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Effect of fermentation conditions of noni (*Morinda citrifolia* L.) juice on glutathione content and lipid oxidation in Vero cells

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Abstract

Morinda citrifolia L. (Rubiaceae) normally known as noni is consumed as traditional medicine for more than 2000 years. In the current work, effects of fresh juice in comparison with fermented juice employing different conditions i.e.sugar supplementation, pure and natural culture fermentation as well as fermentation time on glutathione content and lipid oxidation in Vero cells were studied. The results showed that all noni samples induced glutathione content in Vero cells but at different potential. Either natural or pure culture in the presence of sugar showed a remarked induction of glutathione at concentration of 30 µL/mL whereas the range of 100-200 µL/mL was required in non-sugar supplemented samples. Similarly, inhibition of lipid peroxidation in Vero cells by noni juice was achieved and the superior inhibition potential was noted in the samples with high glutathione content. It can be said that fermentation, especially with sugar supplementation, gave rise to greater noni juice quality compared to fresh juice where pure or natural culture fermentation and time (6 or 12 months) had no significant influence.

Introduction

Morinda citrifolia L., known as noni or "Yor" in Thailand (Chan-Blanco *et al*., 2006). It is native to Southeast Asia and tropical Northern Australia and found distributed from Asia and the Pacific to the Caribbean region (Lim, 2013). In Thailand, the young leave are cooked and eaten as vegetable and the fruits are fermented and consumed as fermented drink. In 1990s, fermented noni juice was applied to medicine and widely consumed in North of America, Europe and Asia which they believe that fermentation process can increase the phytochemical content and, consequently, improve health benefit.

Considering physical characteristics, the color of fresh noni juice is brighter than fermented noni juice which turns to dark brown after fermentation. It is thought that the browning is occurred according to enzymatic reaction. Fermentation also led to decreasing of pH as well as increasing of acidity (Nelson, 2012). Therefore, fresh noni juice is sweeter than fermented juice. pH of fresh juice is approximately 3.72 whereas that in fermented noni juice was lower (≤ 3.5) as a result of lactic acid production during fermentation process. Literature reviews demonstrated that noni can be used as a medical treatment for the patients who suffered from e.g. allergies, sleep disorder, migraine headache, depression, Alzheimer's disease, high pressure and cancer (Nelson, 2012). The effects of fresh noni fruit and extracts from many parts of noni tree such as leaf and root on health benefits have been reported. (Wang *et al.*, 2002;Assi *et al.*, 2015). Fresh noni fruit contains antioxidants that can act as free radical scavenging with 1,1-diphenyl-2-picrylhydrazyl (Su *et al*., 2005), inhibit copper-induce low-density lipoprotein oxidation (Kamiya *et al.*, 2004), induce hydrogen peroxide quenching (Chong *et al.*, 2004) and nitric oxide scavenging (Basu and Hazra, 2006). The phenolic compounds such as coumarins and flavonoids in fresh noni fruit have shown antioxidative and anti-inflammatory properties. For example, flavonoids (quercetin and kampferol) exhibited anti-inflammatory and antioxidant properties. Scopoletin and esculetin have been reported as free radicals-scavenging agent and anti-inflammatory action (Dussossoy *et al*., 2011). As can be seen even though fermented juice has been consumed for many decades, nonetheless, the chemical property of fresh and fermented juice has never been compared which

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this might lead to their different therapeutic property.

When fermentation process is focused, the fermentation method is a household process that employs natural culture fermentation. Therefore, consumer safety is of concern since control of specific type of microorganism is difficult and contamination may result in toxicant production by pathogenic microorganisms. Moreover, scientific evidence is necessitated to prove that fermentation process can actually enhance medicinal activity of noni and effects of natural and pure culture fermentation on these properties should be compared. This study was aimed to reveal the effects of fresh and fermented noni juices produced by different conditions i.e. fermentation ingredient, natural and pure culture fermentation process as well as fermentation time on glutathione content and lipid oxidation in Vero cells.

Materials and Methods

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide(MTT),lipid peroxidation (MDA) and glutathione assay kits were purchased from Merck, Sigma-Aldrich (U.S.A.) and Cayman Chemical, respectively. Perchloric acid was obtained from Loba whereas EDTA was obtained from Ajax. Bio-Rad protein assay kit was obtained from Bio-Rad (U.S.A.). Dulbecco's Modified Eagle Medium and fecal bovine serum were bought from Gibco (Thermo Fisher Scientific).

Fresh noni juice preparation

Noni fruits were purchased from local orchards in Phan district, Chiang Rai. The fully ripe fruits were sorted and washed well with clean water. The fruits were chopped and filtered after blending with electrical blender until homogeneous mixture obtained. Noni juice was then pasteurized at 80°C for 1 min. The fresh juice was subjected to analysis directly or stored in -40°C until use.

Natural fermented noni juice preparation

Fully ripe noni fruits were washed well with clean water and then chopped into small pieces. Twenty percent v/w of 50% w/v sugar syrup was added (in case of sugar supplementation) and the mixture was transferred to fermentation tank and left at room temperature for 6 or 12 months (open the container every 15 days to release gas). After fermentation, fermented juice was filtered, 20% sterilized drinking water was added according to the local recipe and then pasteurized at 80°C for 1 min. The fermented noni samples were stored at -40°C until use.

Preparation of pure culture

Starter culture was prepared by inoculation of *Lactobacillus casei* (TISTR 1340), the selective microorganism for noni fermentation obtaining from TISTR Culture Collection services, Thailand, into sterilized Nutrient broth prior to incubation at 37^0C for 18-24 h before utilization. Thereafter, pure culture noni juice was prepared by washing fully ripen noni fruits well with clean water and then chopped. Twenty percent v/w of hot sugar syrup (50%w/v) was added to chopped fruits and the mixture was boiled for 5 min. After that, the mixture was transferred to sterilized container and cooling shocked by ice cold water bath. When the temperature cooled down to less than 45°C, 10% (w/w) of starter culture was added and fermented for 24 h at room temperature. The pure culture noni juice starter was ready for pure culture fermented noni juice production.

Preparation of pure culture fermented noni juice

The preparation was similar to pure culture noni juice but replaced starter culture with pure culture noni juice (see above). In case of sugar supplementation, 20% v/w of hot sugar syrup (50%w/v) was added before boiling for 5 min and the mixture was left to ferment for 6 or 12 months at room temperature. Finally, the fermented mixture was filtered, 20% of sterilized water was added and pasteurized at 80°C for 1 min. The fermented juice was stored at -40°C until use.

Vero cell culture

After recovery from frozen stock, Vero cells (CLS Cell Lines Service, 605372, Germany) were cultured 2-3 passages to reach their regular growth rate. Briefly, Vero cells were grown in a 25 mL conical flasks at 37^oC, 5% CO_2 and 90% relative humidity in complete growth DMEM medium to obtain enough number of cells. At 80% confluency flask, the cells were tryptonized using 80% Trypsin-EDTA. Viable cell density at 1 x 10^5 cells were plated in each well. Cells were then cultured in complete growth medium and incubated in the condition described above. After incubation, the medium was replaced with fresh medium containing different concentrations of noni samples prior to further incubation for 24 h following with determination on cytotoxicity, glutathione content, and lipid peroxidation.

Cell cytotoxicity assay

In order to choose the concentration ranges of noni juice for glutathione and lipid oxidation studies, cytotoxicity of noni juice was carried out. Colorimetric MTT (Tetrazolium) assay was performed according

to Lima *et al.* (2006). After cells were cultured in the medium containing noni juice (0-250 µL/mL) for 24 h the medium was discarded and 20 µL of 5 mg/mL MTT prepared in phosphate buffered saline (PBS) was added. Incubation for 3 h at 37°C in the dark was required. At the end, MTT was removed and 100 µL DMSO added to start the reaction. The mixture was well mixed and absorbant at 570 nm which indicated viability of cells was read. % cells viability and IC_{50} were calculated. Thereafter, the concentrations further employed in Vero cell culture were less than IC_{50} of each type of the juice.

Glutathione content

Glutathione (GSH) level in Vero cells was determined according to Tieze (1969). GSH is oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) resulting in the formation of GSSG and 5-thio-2-nitrobenzoic acid (TNB). GSSG is then reduced to GSH by glutathione reductase (GR) using reducing equivalent provided by NADPH. In a 6-well culture plate, Vero cells were plated. Fresh medium containing noni juice was added in each well after 24 h incubation followed with another 24 h incubation. Cells were then scraped and centrifuged at 2000 x *g* for 10 min at 4°C. Cells were collected and lysed before supernatant collection. The rate of TNB formation in supernatant was measured spectrophotometrically at 412 nm. Determination was performed in triplicate as described by the test kit (Cayman Chemical, MI).

Lipid peroxidation

According to protocol described by lipid peroxidation (malondialdehyde, MDA) assay kit (Sigma-Aldrich, MO, USA), treated cells with noni juice were homogenized on ice in MDA lysis buffer containing BHT and then centrifuged at 13,000 x *g* for 10 min. Equal volume of 2 N perchloric acid was added, vortexed, and centrifuged to remove precipitated protein. The supernatant was collected in centrifuge tube. 2-thiobarbituric acid solution (TBA) (600 µL) was added into 200 µL supernatant and incubated at 95°C for 60 min. After centrifugation at 3,000 rpm for 10 min, the supernatant (200 µL) was collected and MDA formation was measured using spectrophotometer at 534 nm. The content of MDA was expressed as nmole malondialdehyde (MDA) (Esterbauer and Cheeseman, 1990).

Results and Discussion

Cytotoxicity of noni juice in Vero cell

Toxicity determined by MTT assay was performed to elucidate the safety of noni juice and ensure that the selected doses did not affect cell viability in this study. Table 1 shows that fresh fruit juice (FJ) and juices fermented without sugar for 6 months both pure culture (FPNS6) and natural culture fermentation (FNNS6) had no toxicity to Vero cells at the concentrations ranges 0-250 µL/mL whereas similar IC_{50} values (47.37-56.82 µL/mL) were observed in sugar added fermented products i.e. FN12, FP12, FPS6 and FNS6. Hence, concentration ranges of 0-60 µL/mL were employed in the next study for these samples whereas that of FJ, FPNS6 and FNNS6 were in the ranges of 0-250 µL/mL. The different IC_{50} in these samples was thought to be due to different in chemical compositions especially bioactive compounds presented in each individual such as total phenolic and flavonoid content (as described below). Many phenolic compounds including flavonoid have been reported for their cytotoxicity against many human cancer cells including breast and prostate cancer cells (Sak, 2014). Likewise, the phenolic components isolated from Chiga mushroom showed significant antioxidant activity and marked induction of cancer and normal cell toxicity (Nakajima et al., 2009).The results also infer that FN12, FP12, FPS6 and FNS6 probably are able to inhibit proliferation of other abnormal cells like cancer cells.

Table 1. IC_{50} values for Vero cells obtained from MTT assay following exposure to different types of noni juice for 24 h.

Noni juice	IC_{50} (µL/mL)
FJ	>250
FP12	47.37
FN12	55.68
FPS6	45.50
FPNS6	>250
FNS6	56.82
FNNS6	>250

The samples included fresh noni juice (FJ), 6-month natural fermentation without sugar (FNNS6) and with sugar (FNS6), 6-month pure culture fermentation without sugar (FPNS6) and with sugar (FPS6), 12-month natural fermentation with sugar (FN12) and 12-month pure culture fermentation with sugar (FP12).

Glutathione level in Vero cell

Glutathione is an important natural antioxidant in animal. It is inducible by xenobiotic materials and the high level of glutathione inhibits cellular components damage as a result of oxidation reaction caused by reactive oxygen species. It is hypothesized

that induction of glutathione can be one of the possible health defending mechanisms of noni juice. Therefore, the ability of noni juice on induction of total glutathione was conducted in Vero cell.

It can be seen that fresh juice (FJ) and fermentation without sugar supplement either using pure culture (FPNS6) or natural culture (FNNS6) induced glutathione level in Vero cell but to a lesser extent compared to other samples. Concentration above 100 µL/mL was required to induce glutathione by FJ, FNNS6 and FPNS6 (Figure1a-c). However, the samples containing sugar, FN12, FP12, FPS6, FNS6, induced glutathione at the dose as low as 30 µL/mL (Figure 1d-g). It is thought that induction of glutathione reductase, the enzyme that reduces glutathione disulfide (GSSG) to glutathione (GSH),by noni juice is responsible for these observations (D'Autreaux and Toledano, 2007). Nonetheless, the study carried out in rat showed that administration of deacetylasperulosidic acid (DAA), bioactive compound in noni juice, at the doses 15-60 mg/kg/day for 7 days had no effect on this enzyme (Ma *et al*., 2013) implying that up-regulation of glutathione reductase may not be the case or the induction may be achievable by other bioactive compounds in noni juice.The actual induction mechanism is, however, to be elaborated. Moreover, immunomodulatory effects of these samples have been reported. All noni juice samples significantly increased production of Tumor Necrosis Factor-α (TNF-α) and Interferon-γ (IFN-γ) production in mouse splenocytes and PS6 at concentration of 2.5 µL/mL showed the most promising effects which was also confirmed in the *in vivo* study (Patthanawiboon *et al*., 2016). These results suggested that induction of cellular antioxidant and cytokine production in immune systems are possible disease prevention mechanisms of fermented noni juices.

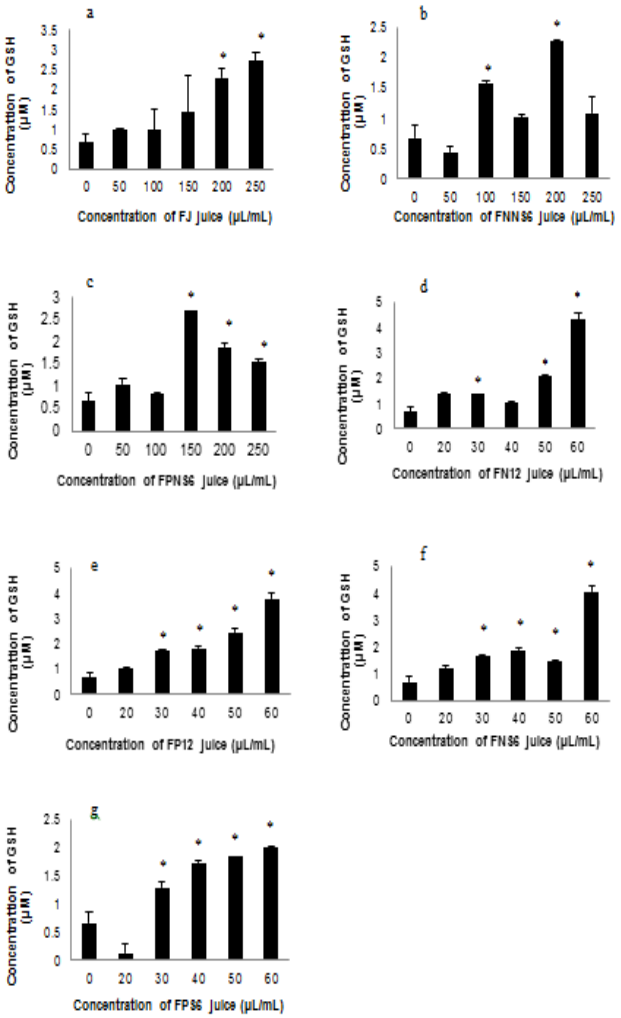

Figure 1. Effect of noni juices on glutathione level in Vero cell where a=FJ, b=FNNS6, c=FPNS6, d=FN12, e=FP12, f=FNS6 and g=FPS6

*,** Mean value was significantly different from control (0 µL/mL) at 95 and 99 % confidence ($P < 0.05$ and 0.01) respectively.

Lipid oxidation inhibition

In relation to induction of glutathione level in Vero cell, the action of noni juice to inhibit oxidation of the lipid in cells was consequently conducted. Vero cells were cultured for 24 h in the presence of various concentrations of noni juice. The amount of malondialdehyde (MDA) accumulated in the supernatant was determined to indicate lipid oxidation level. It was found that all noni samples significantly inhibited lipid oxidation in Vero cells (Figure 2). The most effective samples to inhibit lipid oxidation were sugar containing fermented juice for 6 months, FNS6 and FPS6, at the concentration as low as 20 µL/mL (Figure 2f-g). In accordance with the above findings, sugar was the major fermentation factor that influenced effectiveness of lipid oxidation inhibition while type of culture microorganism had no effect. Fresh noni fruit juice from Tahiti (Tahitian Noni® Juice) was administered to volunteers daily for 1 month. It was revealed that drinking 29.5–118 mL of fresh noni juice significantly reduced MDA-DNA adducts by 44.6–57.4% in smokers. Iridoids were proven to be the bioactive compound that responsible for this result (Wang *et al*., 2002). In addition, inhibition of lipid oxidation by noni juice may be a result of induction of intracellular glutathione as explained above (Figure 1). Balance between free radical and antioxidant is the key of oxidative stress control. Exposure to oxidative stress associates with damage to a wide range of molecular species including lipids and leads to large number of human diseases (Lobo *et al*., 2010). Therefore, elevation of glutathione, natural antioxidant, can retard oxidation of the lipid and promote health

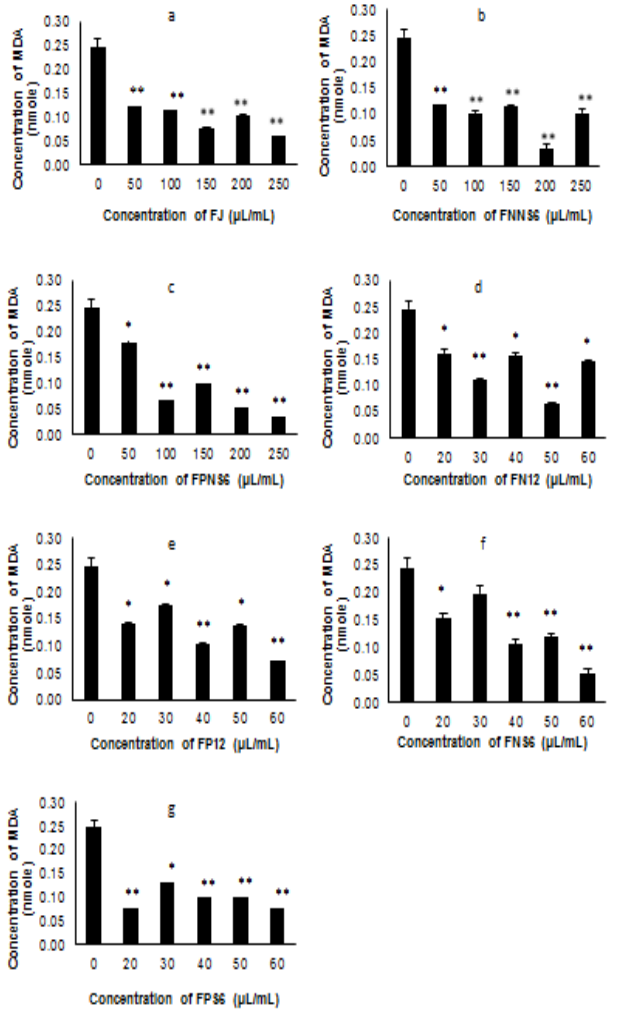

Figure 2. Effect of noni juices on lipid oxidation in Vero cell where a=FJ, b=FNNS6, c=FPNS6, d=FN12, e=FP12, f=FNS6 and g=FPS6

*,** Mean value was significantly different from control (0 µL/mL) at 95 and 99 % confidence ($P < 0.05$ and 0.01) respectively.

protection. Moreover, antioxidant composition in noni samples such as total polyphenol and flavonoid can be one of lipid oxidation inhibition mechanisms. We have reported that fermentation process led to a significant increase in polyphenol content in noni juice but only with sugar supplementation formula whereas type of fermentation microorganism (pure and natural fermentation) and fermentation time between 6 and 12 months had only little effect (Konsue *et al*., 2015). Fresh juice (FJ) and non-sugar supplementation fermented juice both pure (FPNS6) and natural (FNNS6) culture had the lowest total polyphenol content. On the other hand, the higher total polyphenol content was observed in all sugar containing fermentation recipe where the values of FP12, FN12, FPS6 and FNS6 were 133.30±11.67, 133.38±9.87, 174.30±19.40 and 155.13±8.66 mg GAE/ 100 mL, respectively. Similar to total polyphenol results, FJ, FPNS6 and FNNS6 had low flavonoid content than that of FP12, FN12, FPS6 and FNS6 (Konsue *et al*., 2015). Polyphenols react with Folin-Ciocalteu reagent and the blue complex formed is quantified by spectrophotometry (Schofield *et al*., 2001). Even though the method has been validated for its specificity to polyphenol, additive value of TPC can occur as a result of other antioxidant in the samples such as high sugar levels or ascorbic acid (Singleton and Rossi, 1965). Therefore, it was thought that sugar supplementation could enhance TPC value in the sugar supplemented samples. In the current study, total sugar, glucose and fructose in some samples were evaluated. It was noted that total sugar contents were different in FJ (2.32%), FNS6 (12.39%) and FPS6 (12.77%)(data not shown). Likewise, glucose and fructose of FJ were 1.59% and 0.74% whereas that in FNS6 were 3.72% and 8.67% and FPS6 were 3.93% and 8.84%, respectively. However, vitamin C contents in all samples were not markedly different (25.47-43.24 mg/100 mL, data not shown). Therefore, there might be the effects from sugar on TPC content of noni samples. Nonetheless, since significant stronger induction of glutathione content and lipid oxidation inhibition was observed in sugar supplemented juice only, we strongly believed that these were the actual results of higher bioactive compounds in the samples. On the other hand, during fermentation, microorganisms cause plant cell wall breakdown hence releasing of bioactive compounds was encouraged. Sugar is essential component for the growth and metabolic activity of lactic acid bacteria. Study conducted in *L. fermentum, L. reuteri, L. acidophilus* and *L. plantarum* showed that growth of these bacteria in substrate deficiency in sugars contributed to growth limitation (Charalampopoulos *et al*., 2002). This can explain why sugar supplemented fermentation rendered superior amount of bioactive compounds compared with non-sugar supplemented samples and fresh juice, respectively. In addition, lactic acid fermentation has established as a suitable tool for novel applications as functional food dietary supplements or pharmaceutical preparations. Similarly, fermented myrtle berries exhibited a marked antioxidant activity compared to non-fermented berries and this was in agreement with a twice inhibition of linoleic acid peroxidation. These results could be explained by increasing of total phenols, flavonoids and anthocyanins which are the microbial metabolites synthesized during fermentation and/ or are released from the plant during fermentation from cell wall breakdown process (Gobbetti *et al.*, 2010). Moreover, lactic acid bacteria have been employed in production of many bioactive compounds (Di Cagno *et al.*, 2010; Coda *et al*., 2010; Rizzello *et al.*, 2012). It is also pivotal to point out that prolong of

fermentation period to 12 months is not necessitate to enhance these responds. This is in agreement with previous report which showed that *L. casei* produced lactic acid during noni fermentation and it could not survive under low-pH condition after 3 weeks (Wang *et al*., 2009).

Consistent to lipid oxidation reduction ability, we have reported that FRAP values slightly increased in fermented noni juices (Konsue *et al*., 2015). Previous study also reported that Costa Rican noni juice possessed antioxidant properties probably associated with phenolic compounds. Moreover, the sample also showed anti-inflammatory action which was believed to be enhanced by antioxidant effects (Dussossoy *et al.*, 2011).

Conclusion

Fermentation of noni juice in the presence of sugar led to a significant increase glutathione content and a strong lipid oxidation inhibition in the cells. It can be said that sugar supplementation was an important factor influencing chemical quality of fermented noni juice where type of fermentation microorganism and fermentation time did not show significant effect. Finally, induction of glutathione levels and reduction of cellular lipid oxidation are oxidative stress inhibition mechanisms of noni juice which enhance health defending system in human. These effects were pronounced in fermented juice rather than fresh juice.

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