

Toxicity and Antioxidant Tests of *Morinda citrifolia* (noni) Seed Extract

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Abstract: The aim of the current study was to evaluate *Morinda citrifolia* (noni) seed extract, a food ingredient, for potential toxicity and antioxidant activity. Nitrates, nitrites, phytic acid, oxalic acid, as well as aflatoxins B1, B2, G1 and G2 were not detected in the extract. The extract was also non-cytotoxic ($LC_{50} > 1$ mg/mL) in the 24 and 40 h brine shrimp toxicity test. There were no symptoms of toxicity in a subacute (28 day) oral toxicity test in Sprague-Dawley rats. Noni seed extract did not display any genotoxic potential in a primary DNA damage test in *E. coli* PQ37. The extract did exhibit significant antioxidant activity in the Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) tests.

Key words: Antioxidant, *Morinda citrifolia*, natural toxicants, noni, seed, toxicity tests

INTRODUCTION

Morinda citrifolia, commonly known as noni, is a small to medium sized tree that is widely distributed in the tropics. Noni fruit and leaves have been consumed as traditional foods in Southeast Asia and in the Pacific islands (West *et al.*, 2006; West *et al.*, 2007). Whole noni seeds were also eaten (Benthall, 1946; Morton, 1992). Noni fruit juice consumption outside the tropics has increased dramatically during the past decade. In French Polynesia, noni fruit puree and juice constitute the largest volume in agricultural exports, especially to the United States, where they are more than double the quantity of black pearls (Service du Commerce Exterieur de Polynesie Frances, 2008a). In 2005 alone, more than 6,000 metric tons of noni fruit puree was produced, topping copra (Service du Commerce Exterieur de Polynesie Frances, 2008b). In the production of noni fruit puree, the seeds are discarded. Air dried noni seeds constitute 2.5% of the total fruit weight (Nelson, 2005). In French Polynesia alone, the potential annual yield of noni seeds is greater than 150 metric tons.

Noni seeds contain linoleic, oleic and palmitic acids, as well as β -sitosterol, campesterol, and stigmasterol. An essential fatty acid rich vegetable oil is produced from the seeds, which also contains α -tocopherol, δ -tocopherol, and γ -tocopherol (West *et al.*, 2008a). Ricinoleic acid has also been reported to occur in noni seed oil (Daulatabad *et al.*, 1989). Various categories of phytochemicals have been identified in the seed, such as iridoids, lignans, flavonols, and triterpenes (Yang *et al.*, 2009). The iridoids identified in the seeds include deacetylasperulosidic acid, asperulosidic acid, loganic acid, and rhodolatoside. The lignans include americanin, americanin A and americanin D, 3,3'-bisdemethylpinoselinol, 3,4,3',4'-tetrahydroxy-9,7' α -epoxy lignano-7 α ,9'-lactone, and isoprincepin.

Currently, quercetin-3-O- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside is the only flavonol glycoside reportedly identified in noni seeds. Triterpenes in the seed are ursolic acid, daucosterol, and 19-hydroxyl-ursolic acid. Scopoletin, 3-methylbut-3-enyl-6-O- β -D-glucopyranosyl- β -D-glucopyranoside, 4-ethyl-2-hydroxyl-succinate, and 5-hydroxymethyl-2-furancarboxaldehyde were also identified.

The seeds have a hard fibrous coat (Nelson, 2006), and an extract is produced from the seeds which separates the contents from the indigestible fibrous material. The extract possesses antioxidant activity and is used as a food ingredient. The purpose of the present study is to conduct an initial investigation of the safety and antioxidant activity of noni seed extract.

MATERIALS AND METHODS

The study was conducted by the research department of Tahitian Noni International, Inc. during 2006-2009. Noni seeds were collected from discarded material from the fruit finishing process on the island of Tahiti. Several batches of seeds were washed and dried. Dried seeds were then milled and extracted with an aqueous ethanol solution. The extraction solution was then filtered and dried with gum acacia to total solids content of less than 15%, as determined gravimetrically by loss on drying in an oven at 100°C. To avoid potential interferences, an aqueous seed extract was not dried with gum acacia for the primary DNA damage and twenty four hour brine shrimp toxicity tests.

Chemical analyses: Nitrate and nitrite concentrations were measured by a high performance liquid chromatography (HPLC) method (Siu and Henshall, 1998). Noni seed extract was extracted with boiling water,

followed by chloroform extraction, to remove lipids, and precipitation of proteins by centrifuge. Separation and detection were performed with a high-performance anion exchange column and pulsed amperometric detection. Nitrate and nitrite standards were used to quantitate the amounts in the seed extract.

Phytic acid content was also determined by HPLC (Lehrfeld, 1994). Samples were extracted in a sonicator and diluted with water, prior to loading on a silica-based anion exchange column. The column was washed with water and eluted with an aqueous hydrochloric acid solution. The eluate was evaporated to dryness, reconstituted with tetrabutylammonium hydroxide buffer, filtered, and injected into an HPLC with a macroporous polymer anion exchange column. A methanol solvent system was used for the separation, followed by detection with a refractive index detector. Oxalic acid content was determined according to an AOAC method for detection of organic acids in food (AOAC, 2005a). The sample was extracted with a weak sulfuric acid solution, followed by separation on a C18 reverse phase HPLC column. Detection of oxalic acid was performed with a UV detector, with quantitation being made against standards. Aflatoxin content was determined according to an AOAC method (AOAC, 2005b). The sample was extracted with a methanol-water solvent mixture, filtered and then applied to an affinity column with a monoclonal antibody specific for aflatoxins B1, B2, G1, and G2. The column was eluted with methanol, the eluate reacted with bromine, and aflatoxins determined with a fluorescence detector (360 nm excitation and >420 nm cutoff emission).

Primary DNA damage test in *E. coli* PQ37 (SOS-chromotest): The SOS-chromotest in *E. coli* PQ37 was used to determine the potential for noni seed extract to induce primary DNA damage. This test was carried out according to a previously developed method (Fish *et al.*, 1987). *E. coli* PQ37 was incubated at 37°C in the presence of the extract at a concentration of 1000 µg/mL in a 96-well plate. Samples, both with and without S9 mix, were evaluated in triplicate. Following incubation, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was added to the wells to detect β-galactosidase enzyme activity, which is induced during SOS repair of damaged DNA. Nitrophenyl phosphate is also added to the wells to measure alkaline phosphatase activity, an indicator of cell viability. The samples were again incubated and the absorbances of the samples, blanks and controls were measured at 410 and 620 nm with a microplate reader. Vehicle blanks and positive controls, 5 µg/mL 4-nitroquinoline 1-oxide (4NQO) and 100 µg/mL 2-aminoanthracene (2AA), were included in this test. The induction factor of each material was calculated by

dividing the absorbance of the sample at 620 nm by that of the blank, while also correcting for cell viability. Induction factors less than two indicate an absence of genotoxic activity.

Twenty four and forty hour brine shrimp toxicity test:

The 24 h brine shrimp toxicity test has been used for more than 20 years for the assessment of the cytotoxicity of plant materials (Meyer *et al.*, 1982). Replicate samples of the aqueous seed extract were prepared at a concentration of 1 mg/mL in 3% NaCl, pH 8. A green tea infusion was prepared in the same manner to serve as a well known food control. Brine shrimp (*Artemia salina*) eggs were hatched in salt water (3% NaCl) and nauplii were transferred to vials containing 15 mL of test solution. These were incubated for 24 h at room temperature. Following incubation, the numbers of active nauplii were counted and mean percent survival calculated for both samples, with corresponding standard deviations. Additional incubation to 40 h was also carried out to determine the effect of longer exposure times. The concentration resulting in lethality to 50% of the nauplii (LC₅₀) was determined from survival rates. In this test, as well as in the reverse mutation and primary DNA damage tests, comparisons were made with Student's t-test.

Subacute (28 day) oral toxicity test:

Ten nulliparous female Sprague-Dawley rats, age 6-9 weeks, were acclimated for three days at 16-21°C and 12 h light/dark cycle in stainless steel cages with suspended flooring. Feed and water were provided *ad libitum*. The animals were then randomly assigned to a test or control group, with five in each group. Animals in the test group were administered noni seed extract daily at a dose of 1000 mg/kg body weight (b.w.) by gavage for 28 consecutive days. Following the same dose schedule, the control group was administered 0.9% NaCl. The daily gavage volume was 10 mL/kg b.w. Immediately after dosing on day 1, the animals were observed daily for symptoms of toxicity and general health. Animals were weighed prior to the initial dose on day 1 and then weekly until termination of the study. The mean and standard deviations (SD) of the weights of the two groups were calculated and compared statistically with Student's t-test.

Antioxidant assays:

The Oxygen Radical Absorbance Capacity (ORAC) test was performed according to a previously published method (Huang *et al.*, 2002). Noni seed extract was dissolved in 0.75 mM phosphate buffer and filtered. The dissolved extract was combined with fluorescein sodium solution in phosphate buffer and incubated at 37°C in a 96 well microplate reader. 2,2'-Azobis(2-amidinopropane) dihydrochloride in phosphate buffer was added to initiate the reaction. Fluorescence

Table 1: Primary DNA damage assay in *E. coli* PQ37 (SOS-chromotest)

Compound	Concentration (µg/mL)	Induction factors	
		+S9	-S9
Noni seed extract	1000	0.96±0.04	1.07±0.05
4NQO	5	not tested	7.5±0.7*
2AA	100	7.6±1*	not tested

*: p<0.05, compared to blank

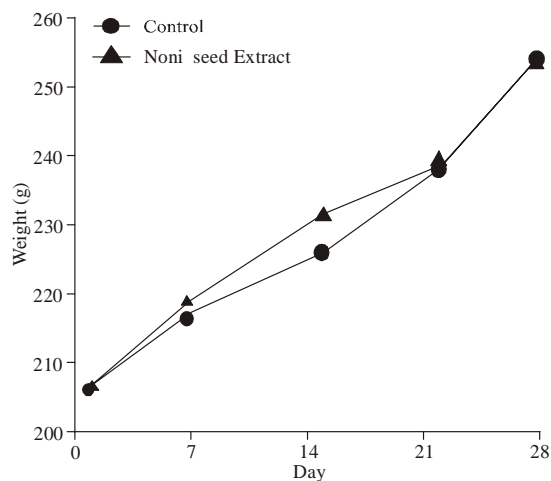


Fig. 1: Weight gain of female rats in 28 day oral toxicity test

common natural toxicants. They also suggest a very low was measured with a microplate reader, with excitation at 485±20 nm and emission at 520±20 nm. Trolox standards were also prepared and assayed according to the same conditions. Fluorescence intensities were measured for 30 minutes and areas under the curve were used to calculate antioxidant activity as µM trolox equivalents/g.

The ferric reducing antioxidant power (FRAP) test was also performed according a previously reported method (Benzie and Strain, 1996). In this test the extract is dissolved in buffer, filtered, and combined with a ferric chloride and 2,4,6-tripyridyl-s-triazine reagent, followed by incubation at 37°C. Absorbance readings were repeatedly made at 593 nm with a spectrophotometer. The change in absorbance was compared to a Fe(II) standard and the results calculated as µmol reduced Fe/g.

RESULTS AND DISCUSSION

The identification of potential natural toxicants and antinutrient substances is an important aspect of the safety evaluation of any plant-derived food. No known antinutrient compounds have been previously identified in noni fruit or leaves. In noni seed extract, nitrates and nitrites were not present at or above detection limits of 1 and 0.5 mg/kg, respectively. Phytic acid was not found in noni seed extract, at a detection limit of 0.1 g/100 g. Oxalic acid was not detected, with a concentration less than 0.1 g/100 g. Aflatoxins B1, B2, G1, and G2 were

Table 2: Survival rates (mean±SD) in the 24 and 40 h brine shrimp toxicity test

Sample	24 h survival (%)	40 h survival (%)
Noni seed extract	84.2±25.8*	66.1±14.0*
Green tea	56.7±16.7*	0

*: LC₅₀ > 1 mg/mL, non-toxic

Table 3: Weights (mean±SD), in g, of female rats in 28 day oral toxicity test

Day	Control	Noni seed extract
1	205.4±6.2	206.4±6.4
7	216.6±4.9	218.8±2.6
15	226.0±13.0	231.4±8.0
22	238.0±12.6	239.0±11.1
28	254.0±13.6	253.5±9.1

also not present in noni seed extract above the 0.5 µg/kg detection limit. These results reveal the absence of some risk for mycotoxin contamination during the harvesting and storage of noni seed, as well as during the production of noni seed extract.

In the SOS-chromotest (Table 1), the mean induction factor of the extract was approximately one, with and without S9 mix, demonstrating that the extract did not induced DNA repair at a frequency greater than the blank. These results do not reveal any genotoxic potential associated with the noni seed extract. The SOS-chromotest results have a high level of agreement (86%) with those from the reverse mutation assay (Legault *et al.*, 1994). Therefore, the SOS-chromotest has some utility in predicting potential mutagenicity, in addition to primary DNA damage.

More than 50% of the brine shrimp nauplii survived after 24 h incubation in 1 mg/mL noni seed aqueous extract or green tea infusion, indicating a lack of cytotoxic potential (LC₅₀ >1 mg/mL). There was no significant difference between the 24 h survival rates, which were 84.2±25.8% for noni seed extract and 56.7±16.7% for green tea, (Table 2). With the incubation times extended to 40 h, an excellent survival rate of 66.1±14.0% was observed with noni seed extract. The 40 h survival rate in green tea, however, was zero.

During the subacute (28 day) oral toxicity test, noni seed extract ingestion did not cause any deaths. Animals in both groups gained weight during the study, (Fig. 1). There were no significant differences in the mean weights of both groups at any time during the study, (Table 3). No symptoms of toxicity were observed in this test. It appears that noni seed extract is well tolerated and is without any apparent toxicity.

The seed extract possesses notable antioxidant activity. The ORAC test revealed that it contained 97.9 µM trolox equivalents/g. The FRAP test demonstrated that the seed extract contained 70.2 µmol reduced Fe/g. This potent antioxidant activity is consistent with previously published *in vitro*, *in vivo*, and human studies of the other parts of the noni plant (Mohd Zin *et al.*, 2002;

West *et al.*, 2009a; Wang *et al.*, 2009). The trolox equivalents/g of noni seed extract, as measured in the ORAC test, is approximately three times greater than polyphenol-rich beverages, such as tea and pomegranate, grape, blueberry, cranberry, açai, and orange juices (Seeram *et al.*, 2008). Comparing the FRAP test result to those of these same beverages, the reducing power of the seed extract is more than eight times greater.

CONCLUSION

Noni seed extract appears to be non-cytotoxic, with an $LC_{50} > 1$ mg/mL. It was also non-toxic in the 28 day oral toxicity test in rats. Natural toxicants were not found in the extract nor were any potential antinutrient substance identified, which was further demonstrated by the appropriate weight gain observed in the 28 day test. The primary DNA damage test did not reveal any genotoxic risk associated with consumption of noni seed extract. These results are consistent with multiple *in vivo* and *in vitro* toxicity tests, as well as human clinical trials of noni fruit, seeds, seed oil, and leaves (West *et al.*, 2007; West *et al.*, 2008a, b; West, 2009; West *et al.*, 2009a, b, c). The extract also possesses significant antioxidant activity. Given this activity, along with the apparent lack of toxicity, noni seed extract may be useful as a healthy food ingredient.

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