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FERMENTED *Morinda citrifolia* LINN JUICE HAS REDUCED ANTIOXIDANT CAPACITY AND DEMONSTRATES CYTOTOXICITY TOWARDS HUMAN BREAST CANCER CELLS

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Abstract

Morinda citrifolia Linn (noni) is a plant belonging to the *Rubiaceae* family. Recently, fermented noni fruit juice has undergone a surge in popularity, driven by its supposed health benefits. The aim of this study was (i) to determine the nutrient composition of fermented noni fruit juice, and (ii) to compare the antioxidant and cytotoxic properties between unfermented (UNJ) and fermented (FNJ) noni fruit juice. The results showed that FNJ is rich in carbohydrates (81.3 g/100 g DW), but low in both protein (5.4 g/100g DW) and fat (4.0 g/100g DW). FNJ also contains high amounts of amino acids (AAs) glutamic acid, aspartic acid and leucine. UNJ exhibited higher free radical scavenging and antioxidant activity than FNJ. UNJ was found to exhibit comparable cytotoxicity across all cells tested. Notably, FNJ demonstrated higher toxicity against normal human breast (184B5) cells (up to 2.6-fold) compared to human breast cancer (MCF7 and MDA-MB-231) cells. To our knowledge, our study is the first to demonstrate that noni fruit juice (both fermented and unfermented) is toxic to normal breast cells. Their toxicity toward normal breast cells and their non-selective targeting may require in-depth *in vivo* study to reveal their potential therapeutic benefits. Caution is warranted as noni fruit juice products from other sources may possess different toxicological and pharmacological profiles.

INTRODUCTION

Cancer is the second leading cause of death globally, with breast cancer being the most common among women [1]. In 2018, global cancer statistics reported that there were approximately 2 million new cases diagnosed worldwide. Many patients are eager to make dietary and lifestyle changes to enhance overall health and increase the probability of long-term survival [2]. One of the common lifestyle behaviours adopted by patients with breast cancer is the use of complementary and alternative medicine (CAM) during or after cancer treatment. According to survey research, the noni plant has been consumed among patients with breast cancer as an herbal intervention for cancer treatment [3].

Noni is the general name given to the species *Morinda citrifolia* Linn (Rubiaceae), which is a plant typically found in the tropics [4]. Different parts of the plant, including the fruit, have been traditionally used as folk remedies for a long list of ailments, including cardiovascular diseases, diabetes and cancer [5]. The crude extracts of various parts of the plant have been reported to contain a significant number of

bioactive components which are therapeutically beneficial for a broad range of pathological conditions [6]. In view of the potentially wide range of health benefits offered by the noni plant, commercial interest has tremendously increased in recent years [5]. Notably, the noni fruit juice is often commercialized fresh or fermented, and either consumed pure or mixed with other juices [7]. The beneficial effects of fermented foods on gut health and its microbiome have resulted in vast interest in the field of food science [8]. Sun *et al.* [9] had previously reported a range of antioxidant and antiproliferative activities found in various berries and citrus fruits; not many reports can be found on their fermented products. In 2018, Wang *et al.* [10] reported higher radical scavenging activities of fermented juice prepared with by-products (pulp and rind) of seed-watermelon juice (*Citrullus lanatus* sp. vulgaris var. megalaspermus). Most recently, Mostafa *et al.* [11] reported on the potential antiproliferative properties of fermented date juice against larynx cancer. The demand for fermented beverages is booming as a trend. Nonetheless, our current knowledge on the chemical properties of fermented noni fruit juice is still limited. More scientific evidence is required to determine if the fermentation process actually enhances the medicinal activities of noni. Also, the effects of natural and pure culture fermentation on these properties warrants investigations.

As a medicinal plant, Hirazumi and colleague were among the first to report that the polysaccharides found in noni fruit juice contain anti-tumour activity that enhances the release of cytokine interferon-gamma (*IFNγ*) from thymocytes [12]. Additionally, noni fruit juice was found to reduce the angiogenic initiation, growth rate and proliferation in human breast tumours [13]. As mentioned earlier, even though the exact mechanism of its cancer-preventative effects remains elusive, a large proportion of cancer patients are consuming noni for the prevention and as nutritional support following conventional chemotherapy [3]. It is hence important that the cytotoxic action of noni fruit juice on human breast cancer cells be examined further. Therefore, this study aims to investigate the nutrient composition of fermented noni fruit juice and to evaluate the *in vitro* antioxidant and cytotoxic properties of the unfermented (UNJ) and fermented (FNJ) noni fruit juice.

MATERIALS AND METHODS

Plant Material

Morinda citrifolia L. (noni) was collected from Alor Gajah, Melaka (Malaysia) in March 2019. The plant was identified, authenticated by Dr. Rahmad Zakaria and a voucher specimen (No. 11784) was submitted to the herbarium at the School of Biological Sciences, Universiti Sains Malaysia, Penang (Malaysia).

Preparation of Unfermented Noni Fruit Juice (UNJ)

One hundred twenty kilograms of fresh noni fruits (ripen) were harvested, washed well with 1% NaCl solution and rinsed with water. The fruits were homogenized with a blender until a homogenous mixture was obtained. The mixture was filtered and stored in a clean container. The final yield after filtration was 56 L, equivalent to 32.7 g/kg in DW after freeze-drying.

Preparation of Fermented Noni Fruit Juice (FNJ)

One hundred twenty kilograms of fresh noni fruits (ripen) were harvested, washed well with 1% salt solution and rinsed with water. The mixture was blended together with 12 L of organic brown rice vinegar using a blender until a homogenous mixture was obtained. The mixture was stored in a clean container for fermentation at room temperature for 7 days. The fermented juice was filtered and subjected to analysis. The final yield after filtration was 81 L, equivalent to 47.3 g/kg in DW after freeze-drying.

Determination of Nutritional Content

The nutritional content and amino acid composition analyses were performed using the Kjeltec™ 8100 Protein Analyzer (Foss, Denmark) and the 1260 Infinity Quaternary LC Fluorescence Detector Agilent (Agilent, USA). Carbohydrate content and energy was determined using in-house method LWI-TEC-F009, LWI-TEC-F007 and LWI-TEC-F008 based on the Methods of Analysis for Nutrition Labeling [14] and Food Regulations 1985 (Ministry of Health Malaysia). Crude protein content was determined according to MS 1194:1991 Methods for Determination of Crude Protein in Foods and Feeds. Amino acid composition was determined using methods described in Yang *et al.* [15]. Fat content was determined using in house LWI-TEC-F003 based on AOAC Official Method 989.05 (Liquid-Liquid Extraction) and AOAC Official Method 920.39 (Soxhlet Extraction).

Evaluation of Antioxidant Capacity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free-Radical-Scavenging Assay

The capacity of the juices to scavenge DPPH was measured according to [16] with some modifications. Briefly, 25 µl of UNJ and FNJ at different concentrations were added to 150 µl of 0.04 mg/mL DPPH solution in methanol. The mixtures were incubated in the dark for 20 min and absorbance was measured at 517 nm using the Multiskan GO Microplate Spectrophotometer (Thermo Scientific, USA). Different concentrations of Trolox (10–60 µg/mL) were used as standards.

The DPPH radical scavenging activity was calculated using the following equation:

DPPH inhibition (%) = Absorbance control – Absorbance sample/Absorbance control x 100

The results were expressed as IC_{50} values (the concentration of noni fruit juice required to produce 50% inhibition of DPPH radical). Quercetin and rutin were used as positive controls. The absorbance of DPPH without juice (blank control) was also measured.

Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing activity of the juices were determined according to Benzie & Strain [17] with minor modifications. The working FRAP reagent was freshly prepared by mixing 300 mmol/L acetate buffer, 10 mmol/L TPTZ solution (2,4,6-tripyridyl-s-triazine) in 40 mmol/L HCl and 20 mmol/L $FeCl_3.6H_2O$ in a ratio of 10:1:1. 300 µl of FRAP reagent was mixed with 5 µl of juice (70 mg/ml), incubated at 37°C for 30 min in the dark. The absorbance was measured at 595 nm and results were expressed as mmol Fe^{2+}/g crude extract based on a calibration curve plotted using $FeSO_4$ solution with different concentrations (0–1 mM). Quercetin and rutin were used as positive controls. The antioxidant capacities of the juices were determined via two independent biological experiments with three technical replicates per experiment.

Cell Culture

Normal human breast cell line (184B5) and human breast cancer cells lines (MCF7 and MDA-MB-231) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM-08459-64; Nacalai Tesque, Japan) supplemented with 10% Fetal Bovine Serum (FBS-FBSEU500; TICO Europe, Netherlands) and 100 units/mL Penicillin/Streptomycin (09367-34; Nacalai Tesque, Japan) in a humidified incubator at 37 °C and 5% CO_2, except for 184B5 where MEGM™ Mammary Epithelial Cell Growth Medium BulletKit™ (Lonza, USA) was used. Sub-culturing and/or media changing were performed every two to three days depending on cell confluence.

Cytotoxicity Assay

The cytotoxic activity of the juice were examined using MTT assay as described by Mosmann [18] with some modifications. Cells were allowed to adhere overnight prior to treatment with FNJ and UNJ at various concentrations. Medium control (blank medium) and cell control (cells without juice treatment) were also included in the same plates. Following 72 h incubation, media was removed, and cells were supplemented with 100 µl fresh media and 20 µl of 5 mg/mL MTT solution in phosphate-buffered saline (Thermo Fisher Scientific, USA). Plates were returned to the incubator (37°C) for a 4 h incubation period. All solutions were then aspirated and 200 µl DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm zeroed against the blank wells (0 cells/well) for each well and the respective IC_{50} values were determined from the percentage cell viability against the juice concentration curve. Results were obtained from three independent experiments; triplicate assays were performed for each run.

Statistical Analysis

Data obtained were expressed as mean ± standard deviation (SD) using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA analysis with LSD post hoc test for multiple comparisons was used to compare mean values. A p-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In our study, FNJ was found to be rich in carbohydrates (81.3 g/100 g DW) but low in proteins (5.4 g/100g DW) and fat (4.0 g/100g DW; Table 1). Glutamic acid, aspartic acid and leucine are the most abundant amino acids (AAs) found in FNJ (Table 2). Asparagine and tryptophan were found to be the least in FNJ. Overall, nutritional analysis of the fermented noni fruit juice revealed significant amounts of carbohydrates and low fat content, in agreement with a previous study by Chunhieng *et al*. [20]. However, the protein content reported in the same study was five-fold higher (25.6 g/ 100 g DW) compared to our analysis [20]. This may be attributed to the differences in growth conditions (i.e. soil, water, etc.), methods of farming and fermentation. Our amino acid composition analysis of FNJ is relatively similar to a previous study [21]. Leucine, an essential amino acid required for human nutrition to support protein synthesis [22,23], was found to be present in high amounts in the noni fruit juice, similar to Lindsay & Golden [21].

Table 1: Proximate composition of fermented noni fruit juice (dry weight)

Composition	
Energy (kcal/100g dry weight)	383
Proximate composition (g/100g dry weight)	
Carbohydrate	81.3
Protein	5.4
Fat	4.0

All values were determined as per described in Section 2.3

Table 2: Amino acid content of fermented noni fruit juice (dry weight)

Amino acid	(mg/kg dry weight)
Alanine	1766
Arginine	1347
Asparagine	ND (<10)
Aspartic acid	3286
Glutamic acid	4367
Glycine	1367
Histidine	536
Isoleucine	914
Leucine	2101
Lysine	858
Methionine	387
Phenylalanine	1087
Proline	747
Serine	1424
Threonine	1190
Tryptophan	ND (<10)
Tyrosine	1036
Valine	1097

All values were determined as per [12].

To counteract the potential threats of oxidative stress, antioxidants found in spices, vegetables and fruits act through several mechanisms, mainly by scavenging and reducing free radicals [24]. Overall, both juices exhibited relatively low FRAP values and DPPH radical scavenging activity in comparison to the positive controls (quercetin and rutin; Table 3). With regards to DPPH radical scavenging ability, UNJ demonstrated a lower IC_{50} (43.78 ± 1.27 mg/ml), indicating greater overall antioxidant activity in comparison to FNJ (IC_{50} = 353.83 ± 33.75 mg/ml). Results obtained from the FRAP assay for both juices were similar to DPPH; UNJ exhibited stronger ferric ion reducing activities (0.0024 ± 0.0002 mmol Fe^{2+}/g) compared to FNJ (0.0009 ± 0.0000 mmol Fe^{2+}/g). Our results revealed that the antioxidant capacity of the noni fruit juice can potentially be influenced by the process of fermentation. UNJ possessed a relatively higher reducing power and scavenging potential than FNJ, indicating that fermentation has reduced its antioxidant capacity. This finding differs from most studies which have suggested that fermentation contributes to increased antioxidant components and antioxidant potential of substrates in fermented plant-based foods [25]. Nonetheless, high phenolic compounds found in unfermented noni fruit juice acting as free radical scavengers and preventing oxidation reactions has been reported by Bramorski et al. [26] and Dussossoy et al. [27], which are consistent with our study.

Table 3: Antioxidant capacities of the unfermented (UNJ) and fermented (FNJ) noni fruit juice

Sample	DPPH IC_{50} (mg/ml)	FRAP (mmol Fe^{2+}/g)
UNJ	43.78 ± 1.27^{a}	0.0024 ± 0.0002^{a}
FNJ	353.83 ± 33.75^{b}	0.0009 ± 0.0000^{b}
Rutin	0.0938 ± 0.0121^{c}	4.493 ± 0.886^{c}
Quercetin	0.0328 ± 0.0056^{d}	20.915 ± 2.484^{d}

All values were expressed as mean ± SD (n = 2). Different superscript letters in the same column indicate the mean values are significantly different, according to one-way ANOVA analysis and post hoc LSD test ($p < 0.05$). Rutin and quercetin were used as positive controls

Over the last decade, the commercialization of noni as herbal food (mostly marketed as noni fruit juice) has made it a widely-traded food supplement worldwide, based on health claims associated with some of its constituents. However, scientific evidence on the therapeutic properties of noni fruit juice is very limited; most of the information regarding its health benefits is anecdotal. The variable amounts of bioactive compounds found in different noni fruit juice may be due to differences in fermentation practices. These include exposure of contents to air and/or to sunlight, the specifications of the fermenting vessel, additives, refrigeration, and duration of fermentation. Moreover, various studies found that the total phenolic content, antioxidant potential and ascorbic acid content in noni fruit itself vary according to the region where the plant is cultivated and the ripening stage of the fruit [28-31].

Previous studies have demonstrated the cytotoxic effects of (i) methanol extracts of unfermented noni fruit, and (ii) the polysaccharide fraction isolated from the noni fruit, against MCF7 cells [32,33]. The former study found that 0.1 mg/mL of crude extract exhibited cytotoxicity against breast cancer (MCF7) at 29%. The latter study found that *M. citrifolia*-treated MCF7 cells exhibited dose-dependent cytotoxicity, with considerable decrease in the levels of Bcl-2 (anti-apoptotic protein) and an increase of p53 levels (pro-apoptotic protein) following a 48hr treatment. However, both studies did not perform experiments on normal breast cells. To determine the cytotoxic action exhibited by UNJ and FNJ in our study, human normal breast (184B5) and breast cancer (MCF7 and MDA-MB-231) cells were incubated with various concentrations of FNJ and UNJ for 72 h. Both FNJ and UNJ exhibited comparable cytotoxicity

towards MCF7 and MDA-MB-231 cells, with IC_{50} values in the range of 6.97-9.25 mg/ml (Table 4). For 184B5 cells, UNJ has a comparable IC_{50} value to that recorded in the two breast cancer cell lines, indicating lack of selectivity. The fold selectivity of UNJ for both breast cancer cell lines over 184B5 (0.86 for MCF7 cells; 0.92 for MDA-MB-231 cells) is higher when compared to FNJ. FNJ has a lower IC_{50} value for 184B5 (3.46 mg/ml) compared to MCF7 and MDA-MB-231. Interestingly, the fold selectivity of FNJ for both breast cancer cell lines over 184B5 is 0.50 and 0.38, respectively. This indicates that it is also cytotoxic to normal cells. However, the cytotoxicity measured for both juices were still lower in comparison to doxorubicin, (IC_{50} less than 0.42 µg/ml across all cell lines). To our knowledge, our study is the first to demonstrate that noni fruit juice (both fermented and unfermented) is toxic to normal breast cells. One possible factor contributing to the cytotoxic effects observed in both juices may be attributed to the presence of compounds that act as toxicants, inducing the overproduction of nitric oxide, reactive oxygen species and subsequent oxidative stress, leading to cell toxicity. This may lead to mitochondrion dysfunction or the production of oxidative products that may damage cell DNA, leading to toxicity. Its toxicity towards normal breast cells and its non-selective targeting of breast cancer cells warrants further investigations using *in vivo* models to evaluate the potential therapeutic benefits as data from several preclinical studies and a human clinical safety study have revealed no adverse health effects, even at high doses [34].

Table 4: Cytotoxic activities of various noni fruit juices

Samples	MDA-MB-231		MCF7		184B5
	IC_{50}	Fold selectivity	IC_{50}	Fold selectivity	
UNJ (mg/ml)	8.69 ± 1.07	0.92	9.25 ± 0.18	0.86	7.96 ± 0.00
FNJ (mg/ml)	9.10 ± 0.26	0.38	6.97 ± 1.44	0.50	3.46 ± 0.08
Doxorubicin* (µg/mL)	0.28 ± 0.07	0.61	0.42 ± 0.01	0.40	0.17 ± 0.05

Human normal breast (184B5) cells and breast cancer (MCF7 and MDA-MB-231) cells were treated with various concentrations of fermented noni fruit extract (FNJ) and unfermented noni fruit extract (UNJ). Effects of extracts on the viability of cells were examined by measuring relative cell viability using MTT assay. All values were expressed as mean ± SD (n = 3). Doxorubicin was used as a cytotoxic reference drug. *IC_{50} values for doxorubicin were adapted from our previous study [19]. Fold selectivity values were determined by dividing IC_{50} of 184B5 cells with the IC_{50} of MDA-MB-231 and MCF7 cells, respectively

Thus far, both animal models and clinical trials reported no toxicity from the consumption of unfermented noni fruit juice [35]. With regards to animal studies, two research reported that noni fruit juice exhibited synergistic or additive beneficial effects with some anticancer agents (i.e. cisplatin and 5-fluorouracil) against Ehrlich ascites carcinoma (EAC) tumour model in mice, suggesting that noni fruit juice may be useful in the treatment of cancer either on its own or in combination with current chemotherapy drugs [36,37]. Additionally, two human intervention studies on smokers also suggested that noni fruit juice may be able to mitigate oxidative damage of DNA and provide protection against tobacco smoke-induced DNA damage in current heavy smokers [38,39]. The anticancer potential and the other beneficial health effects of noni in humans have been reviewed extensively elsewhere [4,7,40]. Although a few *in vitro* and *in vivo* studies suggest an unidentified anticancer activity present to a small degree, clinical data are currently lacking to either support or refute the use of noni plant products, in particular fermented noni fruit juice, as nutritional support for breast cancer patients. We are aware of the shortcomings of our study, primarily the lack of mechanistic insight. However, there is a crucial need to report these findings as they may have a significant impact on educating patients regarding the consumption of such products, especially those that assert unsubstantiated health benefits. Further studies are required to evaluate the safety profile *in vivo* and cytotoxic effects of commercialized fermented noni fruit juice against different cell types and to determine the sensitivity and resistance of different tumour types towards it.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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