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Antioxidative and hepatoprotective activities of lactic acid bacteria fermented calamondin (*Citrus microcarpa*) juice against ethanol injured FL83B mice liver cell

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Abstract

This research was aimed to observe the difference of antioxidant and hepatoprotective capacity against ethanol injury of single- and co-inoculation of lactic acid bacteria and acetic acid bacteria-fermented calamondin juice. Trolox equivalent antioxidant capacity and reducing power was used to observe the antioxidant capacity, while the antioxidant compounds observed were total phenolic and flavonoid compounds. Hepatoprotective activity was observed against ethanol induced injury on FL83B mice liver cell. Lactic acid bacteria fermented calamondins showed higher antioxidant capacity and compound compared to acetic acid bacteria fermented calamondin and lactic acid and acetic acid bacteria co-inoculated calamondin. Regarding hepatoprotective activity, lactic acid bacteria fermented calamondin showed the best protective capacity compared to two other groups, while unfermented calamondin juice had adverse effect on the FL83B liver cell. Lactic acid bacteria fermentation is a potential processing method to be applied on liquid fermentation like calamondin juice to increase the bioactivity of the fruit juice.

Keywords: Lactic acid bacteria, Calamondin, Antioxidant, Hepatoprotective, Liver cell

Introduction

Calamondin (*Citrus microcarpa*), a cultivar related to citrus, bears small-sized fruits with the average diameter of 3.3 cm with the color of glossy green which turns orange or orange–red when it is mature[1]. It has been used to make hot drink in Taiwan for many years because of its potential health benefits. Several flavonoids have been identified from the pulp extracts of calamondin such as: 3',5'-di-C-β-glucopyranosylphloretin (DGPP), naringin, hesperidin, nobiletin, tangeretin and diosmin[2]. DGPP was found in highest quantity; while the other two major flavonoids were naringin and hesperidin[2]. DGPP exhibited good tyrosinase inhibitory activity[3]. Polymethoxyflavones such as nobiletin and tangeretin have several pharmacological properties such as: anti-inflammatory, anticarcinogenic, antiviral, antithrombogenic, and antiatherogenic[4,5].

Fermentation has been known and practiced by humankind since prehistoric times. The appropriate fermentation method can produce healthy food with lower cost and higher amount of peptides as compared to the previous two methods[6]. Moreover, partial cleavage or changes in the glucosides that occur during increases the glucosidase and glucuronidase activities that results in the release of potent antioxidant compounds by transformation of flavonoids[7].

The most common bacteria used in liquid fermentation are acetic acid and lactic acid bacteria. Lactic acid bacteria is gram-positive, non-sporing, catalase negative in the absence of porphorinoids, aerotolerant, acid tolerant, organotrophic, and a strictly fermentative rod or coccus producing lactic acid as a major end product[8]. Various food products are made using these bacteria such as sour dough bread, sorghum beer, yoghurt, fermented milk products and fermented vegetables. Lactic acid bacteria convert the carbohydrate into lactic acid, carbon dioxide and other organic acids, without using oxygen.

Acetic acid bacteria (AAB) are gram negative or gram-variable, non-spore forming, ellipsoidal to rod-shaped cells that can occur in single, pairs or in short chains, and consists of two genera, Acetobacter and Gluconobacter. Vinegar is generally produced by the strains of Acetobacter[9].

They are also involved in the production of other foods such as palm wine, cocoa powder, *nata de coco* (a fermented food from coconut), *pulque* (a beverage from agave) and *kombucha*, a slightly acid and sparkling beverage obtained from tea fermentation by a symbiotic culture of AAB and yeasts[10]. Oxidation of alcohols to sugars is the main characteristic of the AAB.

Beside of shelf life extension, fermentation using lactic acid and acetic acid bacteria can increase the bioactivities of calamondin juice. Therefore, this research was aimed to observe the difference of antioxidant and hepatoprotective capacity against ethanol injury of single- and co-inoculation of lactic acid bacteria and acetic acid bacteria-fermented calamondin juice.

Materials and methods

Materials: FL83B cell (ATCC: CRL-2390) was bought from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Fetal bovine serum (FBS), 10× trypsin (5 g/L trypsin in 2 g/L EDTA) and penicillin and streptomycin mixture containing 10,000 unit/mL penicillin and 10 mg/mL streptomycin were bought from Biological industries (Kibbutz Beit Haemek, Israel). Ham's F-12 K medium (Kaighn's modification of Ham's F-12 Medium) was used in the experiment that contained L-glutamine and analytical grade chemical compounds.

Physicochemical analysis: Refractometer (Hand-held refract meter, N-1E, Atago, Tokyo, Japan) measured the total soluble solid and the result was expressed as °Brix. Color characteristic was determined using Chroma Meter (Color Quest XE, Hunter Associates Laboratory, Inc., Reston, Virginia, U.S.A).

Reducing power assay: Previously described method by Zou et al. was used to determine the reducing power of samples[11]. One ml of sample (1000 µg/ml) in methanol, 0.2 M phosphate buffer (2.5 ml, pH 6.6) and 1% potassium ferricyanide (2.5 ml) was mixed, then incubation was done for 20 min at 50°C. This mixture was added with 2.5 ml of 10% trichloroacetic acid, centrifuged for 10 min at 3000 rpm. The supernatant (2.5 ml) was added with equal volume of distilled water and 1 ml of 1% ferric chloride, and the absorbance was measured at 700 nm. Higher reducing activity was indicated by higher absorbance.

TEAC (Trolox Equivalent Antioxidant Capacity) assay: The method described by Han et al.[12] was used to determine Trolox equivalent antioxidant capacity, which is based the isolated compounds ability against $ABTS^{\bullet+}$. ABTS was mixed with PBS (0.01 M, pH 7.4) to make 7 mM concentration. ABTS stock solution was mixed with 2.45 mM potassium persulfate (final concentration) to prepare $ABTS^{\bullet+}$ and the mixture was kept in the dark at room temperature for 16 h before use. Then, PBS (0.01 M, pH 7.4) was mixed with the $ABTS^{\bullet+}$ solution to an absorbance of 0.70 (±0.02) at 734 nm, and kept at 30 °C for 30 min. $ABTS^{\bullet+}$ solution (2ml) was mixed with ethanol solutions of samples at concentrations of 1000 µg/ml. After allowing to react at room temperature for 20 min, the absorbance was measured at 734 nm. The absorbance value was then compared to the absorbance of the antioxidant capacity obtained by trolox.

Total phenolic and flavonoid determination: The total phenolic content was determined by colorimetry by using the Folin-Ciocalteau method[13]. Co-inoculated soy tempeh (50 µl) was mixed 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 was expressed as gallic acid equivalent (GAE) in micrograms per miligram of concentrate. Flavonoid content measurement will be based on the spectrophotometric determination of complex flavonoid-$AlCl_3$. Methanol (1ml) was mixed with 0.1 ml aliquot of co-inoculated soy tempeh and then 0.05 ml of 5% $AlCl_3$ was added. The absorbance of the mixture was determined after 30 min at 425 nm and compared with a methanol blank. Standard curve was made with quercetin (0–50 µg/mL to determine the total flavonoid content[14].

FL83B Cell Culture: Ham's F-12 K medium that contained 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin was used to culture mouse liver FL83B cells, which was incubated at 37°C in a 5% CO_2 using an incubator NU-4500 (NuAire, Plymouth, MN). Monolayer cells were grown and subculture was done with 1.5 mL of 1× trypsin in phosphate buffered saline (PBS).

Protective capacity: FL83B cells were placed in a 96 well-plate where the concentration in each well was 5×10^4 cells. Before treatment the well-plate was incubated for 24 h at 37°C. Different concentrations of sample (0-1000 µg/mL) were used to treat the cells. After 24 hour, ethanol containing medium was used to wash to induce reduction in 50% cell viability after 6 h additional exposure. Cell viability was determined by adding 10 µL of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/mL) and 100 µL medium into media-removed cells. It was incubated for 4 h at 37°C, after which 100 µL of DMSO was added and further incubated for 10 min. A microplate spectrophotometer (Bio-Tek, VT, USA) measured the absorbance in each well at 570 nm. Cell viability was calculated as (absorbance of treatment/absorbance of control) × 100%. IC_{50} is the concentration of ethanol required to reduce cell viability to 50%.

Statistical analysis: One-way ANOVA was used to analyze the data and difference in significance ($P<0.05$) was determined by Duncan's test, by using SPSS program (version 12.0, St. Armonk, NY, USA).

Results and discussion

Physicochemical properties of fermented calamondin: Table-1 showed physicochemical properties of fermented calamondin compared to unfermented one (calamondin juice). After fermentation, total soluble solid of fermented calamondin decreased from 21.6 to 14.2 for lactic acid fermented calamondin. The lowest decrease was found on acetic acid fermented calamondin which decreased from 21.6 to 16. For lightness (L^* value), lactic acid bacteria fermented calamondin had higher lightness, while acetic acid bacteria tended to decrease the lightness of calamondin juice. The greenish color (a*) tend to reduce after fermentation and the yellowish color (b*) increased significantly.

The highest changes in a* and b* values were found in lactic acid fermented calamondin compared to acetic acid bacteria fermented and lactic acid and acetic acid bacteria co-inoculated calamondin.

Antioxidative capacities and compounds of fermented calamondin: Antioxidant capacity observed by reducing power and trolox equivalent antioxidant capacity (ABTS radical scavenging activity) were shown in Figure-1. All fermented calamondin has higher antioxidant capacity compared to calamondin juice. Moreover, lactic acid bacteria fermented calamondin has higher antioxidant capacity compared to other fermented calamondin. In addition, lactic acid and acetic acid bacteria co-inoculated calamondin showed antioxidant capacity in between lactic acid bacteria fermented calamondin and acetic acid bacteria fermented calamondin.

Antioxidant compounds observed in this study were total phenolic and flavonoid compounds. After fermentation, total phenolic and total flavonoid compounds of calamondin juice increase significantly, especially on lactic acid bacteria fermented calamondin (Figure-2). Acetic acid bacteria fermented calamondin showed the lowest increase in the total phenolic and flavonoid compounds.

Hepatoprotective effect of fermented calamondin on ethanol injured FL83B mice liver cell: Before conducting the hepatoprotective study of fermented calamondin, effect of ethanol on FL83B was observed at different concentration. The result was shown in Figure-3. Cell viability of FL83B liver cell tended to decrease as the increase of ethanol concentration. The lethal concentration needed to kill 50% of the total cell (LC_{50}) calculated from the regression obtained were 5.5% ethanol for 6 h. Therefore, 5.5% ethanol concentration was used as injury standard for hepatoprotective study of fermented calamondin.

Table-1: Physicochemical properties of unfermented and fermented calamondin.

Physicochemical properties	Calamondin juice	Fermented calamondin		
		Lactic acid bacteria-fermented	Acetic acid bacteria-fermented	Lactic acid + acetic acid bacteria-fermented
Total soluble solid (ºBrix)	21.6±0.06[a]	14.20±0.12[d]	16.00±0.12[b]	15.00±0.10[c]
*L** value	16.39±0.02[b]	17.45±0.02[a]	15.49±0.00[c]	14.40±0.04[d]
*a** value	-3.2±0.07[d]	-1.40±0.06[a]	-1.99±0.00[c]	-1.72±0.00[b]
*b** value	1.02±0.04[d]	4.54±0.04[a]	4.44±0.02[b]	4.21±0.03[c]

[a-d]Values (mean ± SD) with different letters were significantly different (P<0.05) based on one way ANOVA with Duncan's post-hoc.

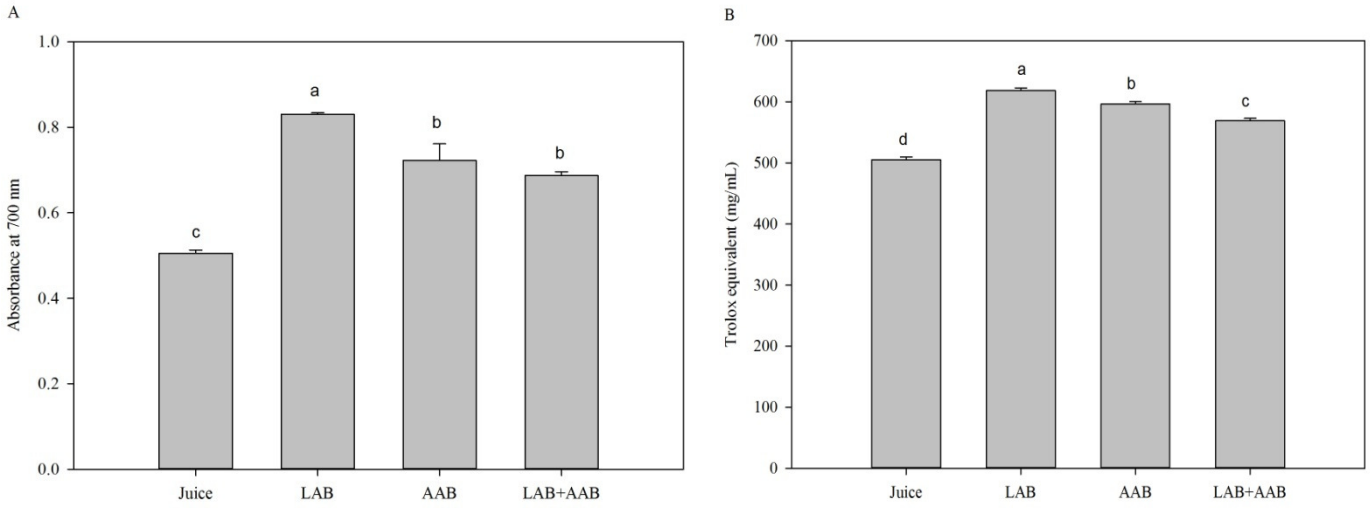

Figure-1: Antioxidant capacities of unfermented and fermented calamondin determined by (A) reducing power and (B) trolox equivalent antioxidant capacity. LAB represents lactic acid bacteria and AAB represents acetic acid bacteria. Values (mean ± SD) with different letters were significantly different (P<0.05) based on one-way ANOVA with Duncan's post-hoc.

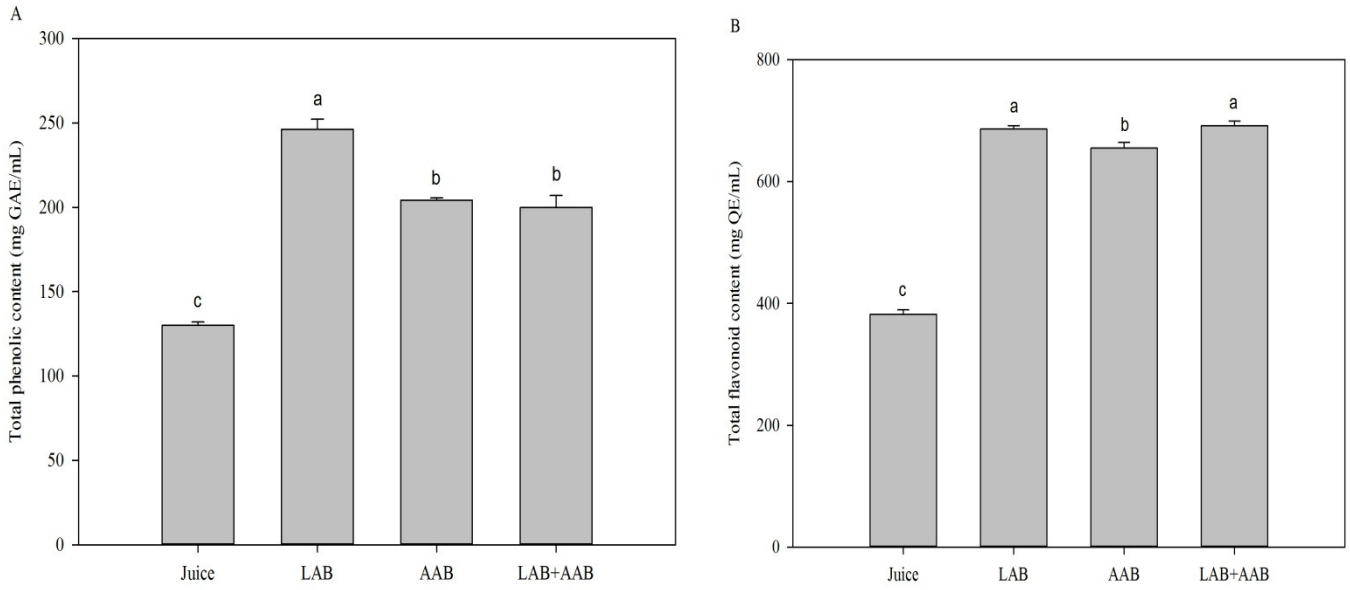

Figure-2: Antioxidant compounds of unfermented and fermented calamondin observed from (A) total phenolic content and (B) total flavonoid content. LAB represents lactic acid bacteria and AAB represents acetic acid bacteria. Values (mean ± SD) with different letters were significantly different (P<0.05) based on one-way ANOVA with Duncan's post-hoc.

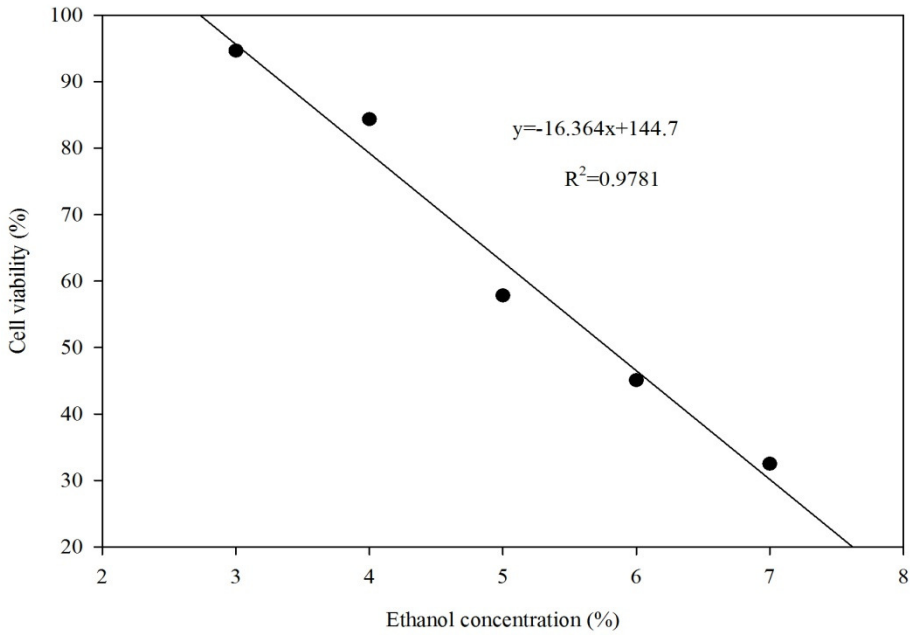

Figure-3: Linear regression of ethanol effect on FL83B cell viability observed by MTT cell viability assay.

The results of hepatoprotective activity showed that calmondin juice tended to injure the cell as it reduced the cell viability of the cell (Figure-4A). At the concentration of 200 µg/mL, the cell viability had significantly reduced compared to control and the reduction was found higher at 1000 µg/mL. Lactic acid bacteria fermented calamondin can protect the FL83B liver cell from ethanol injury as shown that the cell viability increased after the addition of different concentration of lactic acid bacteria fermented calamondin (Figure-4B). Acetic acid bacteria fermented calamondin also showed the hepatoprotective activity against ethanol injured FL83B liver cell (Figure-4C); however, the effect was not as good as lactic acid bacteria fermented calamondin. Moreover, the effect of lactic acid bacteria and acetic acid bacteria co-inoculated calamondin was shown in between those two single inoculated calamondins (Figure-4D). From all the fermented calamondin at different concentrations, fermented calamondin at the concentration of 600 µg/mL had the highest protective ability against ethanol injured FL83B mice liver cell.

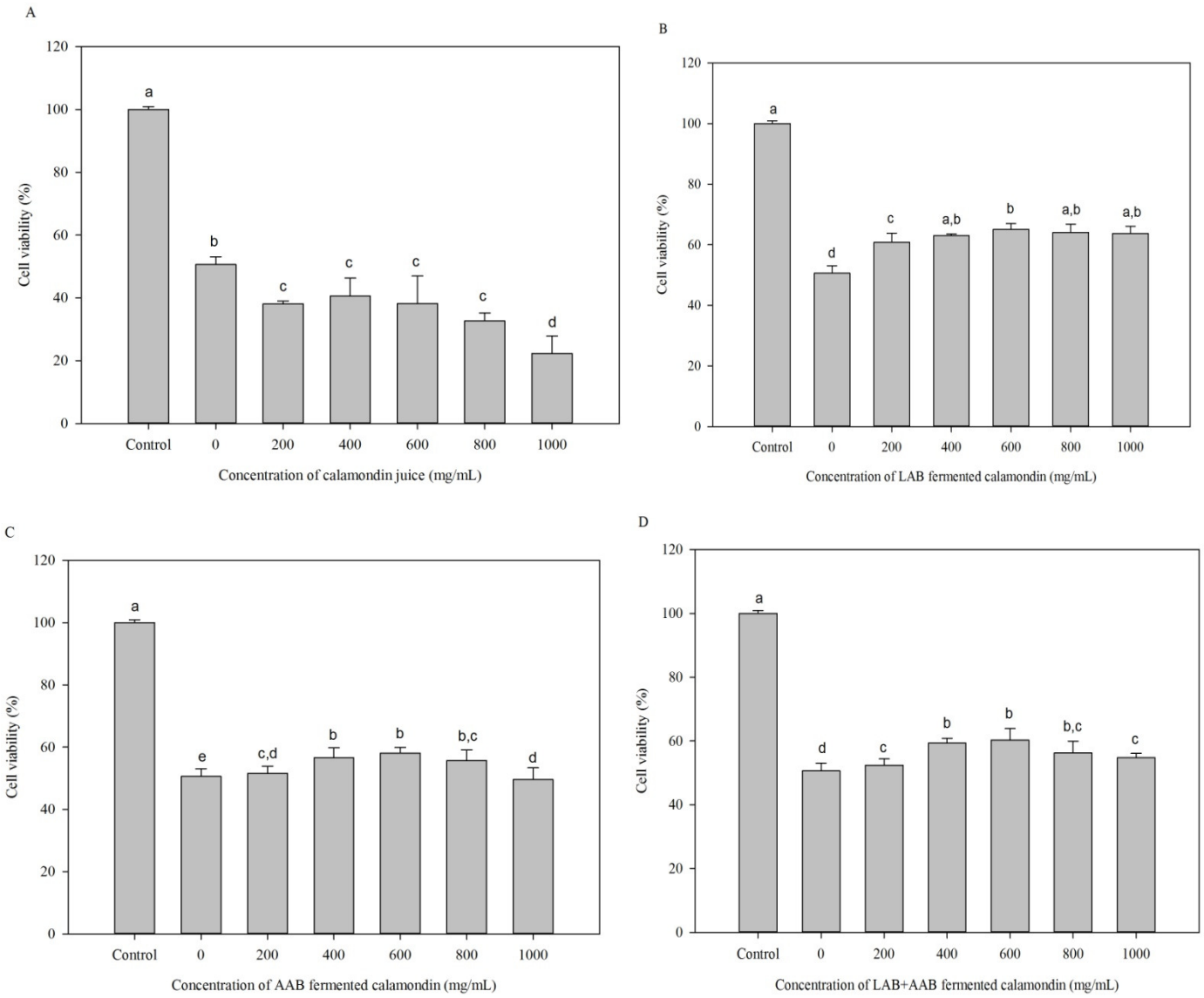

Figure-4: Cell viabilities of FL83B after 24 h treatment with (A) unfermented, (B) lactic acid bacteria fermented, (C) acetic acid bacteria fermented, (D) lactic acid and acetic acid bacteria fermented calamondin on 5.8% ethanol injured FL83B cells. Values (mean ± SD) with different letters were significantly different (P<0.05) based on one-way ANOVA with Duncan's post-hoc.

Unfermented juice tended to have adverse effect on ethanol injured FL83B liver cell. It might due to pH value of the unfermented juice (3.25), while after fermentation, the bioactive compound produced. Lactic acid bacteria can produce β-glucosidase that forms the aglycones bycatalysing the hydrolysis of glucoside isoflavones[15]. These aglycone flavonoids are absorbed faster and in higher amounts than their glucosides by human[16]. This may be the reason of higher hepatoprotective activity of lactic acid fermented calamondin.

Conclusion

Fermented calamondin juice possessed hepatoprotective activity, while unfermented calamondin juice tended to have adverse effect on FL83B liver cells. Beside of hepatoprotective effect, fermentation also increased antioxidant activities and total phenolic compounds. Moreover, lactic acid bacteria can increase the bioactivities of calamondin juice better than acetic acid bacteria.

References

1. Yo S.P. and Lin C.H. (2004). Qualitative and quantitative composition of the flavour components of Taiwan calamondin and Philippine calamansi fruit. *Europ. J. Hort. Sci.*, 69(3), 117-124.
2. Lou S.N., Hsu Y.S. and Ho C.T. (2014). Flavonoid compositions and antioxidant activity of calamondin

extracts prepared using different solvents. *J. Food Drug. Anal.* 22(3), 290-295.

3. Lou S.N., Yu M.W. and Ho C.T. (2012). Tyrosinase inhibitory components of immature calamondin peel. *Food chem.,* 135(3), 1091-1096.

4. Li S., Lo C.Y. and Ho C.T. (2006). Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Citrus sinensis*) peel. *J. Agric. Food Chem.,* 54(12), 4176-4185.

5. Ho C.T., Pan M., Lai C. and Li S. (2012). Polymethoxyflavones as food factors for the management of inflammatory diseases. *Neuropathol.,* 20, 337-341.

6. Okamoto A., Hanagata H., Kawamura Y. and Yanagida F. (1995). Anti-hypertensive substances in fermented soybean, natto. *Plant Foods Hum. Nutr.,* 47(1), 39-47.

7. McCue P. and Shetty K. (2003). Role of carbohydrate-cleaving enzymes in phenolic antioxidant mobilization from whole soybean fermented with *Rhizopus oligosporus*. *Food Biotechnol.,* 17(1), 27-37.

8. König H., Unden G. and Fröhlich J. (2009). Biology of Microorganisms on Grapes, in Must and in Wine. Springer, New York.

9. Sokollek S.J., Hertel C. and Hammes W.P. (1998). Cultivation and preservation of vinegar bacteria. *J Biotechnol.,* 60(3), 195-206.

10. Nanda K., Taniguchi M., Ujike S, Ishihara N., Mori H., Ono H. and Murooka Y. (2001). Characterization of acetic acid bacteria in traditional acetic acid fermentation of rice vinegar (komesu) and unpolished rice vinegar (kurosu) produced in Japan. *Appl. Environ. Microbiol.* 67(2), 986-990.

11. Zou Y., Lu Y. and Wei D. (2004). Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. in vitro. *J. Agric. Food Chem.,* 52(16), 5032-5039.

12. Han J., Weng X. and Bi K. (2008). Antioxidants from a Chinese medicinal herb–Lithospermum erythrorhizon. *Food Chem.,* 106(1), 2-10.

13. Julkunen-Tiitto R. (1985). Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. *J. Agric. Food Chem.,* 33(2), 213-217.

14. Bonvehí J.S. and Coll F.V. (1994). The composition, active components and bacteriostatic activity of propolis in dietetics. *J. Am. Oil Chem. Soc.,* 71(5), 529-532.

15. Chien H.L., Huang H.Y. and Chou C.C. (2006). Transformation of isoflavone phytoestrogens during the fermentation of soymilk with lactic acid bacteria and bifidobacteria. *Food Microbiol.,* 23(8), 772-778.

16. Izumi T., Piskula M.K., Osawa S., Obata A., Tobe K., Saito M., Kataoka S., Kubota Y. and Kikuchi M. (2000). Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *J. Nutr.,* 130(7), 1695-1699.