Sunburn (fohia) Healing Effects of Noni: Is it a Mechanism Involving Its Inhibitory Effects on MMP, COX-2 and Cat-G Enzymes?

Afa K. Palu, Shixin Deng, Brett J. West, Jarakae Jensen and Rachel A. Sabin

ABSTRACT

The aim of the present investigation was to evaluate whether extracts and preparations of noni fruit and leaf, a Polynesian traditional herbal medicine, inhibit MMP, COX-2 and Cat-G enzymes in vitro, as its mechanism of action for healing sun-burn known as fohia in Tonga. Noni leaf ethanolic extract (NLEE) inhibited MMP-1, -2, -3, and -9 enzymes concentration dependently with 0.517, 0.234, 0.184, and 0.302 mg/mL IC50, respectively. Noni fruit juice concentrates (NFJC) in 1 and 5 mg/mL concentrations, inhibited MMP-12 enzymes by 102, and 99%, respectively. NFJC and NLEE inhibited Cat-G enzymes concentration dependently with 0.125, <0.1, and 0.41 mg/mL IC50, respectively. Noni fruit juice fractions 4 and 6 inhibited COX-2 and Cat-G enzymes by 85 and 89%, and 89 and 78%, respectively. Additionally, the noni fruit puree and noni leaf has 1.91 mg/g and 5.77 mg/g of ursolic acid, respectively. NFJC, and NLEE inhibitory effects on MMP, COX-2 and Cat-G enzymes might help explain the traditional usage of the noni fruits and leaves for treatment of fohia skin as alluded to by Polynesian traditional healers. These results warrant further studies into the skin health benefits of noni fruit and leaf to further assess their efficacies and dosages in human subjects suffering from photoaging.

Keywords: Photoprotection, MMP enzymes, cathepsin G, skin health, photoaging, noni.

INTRODUCTION

Morinda citrifolia L., family Rubiaceae, commonly known as noni, has been used in Polynesia for over 2000 years for food, medicine and dyeing of traditional clothes. Its medicinal usage has been purported to include the amelioration of diabetes (suka), gout (kauti), high blood pressure (toto ma-olunga), cancer (kanisā or kahi), boils (hangatāmaki), and other skin and internal ailments (Wang et al., 2002; Palu, 2009; Palu, 2004a). Further, noni fruit juice was consumed after a long voyage to help strengthen and restore vigor to the body (Thaman, 1990). The skin health benefits of the noni fruit and leaf have been alluded to by many traditional healers, especially those in the Islands of Tonga, in the South Pacific (Palu, 2004b). It was a common practice for traditional healers to treat various forms of skin ailments collective known as mahaki kili (skin diseases), before the introduction of modern medicine, including mea fele (skin rash consists of pimples around the torso that when connected result in death), fufua (pimples), mamulu (bruises), lavea (wound), kalokula (redness or inflammation), up to improving fohia (sun burn) due to long exposure to the sun in a hot day.
Improving skin appearances resulting from *fohia* was not a daily practice but was only done on occasions when the skin seemed somewhat *anga kehe* (unhealthy) due to excessive exposure to the sun using the ripe noni fruits and/or noni leaf juice (Palu et al., 2010; Palu, 2004b).

For skin treatment using the noni fruit, you use a very ripe noni fruit that is cut in half, and each halves is rubbed directly on the affected skin area several times and allowed to dry. This is repeated over time until the skin color or skin conditions are considered normal, meaning the way it was before the skin ailments or the *fohia* skin appeared. In contrast, for skin treatment using noni leaf, a semi-matured noni leaf is warmed over a fire and then applied directly onto the affected skin area by pressing it against the skin or the noni leaf is also bounded between two smaller volcanic rocks then the juice from the bounded-non leaf is squeezed directly onto the affected skin area and/or used as a paste on the affected area (Palu et al., 2010; Palu, 2004b). Again, this type of noni leaf skin treatment is repeated over time until the affected area is completely healed.

Even though these traditional healings involved treatment of skin conditions using the noni fruit and noni leaf are well-known among traditional healers, the scientific evidences supporting the skin health benefits have been lacking until now. Interestingly, West and colleagues (2009) previously reported a clinical study that uses a carbomer gel base containing a combined dried noni leaf ethanol extract with fresh noni leaf juice against UVB-Induce erythema model in 25 human volunteers. It was shown that the combined dried noni leaf ethanol extract and fresh noni leaf juice significantly (*p < 0.001*) protected the treated-skin almost 3.5 times greater than the untreated-skin (West et al., 2009). However, the mechanisms for this human clinical effect and the anecdotal evidences from Polynesi of using noni leaf and fruit for treatment of skin conditions including its potential anti-photoaging have not been elucidated.

Our objective in this study was to investigate the effects of noni leaf ethanol extract, noni fruit juice concentrates, and a commercial noni fruit juice product on matrix metalloproteinase and cathepsin G enzymes in vitro to elucidate a potential mechanism for the traditional usage of noni fruit and leaf on *fohia* (sun burn).

**MATERIALS & METHODS**

**Noni samples**

Noni fruit juice commercial product TNOB), noni fruit juice concentrates (NFIC) and noni leaf ethanol extract (NLEE) were supplied by Tropical Resources Inc. Briefly, TNOB is a commercial brand consists mostly of noni fruit puree, and small amount of blueberry and grape concentrates and tropical flavors. NFIC is achieved from heat evaporation of noni fruit juice, without the pulp, yielding about 30-60 brix of noni concentrates. NLEE is the ethanolic extraction of dried noni leaves harvested from Tahiti. Dried noni fruit puree and noni leaves were also obtained from noni fruits and leaves from Tahiti.

**MMP-1 assay**

NLEE (1.0, 0.5, 0.1 mg/mL) and TNOB (10 mg/mL) in various concentrations, in duplicates, were evaluated for their inhibitory effects on MMP-1 enzymes in *in-vitro* bioassays according to established protocols (Johnson et al., 1998; Knight et al., 1992). Briefly, noni samples, in various concentrations, were incubated with the following mixture: MMP-1 enzymes (previously isolated from Human rheumatoid synovial fibroblast), 4 μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ substrate in 1% DMSO and 50 mM MOPS, 10 mM CaCl₂·2H₂O, 10 μM ZnCl₂, 0.05% Brij 35, pH 7.2 incubation buffer. The mixture was pre-incubated for 60 min at 37°C and further incubated for 2 hr. at 37°C. TIMP-2 was used as a reference compound. The amount of enzyme inhibition was quantified using spectrofluorimetric analysis of Mca-Pro-Leu-Gly-NH₂ and reported as % inhibition. Significant criteria were set at ≥ 50% of max stimulation or inhibition.

**MMP-2 assay**

NLEE (1.0, 0.5, 0.1 mg/mL) in various concentrations, in duplicates, was evaluated for its inhibitory effects on MMP-2 enzymes using established protocol (Knight et al., 1992; Olson et al., 1997). Briefly, NLEE in various concentrations was combined with a mixture consisting of: MMP-2 enzymes from Human recombinant, 4 μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ substrate in 1% DMSO and 50 mM MOPS, 10 mM CaCl₂·2H₂O, 10 μM ZnCl₂, 0.05% Brij 35, pH 7.2 incubation buffer. The mixture was pre-incubated for 60 min at 37°C and further incubated for 2 hr. at 37°C. The amount of enzyme inhibition was quantified using spectrofluorimetric analysis of Mca-Pro-Leu-Gly-NH₂ and reported as % inhibition. TIMP-2 was used as a reference compound. Significant criteria were set at ≥ 50% of max stimulation or inhibition.

**MMP-3 assay**

NLEE (1.0, 0.5, 0.1 mg/mL) and TNOB (10 mg/mL) in various concentrations, in duplicates, were evaluated for their inhibitory effects on MMP-3 enzymes using established protocol (Johnson et al., 1998; Knight et al., 1992). Briefly, NLEE&TNOB in various concentrations were combined with a mixture consisting of: MMP-3 enzymes from Human recombinant, 4 μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ substrate in 1% DMSO and 50 mM MOPS, 10 mM CaCl₂·2H₂O, 10 μM ZnCl₂, 0.05% Brij 35, pH 7.2 incubation buffer. The mixture was pre-incubated for 60 min at 37°C and further incubated for 2 hr. at 37°C. The amount of enzyme inhibition was quantified using spectrofluorimetric analysis of Mca-Pro-Leu-Gly-NH₂ and reported as % inhibition. TIMP-2 was used as a reference compound. Significant criteria were set at ≥ 50% of max stimulation or inhibition.

**MMP-7 assay**

TNOB (10 mg/mL) was evaluated for its inhibitory effects on MMP-7 enzymes in-vitro, in duplicates, according to established protocol (Johnson et al., 1998; Knight et al., 1992).
Briefly, TNOB was put in a reaction mixture consisting of: 2 nM Human recombinant (E. coli) MMP-7 enzymes in modified MOPS buffer pH 7.2 for 60 min at 37⁰C. The reaction was initiated by the addition of 4 μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg to the mixture and incubated for 120 min at 37⁰C. The amount of enzyme inhibition was quantified using spectrofluorimetric analysis of Mca-Pro-Leu-Gly and reported as % inhibition. TIMP-2 was used as a reference compound. Significant criteria were set at ≥ 50% of max stimulation or inhibition.

**MMP-9 assay**

NLEE (1.0, 0.5, 0.1 mg/mL) and TNOB (10 mg/mL) in various concentrations, in duplicates, were evaluated for their inhibitory effects on MMP-9 enzymes using established protocol (Johnson et al., 1998; Knight et al., 1992). Briefly, NLEE &TNOB, in various concentrations, were combined with a mixture consisting of: MMP-9 enzymes from human recombinant, 4 μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ substrate in 1% DMSO and 50 mM MOPS, 10 mM CaCl₂·2H₂O, 10 μM ZnCl₂, 0.05% Brij 35, pH 7.2 incubation buffer. The mixture was pre-incubated for 60 min at 37⁰C and further incubated for 2 hr. at 37⁰C. The amount of enzyme inhibition was quantified using spectrofluorimetric analysis of Mca-Pro-Leu-Gly-NH₂ and reported as % inhibition. TIMP-2 was used as a reference compound. Significant criteria were set at ≥ 50% of max stimulation or inhibition.

**MMP-12 assay**

TNOB (1, 5 mg/mL) and NFJC (1, 5 mg/mL) in various concentrations, in duplicates, were evaluated for their inhibitory effects on MMP-12 enzymes in vitro according to established protocol (Belaauaj et al., 2000; Warner et al., 2001). Briefly, TNOB &NFJC in different concentrations were incubated with a mixture consisting of: 2 nM Human recombinant (E. coli) MMP-12 enzymes in modified MOPS buffer, pH 7.2 for 60 min at 37⁰C. The reaction was initiated by the addition of 4 μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg to the mixture and incubated for 120 min at 37⁰C. The amount of enzyme inhibition was quantified using spectrofluorimetric analysis of Mca-Pro-Leu-Gly and reported as % inhibition. TIMP-2 was used as a reference compound. Significant criteria were set at ≥ 50% of max stimulation or inhibition.

**MMP-13 assay**

TNOB (10 mg/mL) was evaluated for its inhibitory effects on MMP-13 enzymes in-vitro, in duplicates, according to established protocol (Johnson et al., 1998; Knight et al., 1992). Briefly, TNOB was put in a reaction mixture consisting of: 2 nM Human recombinant (Sf9 insect cells) MMP-13 enzymes in modified MOPS buffer pH 7.2 for 60 min at 37⁰C. The reaction was initiated by the addition of 4 μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg to the mixture and incubated for 120 min at 37⁰C. The amount of enzyme inhibition was quantified using spectrofluorimetric analysis of Mca-Pro-Leu-Gly and reported as % inhibition. TIMP-2 was used as a reference compound. Significant criteria were set at ≥ 50% of max stimulation or inhibition.

**Cathepsin G assay**

TNOB, NFJC and NLEE in concentrations of 1.0, 0.5, and 0.1 mg/mL were evaluated for their inhibitory effects on cathepsin G (Cat-G) enzymes in in-vitro bioassays, in duplicates, according to an established protocol (Nakajima et al., 1979).

Briefly, different concentrations of TNOB, NFJC and NLEE were incubated with the following reagents in a mixture consisting of: 5 μM Cat-G enzyme (isolated from Human neutrophil) in modified acetate buffer pH 5.5 for 15 min at 25⁰C. Reaction was initiated by addition of 20 μM Suc-Ala-Ala-Pro-Phe-AMC and further incubated for another 30 min at 25⁰C. Chymostatin was used as a control. The enzyme inhibition was quantified by spectrofluorimetric analysis of AMC and reported as percent inhibition.

**Noni Fruit Puree Powder Bioassay Guided Fractionations**

From our fractionation project (Fig. 1): Briefly, two kilograms of freeze-dried noni fruit puree was percolated with 20 L MeOH overnight yielding a methanol fraction. Methanol fraction was evaporated to dryness using a rotary evaporator under reduced pressure (fraction 1).

The dried methanol fraction was combined with 3.0 L of water then later partitioned with 3.0 L petroleum ether (PE) four times yielding a PE partition (fraction 2) and water suspend. The water suspend was partitioned with 3.0 L of ethanol acetate (EtOAc) three times yielding a EtOAc fraction (fraction 3) and a water suspend. The water suspend was further partitioned with 3.0 L of butanol (BuOH) three times yielding a BUOH (fraction 4) and water fractions (fraction 5) which were evaporated to dryness as described above. Fraction 3 (20 g) was further sub-fractionated using a vacuum chromatography column (8 x 50 cm) packed with 800 g of silica gel (200-400 mesh, Sigma-Aldrich, MO, USA, Batch #05116 BD, 60 Å) and further fractionated into several fractions (in which fraction 6 was a part of) using CH₃Cl₂-MeOH.

**Noni Fruit Puree Powder Fractions Effect on COX-2 Enzymes**

Noni fruit fractions obtained fractionation project described above were evaluated for their inhibitory effects on COX-2 enzymes according to established protocol (Riendeau et al., 1997; Warner et al., 1999). Briefly, COX-2 enzyme was isolated from human recombinant insect Sf21 cells. Noni fruit fractions (1-6), in 100 μg/mL concentration, in duplicates, were each put in a mixture containing: 1% DMSO, COX-2 enzymes, 0.3 μM Arachidon acid, and an incubation buffer [100 mM Tris-HCl, pH 7.7, 1 mM Glutathione, 1 μM Hematin, 500 μM phenol]. The mixture was pre-incubated for 15 min at 37⁰C, after which the mixture was further incubated at 37⁰C for 5 min. PGE₂ was quantified using an EIA method. ≥ 50% of maximum stimulation or inhibition was set as criteria for significance.
Freeze-dried Noni Fruit Puree

Percolated with MeOH (20 L)

\[ \text{MeOH Extract (Fraction 1)} \]

Evaporated to dryness, add H\textsubscript{2}O (3 L)
Partitioned with PE (3 L \times 4)

PE Partition (Fraction 2)

H\textsubscript{2}O Suspend
Partitioned with EtOAc (3 L \times 3)

Evaporated to dryness

EtOAc Fraction (Fraction 3)

Sub-fractionation

CH\textsubscript{2}Cl\textsubscript{2}-MeOH (Fraction 6)

BuOH Fraction

H\textsubscript{2}O Fraction (Fraction 5)

Evaporated to dryness

Fig. 1: Fractionation scheme of noni fruit puree powder.

Fig. 2 (A to C): HPLC chromatographs of ursolic acid from dried noni fruit, leaf, and pure ursolic acid. A: ursolic acid; B: Noni leaf; C: Dried noni fruit puree powder. Dried noni fruit puree has 1.91 mg/g while noni leaf has 5.77 mg/g of ursolic acid.
Noni leaf ethanolic extract was evaluated for its inhibitory effects on COX-2 enzymes according to established protocol (Riendeau et al., 1997; Warner et al., 1999). Briefly, COX-2 enzyme was isolated from human recombinant insect Sf21 cells. NLEE in 0.1, 1, 10, 100, and 1000 µg/mL concentration, in duplicates, were each put in a mixture containing 1% DMSO.

COX-2 enzymes, 0.3 µM Arachidonic acid, and an incubation buffer [100 mM Tris-HCl, pH 7.7, 1 mM Glutathione, 1 µM Hemin, 500 µM phenol]. The mixture was pre-incubated for 15 min at 37°C, after which the mixture was further incubated at 37°C for 5 min. PGE2 was quantified using an EIA method. ≥ 50% of maximum stimulation or inhibition was set as criteria for significance.

Noni Fruit Puree Powder Fractions Effects on Cat-G Enzymes

Noni fruit puree powder (100 µg/mL) fractions (1-6) from our fractionation above were evaluated for its inhibitory effect on Cat-G enzymes according to the protocol described above.

Noni Fruit and Leaf Ursolic Acid Quantitation

Dried noni fruit puree and noni leaves were evaluated chemically in order to quantify the amount of ursoic acid present. Methanol (MeOH) and water (H2O), HPLC grade, were obtained from Fisher Scientific Co. (Gardena, CA, USA). Ursolic acid standard was purchased from Chromadex (Irvine, CA, USA). The standard was accurately weighed and then dissolved in an appropriate volume of MeOH to produce corresponding stock standard solutions. Working standard solutions for calibration curves were prepared by diluting the stock solutions with MeOH at different concentrations. All stock and working solutions were maintained at 0 °C in a refrigerator.

Chromatographic separation was performed with a Waters Alliance™ 2690 separations module coupled to a Waters 2996 photodiode array (PDA) detector, utilizing a Waters Atlantis® analytical C18 column (5 µm, 4.6 x 250 mm, Wexford, Ireland) at a flow rate of 1 mL/min. A solvent system [A; MeOH and B; 0.1% of formic acid in H2O (v/v)] was used as the mobile phase. The mobile phase was programmed consecutively in linear gradients as follows: 0-5 min, 5% A; 25-35 min, 95% A. The injection volume was 50 µL.

The column temperature was maintained at 50 °C. The UV spectrum was monitored in the range of 210-400 nm, and HPLC chromatograms were integrated and quantified at 203 nm. Data collection and integration were performed using Waters Millennium® software version 32.

RESULTS

NLEE, TNOB and NLEE effect on MMP enzymes

NLEE inhibited MMP-1, -2, -3, and -9 enzymes (Table 1) with an IC50 of 0.517, 0.234, 0.184, and 0.302 mg/mL respectively. TNOB, in 10 mg/mL concentration, inhibited MMP-1, 3, -7, -9, and -13 enzymes by 87, 85, 87, 72, and 87% respectively (Table 2). Further, TNOB, in 5 and 1 mg/mL concentrations, inhibited MMP-12 enzymes by 93 and 78% respectively while NFJC, in the same concentrations, inhibited MMP-12 enzymes by 99 and 102% respectively (Table 3).

Table 1: Average percent inhibition of MMP-1, -2, -3, and -9 enzymes by various NLEE concentrations in duplicates. IC50 percent inhibition (mg/mL) is also shown for each NLEE inhibitory effects on each MMP enzymes. TIMP-2 was used as a reference compound for all assays with the following IC50: 3.13 nM (MMP-1), 1.33 nM (MMP-2), 4.02 nM (MMP-3), and 3.69 nM (MMP-9).

<table>
<thead>
<tr>
<th>Noni Sample</th>
<th>Conc. (mg/mL)</th>
<th>% Inhibition of Matrix Metalloproteinase Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLEE</td>
<td></td>
<td>MMP-1</td>
</tr>
<tr>
<td>0.1</td>
<td>18</td>
<td>33</td>
</tr>
<tr>
<td>0.5</td>
<td>41</td>
<td>61</td>
</tr>
<tr>
<td>1.0</td>
<td>76</td>
<td>85</td>
</tr>
<tr>
<td>NLEE IC50 (mg/mL)</td>
<td>0.517</td>
<td>0.234</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Noni Sample</th>
<th>Conc. (mg/mL)</th>
<th>% Inhibition of Matrix Metalloproteinase Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNOB</td>
<td></td>
<td>MMP-1</td>
</tr>
<tr>
<td>10</td>
<td>87</td>
<td>85</td>
</tr>
</tbody>
</table>

Table 2: Average percent inhibition of MMP-1, -2, -3, -7, -9, and -13 enzymes by 10 mg/mL concentration of TNOB in duplicates. TIMP-2 was used as a reference compound with an IC50 of 0.0059 μM (MMP-1), 0.0077 μM (MMP-3), 0.0059 μM (MMP-7), 0.0028 μM (MMP-9, MMP-13).

<table>
<thead>
<tr>
<th>Noni Sample</th>
<th>Conc. (mg/mL)</th>
<th>% Inhibition of Matrix Metalloproteinase Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNOB</td>
<td></td>
<td>MMP-1</td>
</tr>
<tr>
<td>0.234</td>
<td>99</td>
<td>93</td>
</tr>
<tr>
<td>NFJC</td>
<td>102</td>
<td>102</td>
</tr>
</tbody>
</table>

Table 3: Average percent inhibition of MMP-12 enzyme by 1 and 5 mg/mL concentrations of TNOB and NFJC in duplicates.TIMP-2 was used as a reference compound with an IC50 of 0.00093 μM.

<table>
<thead>
<tr>
<th>Noni Samples</th>
<th>Concentrations (mg/mL)</th>
<th>% MMP-12 Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNOB</td>
<td>1</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>93</td>
</tr>
<tr>
<td>NFJC</td>
<td>1</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>99</td>
</tr>
</tbody>
</table>

TNOB, NFJC and NLEE effect on Cat-G enzymes

TNOB in 1.0, 0.5, and 0.1 mg/mL concentrations inhibited Cat-G by 94, 90, and 42%, respectively with a 0.125 mg/mL IC50 (Table 4). NFJC in the same concentrations inhibited Cat-G by 103, 101, and 98% respectively with <0.1 mg/mL IC50 (Table 4) and NLEE, in the same concentrations as TNOB and NLEE, inhibited Cat-G by 85, 51, and 16% respectively with a 0.41 mg/mL IC50 while the control compound, chymostatin, had an IC50 of 1.22 µM (Table 4).

Table 4: Average percent inhibition of Cat-G enzymes by various concentrations (1.0, 0.5, 0.1 mg/mL) of noni samples (TNOB, NFJC and NLEE), in duplicates. IC50 inhibitory concentrations of various noni samples are shown. Chymostatin was used as a reference compound with an IC50 of 1.22 µM.

<table>
<thead>
<tr>
<th>Noni Samples</th>
<th>Conc. (mg/mL)</th>
<th>% Inhibition of Cat-G</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNOB</td>
<td>0.1</td>
<td>42</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>90</td>
<td>mg/mL</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>94</td>
<td>mg/mL</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>98</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>NFJC</td>
<td>0.5</td>
<td>101</td>
<td>mg/mL</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>103</td>
<td>mg/mL</td>
</tr>
<tr>
<td>NLEE</td>
<td>0.1</td>
<td>16</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>51</td>
<td>mg/mL</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>85</td>
<td>mg/mL</td>
</tr>
</tbody>
</table>
Noni fruit powder fractions inhibit Cat-G enzymes

Noni fruit fractions (1-6) inhibit Cat-G enzymes by -22, -21, -19, 89, 0, and 78%, respectively.

NLEE effects on COX-2 enzymes

NLEE inhibited COX-2 enzymes by 8, 37, 98, 100, and 99%, respectively with an IC50 of 1.36 μg/mL.

Dried noni fruit puree and noni leaf ursolic acid quantitation

HPLC analysis of the dried noni fruit puree and noni leaf show that dried noni fruit puree has 1.91 mg/g while noni leaf has 5.77 mg/g of ursolic acid as shown in Fig 2.

DISCUSSION

Traditional healing using the noni (Morinda citrifolia L., family Rubiaceae) fruit, root, bark, blossom and leaf is well-known in Polynesia especially in the Islands of Tonga. In fact, the usage of the noni fruit and leaf on boils not coming to a head (hangatamaki ‘ikeike ‘iaihanomata), diabetes (suka), cancer (kanisapekahi), hypertension (toto ma-olunga), skin rash (mea), bruises (mamulu), cuts (lavea), gout (kauntepe langa e hokatanga hui), general body pain (felaunakiki e sinó pe mamahi‘ia) were very common practice before the arrival of westerners and the westernization of medicine in Tonga (Palu, 2004b). As such, a lot of the knowledge of traditional medicinal usage of Tongan medicinal plants is lost and majority of the people are now relying on, and are using westernized medicine for their health care need.

The traditional usage of the noni fruit and leaf for skin health benefits is on the rise due to the advent of Morinda Inc. and the world-wide selling of its noni-based products; availability of results from various bioassays, and other scientific studies including human clinical trials touting noni’s health benefits which seems to support its traditional use. Noni fruit and leaf usage for treatment of human ailments including skin conditions and wound healing have not been scientifically evaluated until recently (Wang et al., 2002; Palu et al., 2010; West et al., 2009; Sang et al., 2001; Sang et al., 2001; Nayak et al., 2009) and the research on the skin health benefits of noni is beginning to emerge (Palu et al., 2010; Sang et al., 2001; Nayak et al., 2009) as potential treatment for various skin health conditions, including but not limited to, UV-induced photoaging.

Photoaging is a manifestation of a complex skin condition resulting from various physiological, biochemical and molecular events that promote this type of skin condition primarily due to repeated ultraviolet light exposure over a period of time. Hence, it is widely believed, based upon the accumulated scientific evidences thus far, that there is a multiple number of signal transduction pathways involved in photo-aging with some known mechanisms, including but not limited to enzymatic reactions, which are involved in various processes leading up to photo-aging. Callaghan and Wilhelm (2008), in their review of ageing, identified various clinical methods used in the assessment of

Table 5. Average percentage inhibition of freeze-dried noni fruit puree fractions 1-6 in 100 μg/mL concentrations, in duplicates, on COX-2 and Cat-G enzymes. Rofecoxib was used as reference compound for COX-2 with a 0.0992 μM IC50 while chymostatin was used as a reference compound for Cat-G with a 1.43 μM IC50.

<table>
<thead>
<tr>
<th>Noni Sample/Conc. (100 μg/mL)</th>
<th>Enzyme</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1</td>
<td>COX-2</td>
<td>3</td>
</tr>
<tr>
<td>Fraction 2</td>
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<td>9</td>
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<td>Fraction 3</td>
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<tr>
<td>Fraction 6</td>
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<tr>
<td>Fraction 1</td>
<td>Cat-G</td>
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<td>Fraction 3</td>
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aging skin that includes cellular and molecular perspectives of skin ageing. Scharfetter-Kochanek and colleagues (2000) identified photo-aging of skin from phenotype to various mechanisms while Seo and colleagues (2003) showed that UV enhanced the expression of COX-2 enzymes in aged human skin in vivo. Further, Varani and colleagues (2001) showed that damaged collagen in photo-aged skin inhibits type I procollagen synthesis while Pentland and colleagues (1990) discovered that enhanced prostaglandin synthesis after ultraviolet injury is mediated by endogenous histamine stimulation. Pillai and colleagues (2005) reviewed the effects of ultraviolet radiation on human skin aging and identified the critical roles that reactive oxygen species, inflammation, and protease activation play on skin aging and devise strategies for prevention of inflammation-induced matrix degradation that includes the inhibition of COX-2 and MMP enzymes as some of the ways to mitigate skin aging. Further, Widyarini and co-workers (2006) identified the estrogen receptor signaling pathway as one of the signal transduction pathways involved in protection against the suppression of the immune system by UV radiation exposure while Bennett and colleagues (2008) showed that the skin immune systems and inflammation may offer protection or promotion of aging depending on the situation. Bergman and colleagues (2003) found a functional activating protein 1 (AP-1) site regulates MMP-2 transcription by cardiac cells leading to aging while Goseki and colleagues (1996) demonstrated the involvement of alkaline phosphatase, cathepsin activities and collagen secretion in aging of human periodontal ligament derived cells.

The photoaging phenomenon has been a hot topic of discussions in the cosmetic industry for over a decade now but it is just becoming an increasingly popular discussion topics in the dietary supplement industries due to the availability of results from inhibitory experiments of various bioassays and human clinical trials that support the usage of certain synthetic ingredients and/or plant extracts that have or may improve skin health conditions (Chiang et al., 2011; Grimm et al., 2004; Lim & Kim, 2007; Talhouk et al., 2007; Gao et al., 2008) relating to photoaging. In fact, these cosmetic ingredients and plant extracts have been shown to have inhibitory effects on enzymes, previously identified to be associated with and contributing to, skin and photoaging. Among those enzymes are the MMPs and Cathepsins enzymes which are members of the protease family that were shown to be upregulated during photo-aging or skin aging (Callaghan & Wilhelm, 2008; Pillai et al., 2005; Widyarini et al., 2006; Li et al., 2004; Chiang et al., 2011; Grimm et al., 2004; Crawford &Matrisian, 1996; Fahlman& Krol, 2009). Therefore, MMP and Cathepsin enzymes present novel targets for pharmaceutical drugs and medicinal plant extracts to ameliorate photoaging. In fact, inhibitors of these enzymes have been shown to help stop or reduce skin and photoaging conditions (Olson et al., 1997; Widyarini et al., 2006; Grimm et al., 2004; Deng et al., 2007; Saludes et al., 2002) or part of the UV-induced skin aging signal transduction pathways which seem to fuel the screening of medicinal plants traditionally used for treatment of skin conditions in Polynesia and elsewhere.

In our study, we found that NLEE inhibited MMP-1, -2, -3, and -9 and the inhibition was concentration dependent. However, the inhibition was more pronounced for MMP-3 as indicated by a lower 0.184 mg/mL IC50 compared to the inhibition of the other MMP enzymes. In fact, the inhibitory effects of NLEE on MMP enzymes can be categorized according to the percent inhibitory effects in vitro, starting from the most potent to the least in the following order: MMP-3>MMP-2>MMP-9>MMP-1 (Table 1). Hence, NLEE is a good candidate to be used for treatment of skin against photoaging and MMP photoaging-related conditions. In like manner, TNOB also inhibits MMP enzymes in the following order from the most potent to the least inhibitory effects: MMP-1>MMP-7>MMP-13>MMP-3>MMP-9 (Table 2).

Additionally, TNOB in 5 and 1 mg/mL concentrations was also shown to inhibit MMP-12 concentration dependently with the maximum amount of enzymes inhibitory effects at the 5 mg/mL concentration. In contrast, NFJC in 5 and 1 mg/mL concentrations also inhibited MMP-12 concentration-dependently but with the most percentage of inhibition at 1 mg/mL instead of 5 mg/mL concentration even though the assay was ran in duplicates and its average percent of inhibition was reported as final percentage of inhibition. This is a phenomenon that we have seen very often in our research with noni for over ten years now. Therefore, we are left to speculate as to why this phenomenon occurs in noni such that in the higher concentrations of noni, as seen in this case with NFJC, the average percent inhibition is lower in the higher concentrations. First NFJC contains higher amounts of noni bioactive compounds or ligands since it is in a concentrated form, leading to increases in various bioactive compounds engaging in competitive inhibition (Takashima et al., 2007) on the active site of the enzyme which resulted in higher percentage of inhibition in the lowest concentration compared to that of the higher concentration. Further, perhaps there is a possibility that one compound binds to the active site of the enzyme while a different compound, that also present in the noni fruit juice concentrates, binds to the allosteric site of the enzymes at the same time thus changing the conformation of the enzymes leading to an alteration of the active site of the enzymes (Friemarke et al., 1994) which ultimately leads to less inhibitory effects at the highest concentrations. This speculation needs further ligand-binding research to evaluate the nature of this binding phenomenon with noni at the highest concentrations.

However, there are other factors that might be involved in the photoaging signal transduction pathways directly or indirectly towards activation of MMP and cathepsin enzymes which are also contributing to photoaging such as the transcription factor AP-1, calpain enzymes, COX-2, 5-LOX and H-1 receptor to name just a few (Han et al., 2004; Ryu et al., 2010; Manev et al., 2000; Gao et al., 2008). Interestingly, Sang and colleagues (2001) have already shown that an unusual iridoid, citrifolinoside, isolated from the
leaves of the noni plant, significantly inhibits UVB-induced AP-1 in cell cultures which might also explain the effects seen in our human clinical trial (West et al., 2009). Further, Su and colleagues (2001) from our lab were the first to discover that noni fruit juice inhibits COX-2 enzymes which are known to be elevated during UV-induced photoaging (Buckman et al., 1998) which might also contribute to the noni leaf protection against UV-induced effects alluded to by West and colleagues (2009). In addition to noni’s inhibitory effects on COX-2 enzymes, Palu and colleagues (2004a) also reported the inhibitory effects of the noni leaves and fruit on 5-LOX enzymes which are also known to be elevated during UV-induce photoaging (Yan et al., 2006). Concomitantly, West and colleagues (2009) also reported that noni leaf ethanol extract also binds to and inhibits histamine H1 receptor. Histamine receptor has also been shown to be involved in the initial transient phase of UV-induced inflammation and histamine receptor antagonist has also been shown to alleviate the UV-induce inflammation (Woodward & Owen, 1982). Further, histamine also augments UVB-induced IL-6 production in the human keratinocytes dose dependently but it was blocked by pyrilamine, a H1 histamine receptor antagonist, which suggest that histamine receptor antagonist will alleviate the effects of sun exposure histamine-related (Shinoda et al., 1998) as it was also shown in the human clinical trial with noni leaf ethanol extract (West et al., 2009).

Further, Sang and colleagues (Sang et al., 2001) and others (Nayak et al., 2009; Takashima et al., 2007) both showed that noni leaf contains various phytochemicals such as ursolic acid, quercetin, kaempferol and rutin that may contribute to the noni leaf anti-photoaging effects while Deng and colleagues (2010) showed that scopoletin, rutin, quercetin, and 5, 15-dimethylorindol were also among the phytochemicals detected in all samples of noni fruits from various countries in the world, although at various concentrations, may also contribute to the noni leaf potential anti-photoaging effects. Additionally, we also quantified the amount of ursolic acid in the dried noni fruit and leaf and found that the amount of ursolic acid in the leaf is about three times more than those found in the dried noni fruit. Perhaps the prominent presence of ursolic acid and other compounds recently identified in the noni leaf contributes to the clinical results reported by West and colleagues (2009) and also offers the potential anti-photoaging effects of noni.

Interestingly, there is a small stream of research on the health effects of ursolic acid on photaging as an anti-photoaging agent. Chen and colleagues (2009) found that UV
t inhibited hWS1 cell viability, lowered elastin biosynthesis, enhanced release of LDH and also up-regulated MMP-1, MMP-2 and catalase enzymes. However, treatment of the hWS1 cells with ursolic acid and arbutin effectively protects cell viability, recovered extracellular elastin levels, suppressed lipid peroxidation, and down-regulate the expression of LDH release. Ursolic acid, in a liposomal form in a commercial product, was shown to increase ceramides and collagen in human skin culture (Yarosh et al., 2000), down-regulate MMP-9 gene in human fibrosarcoma cells (Cha et al., 1998), reduce the expression of UVA-induced elastin mRNA in vitro while preventing yellowish discoloration, wrinkling, and sagging of skin induced by UVA (Kim et al., 2003). Ursolic acid was also shown to significantly suppress the UVA-induced reactive oxygen species production and lipid peroxidation while it also significantly reduced the UVA-induced activation and expression of MMP-2 and p53, known hallmark of photaging (Lee et al., 2003).

Therefore, it is possible that the inhibition of MMP and Cathepsin enzymes by the noni leaf and noni fruit extracts are due to actions of a repertoire of various compounds including ursolic acid, present in noni fruit and leaf, on the molecular level such as the protease enzymes, AP-1 and also the JNK pathways. In fact, Liu and colleagues (2001) showed that noni leaf extract inhibited AP-1 and JNK activities while West and colleagues (2009) showed that the combination of the noni leaf ethanol extract and the noni leaf juice significantly protected the skin from UVB-induced injury to the skin (p ≤ 0.001) compared to the unprotected area of the skin in the human clinical trial involving 25 volunteering subjects.

Further, West and colleagues (2009) also showed that the crude ethanol extract of noni leaves act as a histamine H1 receptor antagonist, accounting for 57% receptor binding inhibition. Interestingly, histamine H1 antagonist was also shown by Yan and colleagues (2006) to have inhibitory effects on ultraviolet exposed skin dermal fibroblasts along with suppression of 5-LOX mRNA expression. Furthermore, Pentland and colleagues (1990) showed that acute ultraviolet light B-induced injury is also associated with dermal mast cell histamine release, leading to fivefold increase in prostaglandin synthesis. However, the increases in the histamine release were also inhibited by the antihistamine pharmaceutical drug Brompheniramine. Therefore, the antagonistic effects of noni leaf on H1 histamine receptor and its anti-UVB induced erythema reported by West and colleagues (2009) showed that noni leaf has the potential to alleviate photoaging effects via its binding inhibition of the H1 histamine receptor. As such, it is quite possible that noni leaf anti-histamine effect is one of its potential mechanisms for its potential anti-photoaging effects, in addition to, its inhibitory effects on MMP and Cat-G enzymes shown here in our study.

These results are significant because it is well-known that AP-1 is activated either directly or indirectly by UV light resulting in activation of some of the MMP enzymes which ultimately resulted in photaging (Benbow & Brinkerhoff, 1997). Hence, M. citrifolia leaves may be useful raw materials in producing products that will be helpful in mitigating UVB-induced injury to the skin, including photaging. Interestingly, scientific evidences from various publications have already shown that quercetin, scopoletin and other phytochemicals from different sources, which are also present in noni leaf and fruit, inhibit MMP and cathepsins enzymes, which are known to contribute to, and are highly expressed in photaging skin (Widyarini et al., 2006; Lin et al., 2008; Inal et al., 2001; Casagrande et al., 2006; Kimura et al., 2009; Bae et al., 2010; Svobodova et al., 2003; Rittie et al., 2006).
Hence, these phytochemical inhibitory effects of the noni fruit and leaf samples on both MMPs and cathepsin enzymes may be the biggest contributors to the inhibitory effects shown here for NFJC, TNOB, and NLEE. Further, more recent scientific evidences also suggest that another member of the protease enzymes family is also involved in mediating the deleterious effects of UVA and UVB on the skin. Molecularly, in addition to UV-induced oxidative inactivation of certain protein tyrosine phosphatases (PTPs), the protease enzyme calpain is also involved in the degradation of the skin cytoskeleton. UV irradiation activates the calpain enzymes and the oxidative alteration of PTPs, of which are contributing factors to photoaging (McCollum et al., 2002). Consequently, both Calpain and Caspases are also activated together by UV irradiation and a calpain inhibitor was shown to decrease caspases activation which seems to suggest that calpain is one of and/or a mediator in caspases activation (Benbow & Brinckerhoff, 1997). Interestingly, Palu (2009) has shown in in-vitro bioassays that TNOB and NFJC significantly inhibited calpain-1 enzymes with a 0.24 mg/mL IC_{50} for NFJC and 0.656 mg/mL IC_{50} for TNOB. The inhibitory effects of noni on calpain enzymes might also serve as another mechanism for potential anti-aging effects of noni. Therefore, the potential for NFJC and TNOB to alleviate UV-induced related conditions including those related to photoaging attributed to calpain should be investigated further in animal and human studies.

Our results show that the noni samples TNOB, NFJC and NLEE inhibit MMP, COX-2 and cathepsin G enzymes in vitro which might account, at least in part, for the skin health benefits of the noni fruit and leaf as alluded to by traditional healers of Polynesia and in the human clinical trial of UVB-induced erythema. Further, the results also offer a supporting mechanism to the potential photo-protective effects of noni fruit and leaf extract. Therefore, a larger human clinical trial is warranted to assess the potential skin health benefits of the noni fruit and leaf on reducing and/or ameliorating photoaging in humans.

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Conflicts of Interest

All authors are employees of Morinda Bioactives Inc.

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