Wound Healing Effects of Noni (*Morinda citrifolia* L.) Leaves: A Mechanism Involving its PDGF/A2A Receptor Ligand Binding and Promotion of Wound Closure

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*Morinda citrifolia* L., (*Rubiaceae*) commonly known as noni, has been used in Polynesia by traditional healers for the treatment of cuts, bruises and wounds. Our objective was to investigate the wound-healing mechanisms of the noni leaf. The investigations of its wound-healing mechanisms were carried out using fresh noni leaf juice (NLJ), noni leaf ethanol extract (NLEE) and its methanol (MFEE) and hexane (HFEE) fractions on the PDGF and A2A receptors in *in vitro* and topically in mice. Fresh noni leaf juice showed significant affinity to PDGF receptors, and displayed 166% binding inhibition of the ligand binding to its receptors, while at the same concentration, it only had 7% inhibition of the ligand binding to the A2A receptors. NLEE, HFEE and MFEE showed significant affinity to A2A receptors, concentration dependently, with IC50 values of 34.1, 42.9 and 86.7 μg/mL, respectively. However, MFEE significantly increased wound closure and reduced the half closure time in mice with a CT50 of 5.4 ± 0.2 days compared with control (*p* < 0.05). These results suggest that noni leaf significantly accelerated wound healing in mice via its ligand binding to the PDGF and A2A receptors as its probable mechanisms of wound-healing and also support its traditional usage for wound-healing in Polynesia.

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INTRODUCTION

*Morinda citrifolia* L., (*Rubiaceae*) commonly known as noni, has been used for thousands of years in the islands of Polynesia as food, for dying traditional clothing and for its medicinal health benefits (Wang *et al.*, 2002). Food use included wrapping fish in the more mature leaves to be cooked in an underground oven known as an ‘*umu*’ in Tonga. Octopuses were also wrapped with the noni leaves then beaten with a stick until the noni-leaves-wrappings are mushy to ensure that the leaf juices completely cover the octopus. The octopus is either washed in the ocean or with clean water before cooking in coconut milk. It is believed that the juice of the leaves helps to tenderize the tough meat of the octopus, resulting in a shorter cooking time and a more chewable meat (Palu, 2004).

As medicine, noni leaves were used to treat *mea fele* – a disease characterized as causing pimples across the torso, which when connected, results in death. The mature noni leaves are mashed between a small and a bigger volcanic rock then is mixed with coconut oil and is applied directly to the infected area (Palu, 2004). The juices of the noni leaves are also used for cuts (*lavea*), bruises (*māmula*), pain (*langa pē *mannahi*) and inflammation (*kulekula*), pimples (*fuofua*) on the face and boils (*hangatāmaki*) that do not come to a head (Little *et al.*, 1964; Leach *et al.*, 1988; Palu, 2009). The noni leaves, preferably not the younger leaves, are mashed using a small volcanic rock, on top of a bigger volcanic rock until they are completely soft and mushy. Then the soft-mashed-noni-leaves are wrapped with a piece of cloth (traditionally, a piece of the coconut bark-like material was used to wrap the soft-mashed-noni-leaves) and the juices squeezed directly onto the area of the body. In some cases, the mashed-noni-leaves are applied directly into the wound or boils and the infected area (Palu, 2004).

Among the plethora of health benefits that traditional healers attributed to the use of noni leaves are antibacterial, healing of broken bones and rheumatic joints (Leach *et al.*, 1988; Little *et al.*, 1964; Palu, 2004; Saludes *et al.*, 2002) and wound-healing properties (Palu, 2004). Even though the wound-healing effects of noni leaves are well-known among the traditional healers and natives of Polynesia, the scientific evidence and mechanisms of action to support their claims have been lacking until now.

Recently, however, Nayak and colleagues (2007) were able to show that ingestion of an ethanol extract of the noni leaves (150 mg/kg/day), in rat’s drinking water, was able to accelerate wound healing in excision and dead space wound models in rats. In their study, the group of rats that had the ethanol extracts of noni leaves in their drinking water showed a significant (71%) reduction in the wound area when compared with controls which only exhibited a 57% reduction (*p* < 0.02). Furthermore, the group of rats receiving the noni leaf ethanol extracts in their drinking water also showed an enhancement in
wound contraction, decreases in epithelialization time, increases in the hydroxproline content and the histological characteristics in their wound model. However, Polynesian traditional healers have been using noni leaves topically and not orally for wound healing. As such, it is imperative scientifically to evaluate the wound-healing effects of noni leaves topically and concomitantly elucidate the possible molecular mechanisms of actions to substantiate the traditional usage.

This study investigated the possible molecular mechanisms of noni leaves’ wound-healing effects by evaluating the radioligand binding effects of the noni leaf juices, and the methanol and the hexane fractions of the alcohol extracts on PDGF and the A_{2A} receptors, which are known to be involved in the recruitment of wound-healing apparatus in vitro. Additionally, the ethanol extract of noni leaves effects on adenosine A_{2A} activity was evaluated in the human platelet rich plasma aggregation assay. Finally, in order to evaluate the wound-healing effects in vivo, the effects of the methanol fraction were evaluated, as a topical applicant, in a mouse cutaneous injury assay, with respect to wound-closure and reduction in half closure time in order to validate the traditional use of noni leaves in Polynesia for the promotion of wound healing.

**MATERIALS AND METHODS**

**Plant materials.** The leaves of *M. citrifolia* were harvested from noni trees growing in Tahiti and identified by one of the authors who is a botanist and a native of Tonga who had collected medicinal plants, including noni fruits, leaves, flowers, roots and stems, for traditional healing practices over 30 years, from Polynesia. Briefly, mature noni leaves were picked and washed with water then they were dried in a copra-dryer before being shipped to our laboratory. Additionally, fresh green mature noni leaves were also harvested and immediately frozen then they were shipped frozen to our laboratory and kept frozen in a –20°C freezer.

**Preparation of noni leaf extracts and noni leaf juices.** Briefly, dried noni leaves powder (200 g) was percolated with 1.0 L of EtOH overnight then its supernatant was removed after centrifugation. The supernatant’s volume was reduced to dryness using a vacuum rotary evaporator yielding 20.41 g. A portion, 10.59 g, of the dried extracts was successively partitioned, three times, with a mixture of 200 mL hexane and 90% MeOH yielding a hexane fraction (HFEE) and a MeOH fraction (MFEE). The HFEE and MFEE fractions were reduced to dryness using a rotor evaporator under vacuum. Frozen noni leaves were thawed overnight and noni leaves were processed through a mechanical press yielding juices. Noni leaf juices were pasteurized using an FT74 HTST/UFHT System (Armfield Inc. NJ, USA) then allowed to cool down to room temperature before being stored in the refrigerator until testing started.

**PDGF receptor radioligand binding assay.** The effect of pasteurized noni leaf juice (NLJ) on ligand binding affinities on PDGF receptors was evaluated according to an established protocol (Williams et al., 1984). Briefly, PDGF receptors were isolated from mouse 3T3 cells. A 0.02 nM [^{25}S]PDGF-BB (PDGF) was used as a ligand. NLJ in 1000 μg/mL was dissolved in 1% DMSO. It was then mixed with the ligand and incubated in DMEM +10% FBS buffer. A 0.1 nM PDGF-BB (PDGF), a non-specific ligand, was added to the mixture and incubated for 45 min at 25°C. The ligand binding effects of NLJ was evaluated using a quantitation method and ≥50% of stimulation or inhibition of ligand binding was considered significant.

**Adenosine A_{2A} tissue assay effects of noni leaf ethanol extracts.** The ethanol extracts of the noni leaves (NLEE) were also evaluated for its A_{2A} activity in human platelet rich plasma aggregation assay according to a protocol described previously by Gurden et al. (1993). Adenosine A_{2A} source was human 60 ± 10 kg platelet rich plasma. Briefly, a reaction mixture was set up consisting of NLEE at 100, 300, 1000 and 3000 μg/mL concentrations, in duplicates, and the adenosine A_{2A} were incubated in incubation buffer [citrate-phosphate-dextrose solution with adenine (CPD-A) treated platelet rich plasma] and a vehicle containing a 0.025 mL distilled water. The administration volume was 25 μL of the NLEE–vehicle–buffer mixture while the bath volume was 0.5 mL. The reaction mixture was incubated for 5 min at 37°C and the results were quantified using optical density changes as a quantitation method after 5 min from the start of the reactions. CGS-21680, a known agonist of A_{2A} receptors, was used as a control. Significance criteria were set as either ≥50% inhibition of aggregation relative to CGS-21680-induced inhibition or ≥50% reduction of CGS-21680-induced response.

**A_{2A} receptor radioligand binding assays of NLJ, NLEE, HFEE and MFEE.** The effects of NLJ, NLEE, HFEE and MFEE on the adenosine A_{2A} receptors was evaluated using a protocol described previously (Varani et al., 1996). Briefly, the A_{2A} receptors were isolated from human recombinant HEK-293 cells. NLEE, HFEE and MFEE, in 10, 25, 50, 100 and 200 μg/mL and NLJ in 1 mg/mL were dissolved in 1% DMSO. A mixture of each noni sample with DMSO and an A_{2A} receptor ligand consisting of 0.05 μM [^{3}H] CGS-21680 was combined with a 50 μM NECA, non-specific ligand, and incubated in an incubation buffer [50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM EDTA, 2 U/mL adenosine deaminase] for 90 min at 25°C. The radioligand bindings were evaluated using a quantitation method and ≥50% of maximum stimulation or inhibition of ligand binding was considered significant.

**Animals.** Twenty five healthy ICR derived male mice (weighing 24 ± 2 g) with no prior drug or wound healing experiments were used for the present study. The animals were maintained in a controlled temperature (23–24°C) and humidity (50–60%) environment with 12 h light–dark cycle for at least 1 week before the experiment. They were also fed with a commercial standard lab chow for mice (LabDiet Rodent Diet, PMI Nutrition International, USA). All aspects of housing the animals, experimentation and disposal of animals were performed in accordance with the established guidelines outlined in the International Guiding Principles for Biomedical Research Involving Animals (CIOMS Publication No. ISBN 92 90360194, 1985).
For the cutaneous injury model, the animals were divided into five groups consisting of five animals in each group: group 1 – vehicle (20 μL of 0.5% EtOH); group II – 1000 μg MFE; group III – 300 μg MFE; group IV – 100 μg MFE; group V (control) – CGS-21680-hydrochloride (Tocris, USA). The MFE extracts, ethanol and CGS-21680-hydrochloride were administered once daily for 10 consecutive days at the same time.

Wound healing activity. The MFE extract was evaluated for potential wound healing effect in mice using a protocol described previously (Montesinos et al., 1997). Briefly, under hexobarbital (90 mg/kg, i.p.) anesthesia, the shoulder and back region of each animal were shaved. A sharp punch (ID 12 mm) was applied to remove the skin, including panniculus, carnosus and adherent tissues. The wound area was measured by use of an image analyser (Life Science Resources Vista Version 3.0), and traced onto clear plastic sheets on days 1, 3, 5, 7, 9 and 11. A 20 μL of 0.5% ethanol (vehicle) (Merck, Germany), CGS-21680-hydrochloride (10 μg/mouse) (Tocris, USA) and MFE at 1000, 300 and 100 μg were administered topically, immediately following induced cutaneous injury, then twice/day, for 10 consecutive days.

The percent closure of the wound, and its CT₅₀ were determined by linear regression using GraphPad Prism (Graph Pad Software USA). Comparisons between treated and the vehicle groups at each measurement time point on days 1, 3, 5, 7, 9 and 11 were determined using unpaired Student’s t-test. Differences were considered statistical significant at a p < 0.05 level.

RESULTS

PDGF receptor radioligand binding study

The radioligand binding effects of noni leaf juice on PDGF receptors, under duplicate sample concentrations of 1 mg/mL, averaged a 166% inhibition of ligand binding of the agonist [125I] PDGF-BB to the PDGF receptors.

A₂A receptor radioligand binding studies of NLJ, NLEE, HFEE and MFE

The radioligand binding effects of noni leaf juice on A₂A receptors, under duplicate sample concentrations of 1 mg/mL, averaged 7% inhibition of ligand binding of the agonist [³H] CGS-21680 to the A₂A receptors. The percentage of HFEE inhibition of radioligand binding of [³H] CGS-21680 at duplicate concentrations of 10, 25, 50, 100 and 200 μg/mL, were determined using unpaired Student’s t-test. Differences were considered statistically significant at a p < 0.05 level.

Adenosine A₂A tissue assay effects of noni leaf ethanol extracts

The aggregation effects of NLEE on the adenosine A₂A tissue averaged 2% [100 μg/mL], 28% [300 μg/mL], 91% [1000 μg/mL] and 100% [3 mg/mL] with an EC₅₀ of 425 μg/mL.
Noni leaf ethanol extracts effect on wound healing in mice

The effects of MFEE on the promotion of wound closure (WC) and healing in the mouse cutaneous injury assay are shown in Table 1 as average percentages. The mean percentage of wound closure for the MFEE 100 μg group was similar to that of the positive control group (compound CGS-21680) and were both higher than the mean percentages for MFEE 1000 and 300 μg/mL concentrations and the vehicle control groups. The dosage used in the vehicle was 20 μL ethanol (0.5%) per mouse. The results are averages of five mice from each group on days 3, 5, 7, 9 and 11, after topical applications of MFEE (Table 1).

DISCUSSION

Wound healing, a four step process (coagulation, inflammation, cell migration and growth, remodeling), is complex, yet tightly regulated. It involves other factors such as enzymes and receptors (Gurden et al., 1993; Komarcevic, 2000; Montesinos et al., 1997; Werner and Grose, 2002) from an arsenal of growth factors and cytokines that participate in wound healing. Among the receptors established to be involved in wound healing are the A2A receptors. It has been shown that the activation of the A2A receptors leads to faster recruitment of the wound-healing apparatus to the site of wounds. Concomitantly, the healing and closure of the wound increases as a result of A2A receptor activations and the other molecular pathways involved in wound healing that are influenced by its activation (Morello et al., 2009; Victor-Vega et al., 2002; Broughton et al., 2006). Similarly, the other receptor that has also been established to be involved in wound healing is the platelet-derived growth factor, simply known as PDGF. PDGF and other cytokines are crucial in recruiting neutrophils to the wound site to remove contaminating bacteria, induces cell migration, induce fibroblast osteopontin expression and initiate phenotypic changes in cells thus enabling the conversion of fibroblasts into myofibroblasts which facilitate wound closure in various tissues including skin (Uhl et al., 2003; Mori et al., 2008; Jiang et al., 2008; Rajkumar et al., 2006; Gope and Gope, 2009; Barrientos et al., 2008; Mogford et al., 2009; Brueckmann et al., 2007; Schultz and Wysocki, 2009).

The topical wound-healing effect of noni leaves is well known among the natives and traditional healers in Polynesia but its molecular mechanisms have not been elucidated. The effects of the noni leaf juices on both the PDGF and the A2A receptors were surprisingly different. In fact, it seems that noni leaf juices ligand binding is more pronounced on the PDGF receptors than the A2A receptors. In the same concentrations (1 mg/mL), noni leaf juices increased ligand binding with an average of 166% on the PDGF receptors while it was only 7% for the A2A receptors. Hence noni leaf juice seems to be an agonist of ligand binding on the PDGF receptors. However, the ethanol extract (NLEE) and the methanol (MFEE) and hexane fractions (HFEE) from the fractionation of the alcohol extract of the dried noni leaves seems to indicate that they do increase ligand binding on the A2A receptors differently from that of the noni leaf juices, which are commonly used by the natives and traditional healers. In fact, the radioligand binding effects of MFEE on the A2A receptors increased from 10% (10 μg/mL) to 71% (200 μg/mL), while HFEE increased from 19% (10 μg/mL) to 87% (200 μg/mL) and NLEE also increased from 24% (10 μg/mL) to 100% (200 μg/mL), all in a concentration-dependent manner. In like manner, NLEE exhibited significant ability to inhibit platelet aggregation in a concentration-dependent manner with significant inhibition at concentrations ≥1000 μg/mL and with a 425 μg/mL EC50. In contrast, the wound healing effects of MFEE were surprisingly different from that of its ligand-binding effects on the A2A receptors. The wound-healing effects of the MFEE were not concentration-dependent as shown in the mouse cutaneous injury assay (Table 1). In fact, the MFEE in 100 μg/mL concentration increased wound closure significantly compared with that of the 300 and 1000 μg/mL concentrations. This is due to a much higher wound closure rate (5.4 ± 0.2 days CT50) for the 100 μg/mL MFEE, compared with the 1000 μg/mL MFEE (6.1 ± 0.2 days CT50) and the vehicle group (6.5 ± 0.2 days CT50). Additionally, the positive control compound CGS-21680-hydrochloride was equally as effective as the 100 μg/mL MFEE with a wound closure rate of 5.4 ± 0.4 days CT50 (p < 0.05). This could be due to the ability of the MFEE fraction to recruit other factors involved in the wound-healing processes in vivo, which are not accounted for in the A2A radioligand binding in in vitro bioassays, in addition to its ability to activate the adenosine A2A and the PDGF receptors. Such a phenomenon could explain why MFEE in 100 μg/mL concentration did not appear to potently activate the adenosine receptors, while it potently activated the PDGF receptors, and concomitantly accelerates the wound healing and closure effect (Table 1).

Table 1. Mean percentage of wound closure (WC) effect of HFEE topically treated mice 3–11 days post treatment compared with vehicle treated groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose/Mouse</th>
<th>WC 3</th>
<th>WC 5</th>
<th>WC 7</th>
<th>WC 9</th>
<th>WC 11</th>
<th>CT50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>20 μL</td>
<td>26.5</td>
<td>48.0</td>
<td>59.3</td>
<td>66.2</td>
<td>75.9</td>
<td>6.5</td>
</tr>
<tr>
<td>HFEE 1</td>
<td>1000 μg</td>
<td>40.9*</td>
<td>47.8</td>
<td>59.4</td>
<td>71.9</td>
<td>76.9</td>
<td>6.1</td>
</tr>
<tr>
<td>HFEE 2</td>
<td>300 μg</td>
<td>41.0</td>
<td>53.9</td>
<td>64.7</td>
<td>78*</td>
<td>84.7*</td>
<td>5.6</td>
</tr>
<tr>
<td>HFEE 3</td>
<td>100 μg</td>
<td>36.1</td>
<td>52.2</td>
<td>70.2*</td>
<td>82.0*</td>
<td>92.2*</td>
<td>5.4*</td>
</tr>
<tr>
<td>CGS-21680</td>
<td>10 μg</td>
<td>39.5</td>
<td>53.3</td>
<td>69.7*</td>
<td>81.4*</td>
<td>90.7*</td>
<td>5.4*</td>
</tr>
</tbody>
</table>

*p < 0.05
ated the wound closure rate as evidenced by its 5.4 CT₅₀. Hence, the wound healing effects of MFEE were more pronounced at the 100 μg/mL concentration compared with 300 and 1000 μg/mL concentrations and the vehicle control.

The data demonstrate that noni leaf juice, NLEE, HFEE and MFEE promote wound healing by increasing the ligand bindings at the A₂A and PDGF receptors and also increase the wound closure rate as its mechanisms of action. Additionally, our results provide a possible molecular mechanism for the traditional usage of noni leaves for wound healing in Polynesia. In light of our data from topical uses of the noni leaf hexane fraction of the ethanol extracts, a clinical trial is warranted to assess the efficacy of using MFEE topically to accelerate wound healing in humans.

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Declaration of Interest

All authors are employees of Tahitian Noni International, Inc. The authors alone are responsible for the content and writing of the paper.

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