullet+}$ was made by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixtures to stand in the dark at room temperature for 16 h before use. The $ABTS^{\bullet+}$ solution was diluted with PBS (0.01 M, pH 7.4) to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C for 30 min. Ethanol solutions (2.0 ml) of the samples at concentrations of 1000 µg/ml were mixed with 2.0 ml of diluted $ABTS^{\bullet+}$ solution. After reaction at room temperature for 20 min, the absorbance at 734 nm was measured. Lower absorbance of the reaction mixture indicated higher $ABTS^{\bullet+}$ scavenging activity. The capability to scavenge the $ABTS^{\bullet+}$ was calculated using the following formula, where absorbances blank sample was absorbance of 2.0 ml of PBS plus 2 ml of the sample at different concentrations, absorbances control was absorbance of 2.0 ml of diluted $ABTS^{\bullet+}$ solution plus 2 ml of ethanol and absorbances blank control was absorbance of 2.0 ml of PBS plus 2 ml of ethanol. ABTS (%) was calculated by
{1-(Absorbance sample-Absorbance blank sample)/(Absorbance control-Absorbance blank control)}x 100%.

Reducing power assay: The reducing power of the samples was determined according to the method described by Zou et al.[10]. One ml of sample (1000 µg/ml) in methanol was mixed with 0.2 M phosphate buffer (2.5 ml, pH 6.6) and 1% potassium ferricyanide (2.5 ml), and the mixture was then incubated at 50°C for 20 min. Afterward, 2.5 ml of 10% trichloroacetic acid was added to the reaction mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml distilled water and 1 ml of 1% ferric chloride. The absorbance was measured at 700 nm, where higher absorbance indicated a higher reducing activity.

FRAP (ferric reducing ability of plasma): Ferric reducing ability of plasma (FRAP) is a method based on ferric-trotripyridyltriazine (Fe^{III}-TPTZ) complex reduction into ferrous (Fe^{II}) form, which develops an intense blue color with an absorption maximum at 593 nm[11]. FRAP assay was conducted by following Niemeyer and Metzler[12] method with slight modification. FRAP reagent was prepared fresh by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-tripyridyl-s-triazine (10 mM), and $FeCl_3.6H_2O$ (20 mM) in a 10:1:1 (v:v:v) ratio and then pre-warmed to 37°C before use. For measurements, 0.04 ml of extract was mixed with 0.12 mL distilled water and 1.2 ml of FRAP reagent and then incubated at 37°C for 5 min. Absorbance of the mixture was then read at 593 nm. Reducing power was presented in µmole Fe^{2+} equivalents, calculated from a standard curve prepared with 0 to 0.40 mM $FeSO_4$•7H2O.

TBARS (Thiobarbituric acid reactive substances): A modified thiobarbituric acid-reactive species (TBARS) assay[13] was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid rich media[14]. Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm[15].

Egg homogenate (0.5 ml of 10% v/v) and 0.1 ml of extract were added to a test tube and made up to 1 ml with distilled water. 0.05 ml of FeSO4 (0.07 M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 ml of 0.8% (w/v) TBA in 1.1% sodium dodecyl sulphate and 0.05 ml 20%

TCA were added and the resulting mixture was vortexed and then heated at 95ºC for 60 min. The generated color was measured at 532 nm. Inhibition of lipid peroxidations (%) by concentrates were calculated with formula: (C-E)/C x 100%; where C is the absorbance value of the fully oxidized control and E is ($Abs_{532+TBA}$ -$Abs_{532-TBA}$).

Determination of phenolic compounds contents: Determination of total phenolic content: The Folin–Ciocalteau method was conducted for the colorimetric estimation of total phenolic content[16]. Each concentrate (50 µL) was dissolved with 2 mL of distilled water, oxidized by 1 mL of Folin–Ciocalteau's reagents (2 N), and neutralized with 5 mL saturated sodium carbonate (20%). After incubation for 20 min in room temperature, the absorbance of the resulting blue color was measured at 735 nm with gallic acid as standard. Results were expressed as gallic acid equivalent (GAE) in micrograms per miligram of concentrate.

Determination of total flavonoid content: Flavonoid content measurement was based on the spectrophotometric determination of complex flavonoid-$AlCl_3$[17]. Briefly, 0.1 mL aliquot of appropriately diluted lemon and Japanese apricot juice concentrate was mixed with 1 mL of methanol and subsequently with 0.05 mL of a 5% $AlCl_3$ solution. After 30 min, absorbance of the mixture was determined at 425 nm versus a prepared methanol blank. The total flavonoid content was determined using a standard curve with quercetin (0–50 µg/ml). Result was expressed as microgram of quercetin equivalents (µg QE) per milligram sample[18].

LC-MS assay for mumefural identification: The mobile phase consisted of 0.1% formic acid in 5% acetonitrile solution (solvent A) and 0.1% formic acid in 95% acetonitrile solution (solvent B). The LC was carried out by step-wise gradient as follow: 100% A and 0% B at 0 sec, 0% A and 100% B at 80 second and 100% A and 0% at 90 second, with a flow rate of 0.2 mL/min for total running time of 60 min. The injection volume was 5.0 µL and the column temperature was 25°C. The ESI source conditions in the positive ionization mode were as follows: capillary voltage 28 V, spray voltage 4 kV, tube lens offset 10 V, capillary temperature of 300°C. The sheath and aux/sweep gas flows were 50 arb and 5 arb, respectively.

Cell culture: FL83B Cell Culture: The experiments were performed in FL83B cells (mouse liver cell line). FL83B was grown in Kaighn's modification of Ham's F12 medium with L-glutamine (F-12K) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% of 10,000 unit/mL penicillin with 10 mg/mL streptomycin. Culture incubation was done at 37°C with 5% CO_2. The cells were grown as monolayer and subcultures were performed with 1.5 mL of 1x trypsin (diluted from 10x trypsin solution with phosphate buffered saline or PBS) after washed with PBS when cell growth reached confluence.

Cell Viability Assessment: Cell suspension (200 µL) was added to each well of a flat-bottom 96 well plate at a density of $1x10^5$ cells/mL. Cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 24 h. Afterwards, the cells were injured by different concentration of ethanol (for IC_{50} determination) or lemon and Japanese apricot concentrates (for cytotoxicity assay) for 6 h. For protection activity, different concentration of lemon and Japanese apricot juice concentrates were added into the adhered cell. Lemon and Japanese apricot juice concentrates were administered for 24 h and ethanol was added to induce cell injury causing reduction of 50% cells (IC_{50}).

The cell viability was assessed by adding 10 µL of MTT solution (5 mg/mL) and 100 µL medium into media-removed cells. After incubation at 37°C for 4 h, 100 µL of DMSO was added and incubated for another 10 min and absorbance was measured at 570 nm by microplate spectrophotometer (Biotek, USA). Cell viability was calculated by (OD_{contol}-OD_{sample})/$OD_{control}$ x 100. An IC_{50} value denotes the concentration of sample which is required to reduce 50% of cell viability.

Statistical analysis: Experiments were done in three replication and the data were expressed as mean ± standard deviation (SD). Statistical analysis were performed by student t-test by SPSS program where result denoting * indicated that the results are significantly different ($p<0.05$). Graphics shown were made by SigmaPlot 10.0 graphics software.

Results and Discussion

Antioxidant capacity and phenolic content determination: Five antioxidant capacity assays were conducted in this research, which were DPPH radical scavenging activity, trolox equivalent antioxidant capacity, reducing power, ferric reducing ability of plasma and thiobarbituric acid reactive substances assay. Antioxidant properties observed from lemon juice concentrate was found to be higher than Japanese apricot juice concentrate (table-1). This finding is also supported by phenolic content results. Total phenolic and flavonoid content revealed that lemon juice concentrate consisted of higher amount of these compounds compared to Japanese apricot juice concentrate. Phenolic compounds may be one of contributor for higher antioxidant capacity of lemon juice concentrate.

LC-MS assay for mumefural determination: LC-MS assay was carried out to determine the presence of mumefural, a bioactive compound found in both Japanese apricot juice concentrate and lemon juice concentrate. Molecular weight of mumefural is 301.2 g/mol, so m/z of mumefural will be observed in 302.2 m/z [M+H]. After total chromatogram was determined, the selective ions which have m/z in 300.5-301.5 range were chosen and their spectrum was observed by mass spectrometry. Figure-1 shows total and selected ion chromatogram of lemon juice concentrate, while figure-2 shows total and selected ion chromatogram of Japanese apricot juice concentrate.

Table-1
Antioxidant capacity and total phenolic content of lemon juice concentrate compared to Japanese apricot juice concentrate

Antioxidant capacity	Lemon juice concentrate	Japanese apricot juice concentrates
DPPH scavenging activity (%)[1]	74.10 ± 3.09	66.00 ± 4.62*
Reducing power (absorbance)[1]	1.70 ± 0.07	0.62 ± 0.05*
Ferric reducing ability (µmol/L)[1]	1039.60 ± 52.00	274.27 ± 18.15*
Lipid peroxidation inhibition (%)[1]	11.53 ± 1.44	4.84 ± 1.47*
Trolox equivalent antioxidant capacity (TEAC) (mg/g)	395.38 ± 2.18	129.67 ± 2.97*
Total phenolic (mg gallic acid equivalent (GAE)/g)	191.67 ± 41.67	108.33 ± 13.89*
Total flavonoid (mg quercetin equivalent (QE)/g)	32.00 ± 1.33	9.11 ± 6.67*

[1]Used sample concentrations are 1 mg/mL, *Data are significantly different at P<0.05 (n=3) analyzed by t-test

Table-2
Different concentrations of lemon and Japanese apricot fruit concentrates on FL83B cytotoxicity test

Sample	Concentration (µg/mL)	Cell viability (%)
Control		100.0 ± 7.7
Lemon juice concentrate	200	117.7 ± 13.5
	400	110.3 ± 7.9
	800	103.7 ± 14.8
Japanese apricot juice concentrates	200	114.1 ± 10.9
	400	110.2 ± 13.7
	800	92.0 ± 12.4

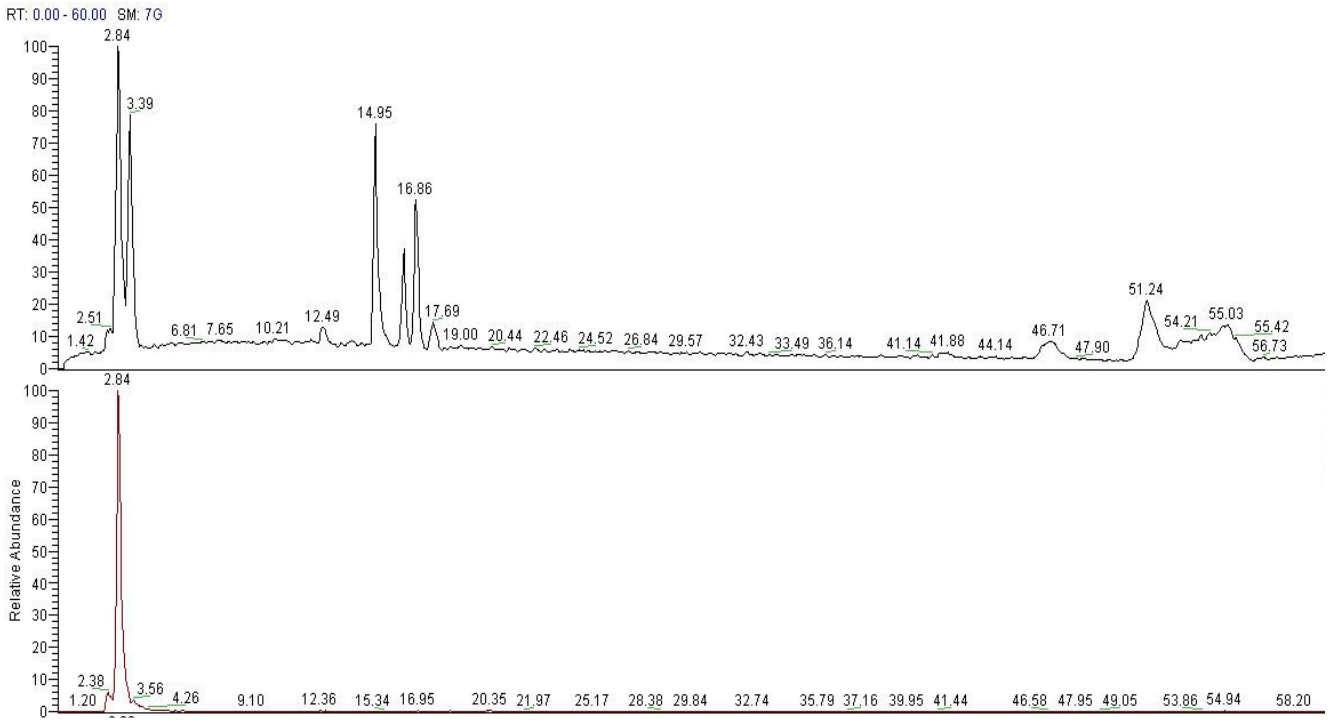

Figure-1
LC chromatogram of lemon juice concentrate
(a) LC total chromatogram and (b) LC selected ion chromatogram of m/z 300.5-301.5.

The chromatograms result showed more peaks in lemon juice concentrate compared to Japanese apricot concentrate, which indicated that more compounds were present in lemon juice concentrate. Chromatogram peak consisting compounds in the range of 300.5-301.5 m/z came out at retention time of 2.84 and 2.92 for lemon juice concentrate and Japanese apricot juice concentrate, respectively. These peaks were further observed by mass spectrometry as shown in figure-3. The relative abundance of lemon juice concentrate was 42% and Japanese apricot juice concentrate was 70%.

Hepatoprotective activity on ethanol-induced injury: Compound cytotoxicity on the FL83B cell should be conducted before hepatoprotective assay to ensure the compound's concentrations used in this research did not cause any harm to the cell. The results revealed that no significant cytotoxicity was observed in both concentrates up to the concentration of 800 µg/mL, but at the highest concentration, Japanese apricot concentrate showed little reduction in cell viability as shown in table-2. Therefore, concentrations used for measuring hepatoprotective activity were not higher than 600 µg/mL.

Concentration dependence activity was found in ethanol induced injury on FL83B cell as shown in figure-4. LC_{50} (concentration of the ethanol needed to kill 50% of the total cells) which was found to be 5.5% in this experiment. Hepatoprotective activity was carried out first by exposing FL83B mice liver cells with the concentrate followed by ethanol injury at the concentration of 5.5% (LC_{50}). Result revealed that lemon juice concentrate at the concentration of 600 µg/mL showed significant protection ability on injured cells and was able to increase the cell viability from 50% to 56.7% in figure-5. Japanese apricot juice concentrate showed less protection compared to lemon juice concentrate, which increased the cell viability until 56.0%.

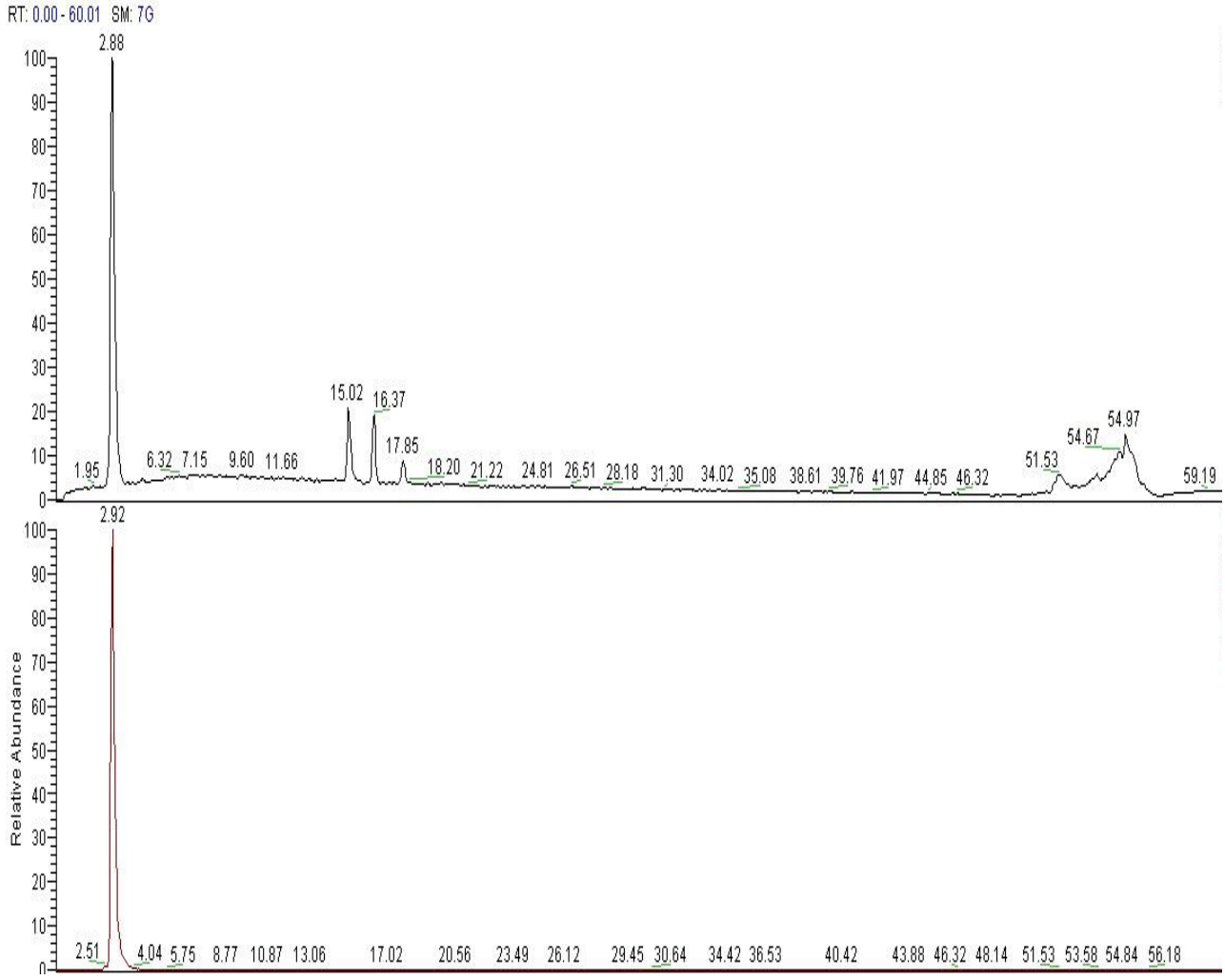

Figure-2
LC chromatogram of Japanese apricot juice concentrate.
(a) LC total chromatogram and (b) LC selected ion chromatogram of m/z 300.5-301.5

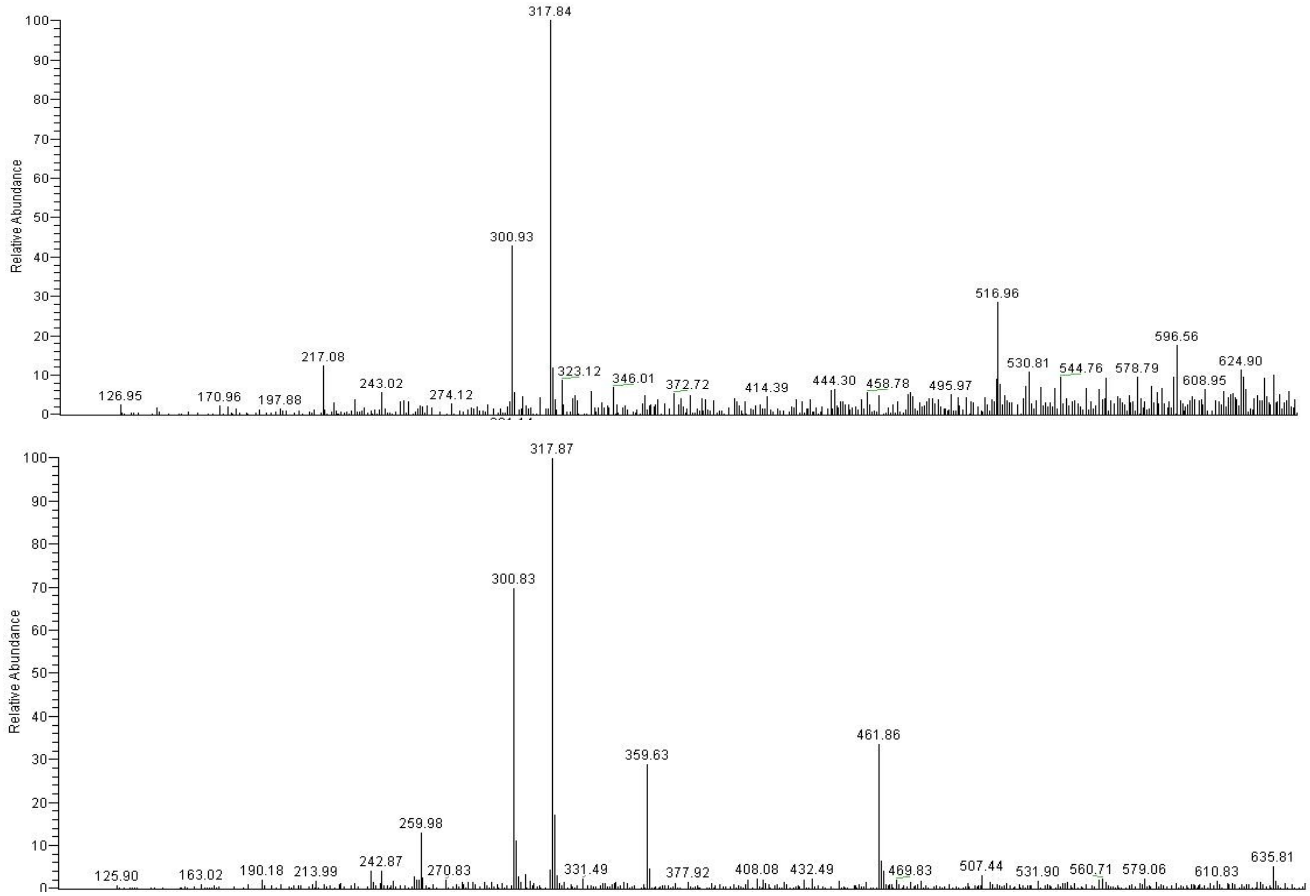

Figure-3
MS spectra of mumefural (m/z 301.2) from
(a) lemon juice concentrate and (b) Japanese apricot juice concentrate

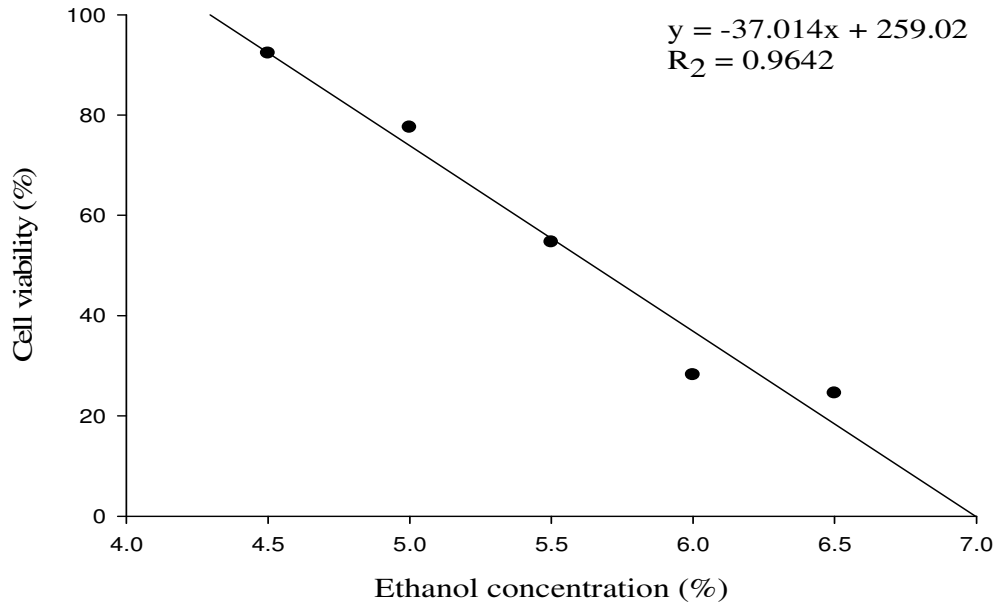

Figure-4
IC_{50} value of different ethanol concentration injury for 6 hours on FL83B cell viability

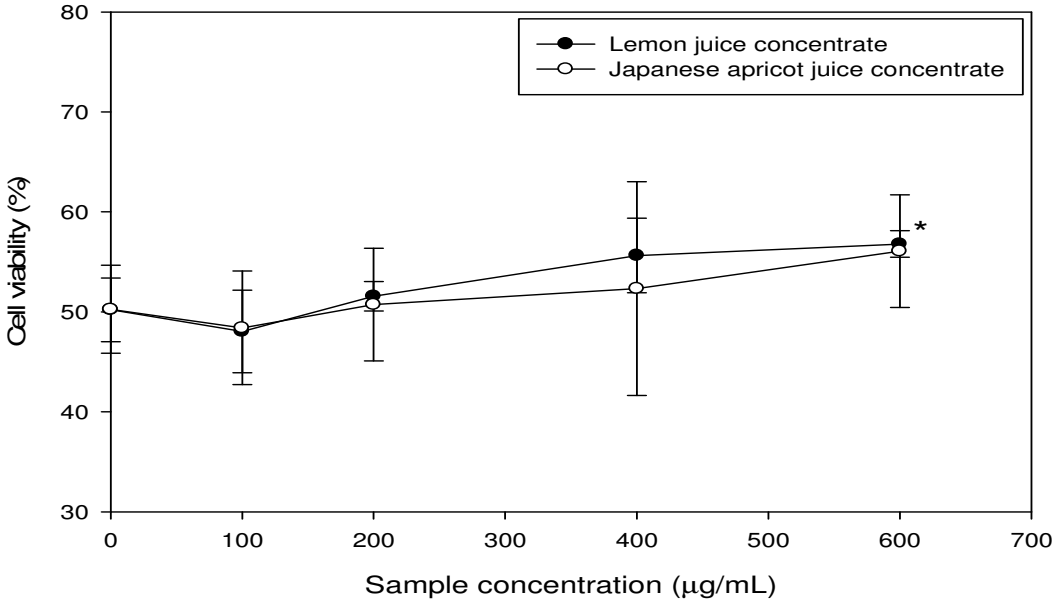

Figure-5

FL83B cell viability after 24 h incubation of Different concentrations of lemon and Japanese apricot juice concentrates followed by 5.5% ethanol injury for 6 hours. *Data are significantly different at P<0.05 (n=3) analyzed by t-test

Discussion: The hepatotoxicity of alcohol results from the hepatic oxidative metabolism, which involves alcohol dehydrogenase (ADH)-mediated excessive generation of NADH (nicotinamide adenine dinucleotide reduced form), and acetaldehyde, the main oxidative metabolite of ethanol. Acetaldehyde may also result from microsomal ethanol oxidizing system (MEOS) containing an inducible form of cytochrome P4502El (CYP2El), which is able to produce acetaldehyde from ethanol[19]. Ethanol metabolism via the NADPH-dependent CYP2EI oxidizing pathway is responsible for the generation of a great amount of reactive oxygen species (ROS), which is able to induce several damaging events in liver tissue, including the peroxidation of cell membrane phospholipids (lipid peroxidation) which plays a pivotal role in the pathogenesis of alcoholic liver injury.

Antioxidant properties observed from lemon juice concentrate, which were DPPH radical scavenging activity, trolox equivalent antioxidant capacity, reducing power, ferric reducing ability of plasma and thiobarbituric acid reactive substances assay, were higher than Japanese apricot juice concentrate. Moreover, this finding was also supported by higher total phenolic and flavonoid content found in lemon juice concentrate. A strong positive correlation of citrus's total phenolic content and FRAP antioxidant properties values ($R^2 = 0.9090$) was also observed in research done by Abd Ghafar et al.[20].

In the cell culture study, 600 µg/mL of lemon concentrate extract showed significant protection ability on ethanol injured cell, a little higher protection compared to Japanese apricot juice concentrate. Since oxidative stress is also one of the damage caused by ethanol injury, antioxidant properties of lemon juice concentrate may have played the protective role on ethanol induced injury.

Mumefural compound was identified both in lemon juice and Japanese apricot concentrate by using liquid chromatography-mass spectrometry. Abundance of mumefural compound (300.83 m/z in lemon juice concentrate and 300.93 m/z in Japanese apricot juice concentrate) was found. Lemon juice concentrate was found to have less mumefural (42%) compound compared to Japanese apricot juice concentrate (70%). Therefore, hepatoprotective activity of lemon might not be only because of mumefural compound. Other compounds, such as phenolic compounds, flavonoid compounds and organic acids, might have contributed on hepatoprotective activity of lemon juice concentrate and Japanese apricot juice concentrate.

Conclusion

DPPH radical scavenging activity, trolox equivalent antioxidant capacity, reducing power, ferric reducing ability of plasma and thiobarbituric acid reactive substances assay of lemon juice concentrate were higher than Japanese apricot juice concentrate. Moreover, this finding was also supported by higher total phenolic and flavonoid content found in lemon juice concentrate. Mumefural, compound reported to have bioactivity and found in Japanese apricot concentrate, was also found in lemon juice concentrate by using liquid chromatography-mass spectrometry determination. In the mice liver cell FL83B, 600

µg/mL of lemon concentrate extract showed significant protective ability on ethanol injured cells, which was a little bit higher compared to Japanese apricot juice concentrate.

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