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Studies on anti-inflammatory and analgesic activities of betel nut in rodents

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Abstract

Ethnopharmacological relevance: Areca catechu, commonly known as betel nut, is very famous for its medicinal use in multiple disorders. It is also popular as a remedy against inflammatory disorders in the Unani (Greco-Arab) system of medicine.

Objective of the study: This study was aimed at investigating the anti-inflammatory and analgesic activities of the crude extract of Areca catechu and its respective fractions.

Materials and methods: Paw edema, formalin-induced nociception and acetic acid-induced writhing assays were carried out in vivo. Free radical scavenging activity of the plant extract was performed in vitro.

Results: Preliminary experiments using a single dose (100 mg/kg) of Areca catechu and its respective fractions demonstrated an anti-inflammatory effect on carrageenan-induced edema in mice and rats, the aqueous fraction of the extract being distinctly more effective. When studied on prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), arachidonic acid, histamine, or serotonin (5HT)-induced edema in rats, Areca catechu and its aqueous fraction markedly repressed only the PGE\textsubscript{2} and arachidonic acid-induced inflammation. When studied for analgesic activity, the crude extract and its aqueous fraction produced a dose-dependent (10–100 mg/kg) inhibitory effect on formalin-induced nociception in mice and acetic acid-induced writhing in rats, similar to aspirin. In DPPH assay, Areca catechu and its aqueous fraction exhibited free radical scavenging activity with respective IC\textsubscript{50} values of 5.34 \(\mu\text{g/ml}\) (4.93–5.78, CI; 95%, \(n = 5\)) and 7.28 \(\mu\text{g/ml}\) (6.04–7.95, \(n = 4\)), like that of rutin with IC\textsubscript{50} value of 4.75 \(\mu\text{g/ml}\) (3.89–5.42, \(n = 4\)).

Conclusion: These results indicate the anti-inflammatory and analgesic effects of Areca catechu and provide a rationale for its medicinal use in inflammatory disorders.

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1. Introduction

Areca catechu Linn. is a member of the Palmaceae family. It is known as Areca nut/Betel nut in English. In Pakistan, Betel nut is referred to as Chalia or Supari (Usmanghani et al., 1997). It is distributed throughout the world and widely cultivated in South-Asian countries (Gupta and Warnakulasuriya, 2002). For centuries, people living in different areas of the world have been using it for masticatory purposes. Betel nut is used in folk medicine for the treatment of multiple disorders, such as inflammation (gingivitis, conjunctivitis, edema, etc.), flatulence, diarrhea and dysentery, hyper-emesis in pregnancy, dysuria and high blood pressure (Nadkarni, 1976; Duke et al., 2002). It also possesses astringent, analgesic and aphrodisiac properties (Kapoor, 1990). Areca catechu powder has been applied topically as an anti-inflammatory remedy or the nut is made into ash for local application. It is also used as collyrium, in a more refined form, to get relief in conjunctivitis and epiphoria (Usmanghani et al., 1997). Moreover, a number of compound preparations containing Areca catechu such as Ma'jun Supari and Ma'jun Mochrus are applied topically in wound healing (Awan, 1960; Usmanghani et al., 1997).

Betel nut is reported to contain many chemical entities including, alkaloid (arencaidine, arecoline, guvacine and guvacoline), carbohydrates, fats, proteins and tannins (Farnsworth, 1976), β-sitosterol, gallic-acid and thiamine (Duke, 1992). Areca catechu has been widely studied for its anti-oxidant and abortifacient (Shrestha et al., 2010), hepatoprotective (Pithayanukul et al., 2009), cardiac-suppressive (Ghayur and Gilani, 2007), anti-oxidant (Lai et al., 2007), antibiotic (Wang and Huang, 2005), cholinomimetic and acetylcholinesterase inhibitory (Gilani et al., 2004), hypcholesterolemic (Park et al., 2002), platelet aggregation modulatory (Jeng et al., 2002), anti-aging (Lee and Choi, 2007), anti-inflammatory (Shrestha et al., 2010), anti-microbial (Lai et al., 2007), anti-parasitic (Khurana et al., 2007), anti-oxidant (Shrestha et al., 2010), anti-inflammatory (Shrestha et al., 2010), anti-microbial (Lai et al., 2007), anti-parasitic (Khurana et al., 2007), anti-oxidant (Shrestha et al., 2010), anti-inflammatory (Shrestha et al., 2010), anti-microbial (Lai et al., 2007), anti-parasitic (Khurana et al., 2007)
On the other hand, there are some reports showing dose-related beneficial and ill-effects of areca nut extract and its one of the alkaloids, i.e., arecoline (Lee and Choi, 1999; Chang et al., 2004; Huang et al., 2010; Liu et al., 2011). In contrast, it has also been reported that the alkaloids of betel nut are not the cause of cancer but that the excessive exposure of lime, which is one of the principal ingredients of betel quid (areca nut, lime, leaf) contributes much to this (Smythies, 1977; Kashyap et al., 2008). Moreover, different studies have shown the contaminated and aflatoxin infected nut chewing as a major cause of numerous diseases in third-world countries (Raisuddin and Misra, 1991; Srivatanakul et al., 1991). This is also common with other medicinal plants such as, tea; while the tannins in tea are known to be carcinogenic, the proven usefulness of polyphenols is unquestionable for their cardiovascular protective effects (Weisburger and Chung, 2002).

Despite the presence of some reports on the pro/anti-inflammatory and anti-oxidant activities of Areca catechu extract of some other regions (Lee and Choi, 1999; Chang et al., 2004; Huang et al., 2010), no study is available on Areca catechu of Pakistani origin demonstrating its anti-inflammatory or analgesic properties, even though, in the traditional system of medicine, it has been used to relieve inflammation and pain for a long time (Kapoor, 1990; Duke et al., 2002). This investigation demonstrated the anti-inflammatory effect of betel nut, mediated through cyclooxygenase and lipoxygenase inhibitory pathways, and through degradation and/or inactivation of prostaglandin E2 (PGE2), for its perceived medicinal use in inflammatory disorders. Moreover, this study also provides the first evidence for its analgesic effect in rodents.

2. Materials and methods

2.1. Plant material and preparation of the crude extract

Areca catechu nuts were purchased from a local market and authenticated by Mr. Abrar from the Department of Botany, University of Karachi. A voucher sample was submitted to the Karachi University Herbarium (KUH) with reference no. 67278. Betel nuts (1 kg) were ground into powder and soaked for a period of 6 days in 70% methanol. The filtrate was evaporated to produce a dark brown pasty extract using a rotary evaporator (Buchii model RE-111, Switzerland) under reduced pressure, and producing 266 g of Areca catechu extract (26.6%). The crude extract (241 g) was partitioned between water and hexane (1:1). Evaporation of hexane produced a gum-like material (hexane fraction, weighing 0.5 g). The aqueous layer was further partitioned with chloroform, n-butanol, dichloromethane and ethyl acetate respectively, and obtained 12 g chloroform fraction, 18 g n-butanol fraction, 15 g dichloromethane fraction, 8 g ethyl acetate fraction. The remaining aqueous layer yielded 180 g as aqueous fraction (Dar and Khatoon, 2000). However, 7.5 g of the extract was lost during the process of fractionation, which is not more than 3% of the total extract used for fractionation. Moreover, the chloroform, n-butanol and dichloromethane fractions were not found to be effective in any of the assays carried out in this study.

2.2. Phytochemical analysis

Preliminary phytochemical screening of the crude extract and its aqueous fraction was carried out qualitatively for the presence of alkaloids, anthraquinones, coumarins, flavonoids, saponins, sterols, tannins and terpenes by the following standard methods (Edeoga et al., 2005).

2.3. Drugs and animals

Acetic acid, arachidonic acid, caffeine, chlorpheniramine maleate, 1,1-diphenyl-2-picrylhydrazyl, formalin, histamine phosphate, lambda carrageenan, methysergide maleate, prostaglandin E2 (PGE2), serotonin and rutin were purchased from Sigma–Aldrich, St. Louis, Missouri, USA, while aspirin was purchased from Reckitt and Colman, Pakistan. Oral treatments were given in a volume of 5 ml/kg and 10 ml/kg to rats and mice, respectively. All the test compounds were solubilized in physiological saline except organic fractions (hexane and ethyl acetate), which were dissolved in 10% DMSO.

BALB/c mice (20–30 g) and Sprague Dawley (SD) rats (120–180 g) of either sex, provided by the animal house of the Aga Khan University, Karachi, were used. All animals were kept under standard conditions with normal light cycle (12 h), with free access to food and water. These experiments were carried out in accordance with ethical guidelines of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (NRC, 1996).

2.4. Carrageenan-induced paw edema in mice

The anti-inflammatory activity of Areca catechu and its fractions were determined using the model of carrageenan-induced paw edema (Winter et al., 1962). Mice of either sex were divided into six groups (n = 6 each). All mice were orally treated with saline (negative control) or aspirin (positive control) or Areca catechu extract or hexane fraction or ethyl acetate fraction or aqueous fraction at a dose of 100 mg/kg. Following 30 min of oral treatment, 30 µl of 1% carrageenan was administered into the sub-plantar side of the right hind paw of all the animals. After 3rd h of carrageenan administration, all animals were killed by cervical dislocation and their hind paws were immediately amputated at the ankle joint and weighed simultaneously. The weight of the left hind paw (control) was subtracted from the weight of the right hind paw (test) to obtain the weight of the resultant edema. Percent inhibition of paw edema was calculated using the following formula:

\[
\text{Percent inhibition} = \frac{\text{EW}_C - \text{EW}_T}{\text{EW}_C} \times 100
\]

where

- \(\text{EW}_C\) = edema weight,
- \(\text{EW}_C = \text{edema weight of right paw (control),}\)
- \(\text{EW}_T = \text{edema weight of left paw (test).}\)

2.5. Carrageenan-induced paw edema in rats

SD rats of either sex were divided into ten different groups (n = 6 each) and were treated orally with saline (negative control), different doses (10, 50 and 100 mg/kg) of aspirin (positive control) or Areca catechu or its aqueous fraction. After 30 min of treatment, 50 µl of 1% carrageenan was administered into the sub-plantar side of the right hind paw. The volume of the right hind paw was measured (Winter et al., 1962) using a plethysmometer (ITTC Inc. Life Sciences, Woodlands Hills, CA). The paw volume was measured before and at 1st, 2nd, 3rd and 4th h after carrageenan administration. The edema volume of paw and percent inhibition of edema were calculated using the following formulas:

\[
\text{EV}_C = \text{PV}_A - \text{PV}_I
\]
EV = edema volume, 
PVI = paw volume before carrageenan administration (i.e. initial paw volume). 
PVA = paw volume after carrageenan administration.

\[
\text{Percent inhibition} = \frac{EV_C - EV_I}{EV_C} \times 100
\]

EV_C = edema volume of control animal, 
EV_I = edema volume of test animal.

2.6. Prostaglandin E_2-induced paw edema in rats

Rats (SD) of either sex were divided into eight different groups (n=6 each). Rats were treated intraperitoneally with saline (control) or Areca catechu extract (10, 50 and 100 mg/kg) or its aqueous fraction (10, 50 and 100 mg/kg) or aspirin. After 30 min of treatment, 100 μl of prostaglandin E_2 (0.01 μg/ml) was administered into the sub-plantar side of the right hind paw of each rat and the paw edema was determined (Parmar and Gosh, 1978).

2.7. Arachidonic acid-induced paw edema in rats

Rats (SD) of either sex were divided into five different groups (n=6 each). Rats were treated intraperitoneally with saline (control) or aspirin or caffeic acid or areca extract or aqueous fraction (100 mg/kg). After 30 min of treatment, 100 μl of 0.5% arachidonic acid was administered into the sub-plantar side of the right hind paw of each rat, and the edema volume was determined as described by Di Martino et al. (1987).

2.8. Histamine induced paw edema in rats

In this assay, SD rats were divided into four groups (n=6 each), which were orally treated with saline (negative control) or chlorpheniramine (positive control 25 mg/kg), or Areca catechu extract (100 mg/kg) or its aqueous fraction (100 mg/kg). After 30 min of oral administration, 100 μl (1 mg/ml) of histamine was injected into the sub-plantar side of the right hind paw of the rats and paw edema was determined (Parmar and Gosh, 1978).

2.9. Serotonin induced paw edema in rats

Rats (SD) were divided into the four groups (n=6 each); which were orally treated with saline (negative control) or methysergide (positive control, 25 mg/kg) or Areca catechu extract (100 mg/kg) or its aqueous fraction (100 mg/kg). After 30 min of oral administration, 100 μl (1 mg/ml) of serotonin was injected into the sub-plantar side of the right hind paw of rats and paw edema was determined (Parmar and Gosh, 1978).

2.10. Formalin-induced noicception in mice

The antinociceptive activity of test material was assessed using the formalin assay (Hunskaar and Hole, 1987). A total of ten different groups of mice (n=6 each) were treated orally with saline (control) or different doses (10, 50 and 100 mg/kg) of aspirin or Areca catechu extract or its aqueous fraction. After 1 h of oral treatment, 20 μl of 1% formalin was injected into the sub-plantar space of the right hind paw and then the animals were placed in a transparent plastic observation chamber. The duration of paw licking was recorded between 0-5 min (1st phase) and 15-45 min (2nd phase). Percent inhibition of noiception during 1st and 2nd phases was determined by comparing the paw licking time with the respective positive control.

2.11. Acetic acid-induced writhing in mice

The test was performed as described earlier (Collier et al., 1968). In this experiment the mice were divided into ten different groups (n=6 each), which were treated orally with saline (negative control) or different doses (10, 50 and 100 mg/kg) of aspirin (positive control) or Areca catechu extract or its aqueous fraction. After 30 min of the oral treatment, 0.8% acetic acid was administered through intra-peritoneal route. Immediately after injection of acetic acid, each animal was placed in a transparent plastic observation chamber and the number of writhing movements was counted for 30 min commencing after injection of acetic acid. Percent inhibition of the number of writhing movements was calculated and compared with positive control.

2.12. Free radical scavenging activity

Free radical scavenging activity was determined by following the previously performed assay with a slight modification (Luciana et al., 2001). The experiment was divided into two groups, the positive control group was treated with rutin and the experiment groups were treated with Areca catechu extract or its aqueous fraction (1 mg/ml). Plant extract and its aqueous fraction were diluted to final concentrations of 1, 5, 10 and 15 μg/ml in methanol and finally the negative control group, methanol (300 μl) along with test solution (800 μl of methanol) was used as a blank. The reaction mixture containing 800 μl of test sample and 300 μl of 1,1-diphenyl-2-picryl-hydrazil (DPPH) was incubated at room temperature for 30 min, and the absorbance was measured at 518 nm. The absorbance of the test sample was subtracted from that of the blank and the percent antioxidant activity was calculated. Each experiment was performed in duplicates. The percentage inhibition was calculated by the following equation:

\[
\% \text{ DPPH radical inhibition} = \frac{1 - (\text{control absorbance} - \text{sample absorbance})}{\text{control}} \times 100
\]

The DPPH solution was used as a control. The concentration–response curves were plotted as concentration of extracts in μg/ml against percent of free radical scavenging activity for the calculation of EC\text{50} values along with ±SEM.

2.13. Data analysis

Results are expressed as the mean ± SEM (n= number of observations) and the median inhibitory concentration (IC\text{50}) values with 95% confidence intervals. p<0.05 was considered significantly different using one-way ANOVA followed by Dunnett’s test or unpaired t-test. The concentration–response curves (CRCs) were analyzed by non-linear regression using GraphPAD program (GraphPAD, San Diego, California, USA).

3. Results

3.1. Phytochemical analysis

Preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, saponins, sterols, tannins and terpenes in Areca catechu extract and in its aqueous fraction.

3.2. Carrageenan-induced paw edema in mice

Areca catechu extract, its aqueous (A. fraction), ethyl acetate (E. fraction) and hexane (H. fraction) fractions showed significant reduction against carrageenan-induced paw edema in mice with
percent inhibition of 52 ± 3.8, 76 ± 4.6, 33 ± 3.5, 16 ± 2.4 of 100% of carrageen control, similar to aspirin (Fig. 1). The order of activity in the test substance was observed as aqueous fraction > Areca catechu extract > ethyl acetate fraction > hexane fraction.

### 3.3. Carrageenan-induced paw edema in rats

Areca catechu extract and its aqueous fraction demonstrated dose-dependent (50 and 100 mg/kg) and persistent inhibition against carrageenan (CG) induced paw edema in rats as seen in Table 2, while a separate group of mice pretreated with aspirin (100 mg/kg) did not show any protective effect on CG-induced rat paw edema (data not shown).

### 3.4. Prostaglandin E2-induced paw edema in rats

Areca catechu extract and its aqueous fraction demonstrated dose-dependent (50 and 100 mg/kg) and persistent inhibition against prostaglandin E2 (PGE2)-induced paw edema in rats as seen in Table 2, while a separate group of mice pretreated with aspirin (100 mg/kg) did not show any protective effect on PGE2-induced rat paw edema (data not shown).

### 3.5. Arachidonic acid-induced paw edema in rats

Areca catechu extract and its aqueous fraction showed marked inhibition of arachidonic acid-induced paw edema in rats at a dose of 100 mg/kg, similar to caffeic acid (Table 3), while it showed no effect on lower doses (data not shown). The percent inhibitory order of edema at 100 mg/kg was caffeic acid > aqueous fraction > Areca catechu extract (Table 3).

### 3.6. Histamine-induced paw edema in rats

When tested against histamine-induced paw edema, Areca catechu and its aqueous fraction (100 mg/kg) failed to produce any significant inhibition of edema when compared with the inhibitory effect of chlorpheniramine (Table 4).

### 3.7. Serotonin-induced paw edema in rats

There was no consequential inhibition of edema in this assay either by Areca catechu extract or with its aqueous fraction (100 mg/kg) when compared with methysergide treated group (Table 5).

---

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Areca catechu extract</td>
<td>10</td>
<td>4.8 ± 0.44a</td>
<td>23.7 ± 1.97**</td>
<td>25.4 ± 1.09**</td>
<td>21.8 ± 1.77**</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td>10</td>
<td>6.3 ± 2.43a</td>
<td>32.2 ± 1.52**</td>
<td>30.1 ± 2.99**</td>
<td>29.6 ± 2.68**</td>
</tr>
<tr>
<td>Aspirin</td>
<td>10</td>
<td>4.1 ± 1.79a</td>
<td>6.7 ± 2.14a</td>
<td>5.4 ± 1.59a</td>
<td>4.2 ± 1.69a</td>
</tr>
<tr>
<td>Areca catechu extract</td>
<td>50</td>
<td>9.4 ± 1.10a</td>
<td>40.8 ± 1.96**</td>
<td>42.6 ± 1.87**</td>
<td>43.1 ± 1.69**</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td>50</td>
<td>8.7 ± 2.37a</td>
<td>62.4 ± 1.72**</td>
<td>63.7 ± 0.58***</td>
<td>62.0 ± 0.94***</td>
</tr>
<tr>
<td>Aspirin</td>
<td>50</td>
<td>7.4 ± 2.68a</td>
<td>10.8 ± 1.90a</td>
<td>12.3 ± 2.98a</td>
<td>8.4 ± 2.39a</td>
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<tr>
<td>Areca catechu extract</td>
<td>100</td>
<td>9.0 ± 1.66a</td>
<td>59.5 ± 1.80***</td>
<td>60.4 ± 1.05***</td>
<td>61.1 ± 2.41***</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td>100</td>
<td>7.2 ± 2.01a</td>
<td>80.2 ± 0.76***</td>
<td>83.4 ± 0.28***</td>
<td>84.0 ± 0.19***</td>
</tr>
<tr>
<td>Aspirin</td>
<td>100</td>
<td>11.8 ± 2.93a</td>
<td>47.2 ± 1.91**</td>
<td>45.6 ± 2.01**</td>
<td>43.9 ± 2.47**</td>
</tr>
</tbody>
</table>

Each value shown represents mean ± SEM (n = 4–6).

**p < 0.01, ***p < 0.001 and n.s. (non-significant) versus only carrageenan treated group (one-way ANOVA, followed by Dunnett’s test).

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Areca catechu extract</td>
<td>10</td>
<td>1.5 ± 0.64a</td>
<td>2 ± 0.41a</td>
<td>1.2 ± 0.63a</td>
<td>3 ± 0.70a</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td>10</td>
<td>1.9 ± 0.20a</td>
<td>2.5 ± 0.41a</td>
<td>3 ± 0.58a</td>
<td>2.9 ± 0.58a</td>
</tr>
<tr>
<td>Areca catechu extract</td>
<td>50</td>
<td>15.2 ± 1.31a</td>
<td>18 ± 2.29a</td>
<td>16 ± 1.5a</td>
<td>17 ± 2.05a</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td>50</td>
<td>51.2 ± 2.1***</td>
<td>49 ± 1.65***</td>
<td>52 ± 1.3***</td>
<td>55 ± 1.94***</td>
</tr>
<tr>
<td>Areca catechu extract</td>
<td>100</td>
<td>62 ± 1.35***</td>
<td>61 ± 2.87***</td>
<td>63 ± 1.3***</td>
<td>62 ± 1.2***</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td>100</td>
<td>86 ± 3.63***</td>
<td>86 ± 1.82***</td>
<td>88 ± 3.03***</td>
<td>87 ± 2.94***</td>
</tr>
</tbody>
</table>

Each value shown represents mean ± SEM (n = 4–6).

* p < 0.05, **p < 0.001 and n.s. (non-significant) versus only prostaglandin E2 treated group (one-way ANOVA, followed by Dunnett’s test).
3.9. Acetic acid-induced writhing in rats

Effect of *Areca catechu* treated group (one-way ANOVA, followed by Dunnett’s test).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Percent inhibition of edema volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1h</td>
</tr>
<tr>
<td><em>Areca catechu</em> 100</td>
<td></td>
<td>17 ± 1.2*</td>
</tr>
<tr>
<td>Aqueous fraction 100</td>
<td></td>
<td>47 ± 1.21***</td>
</tr>
<tr>
<td>Caffeic acid 100</td>
<td></td>
<td>63 ± 2.32***</td>
</tr>
<tr>
<td>Aspirin 100</td>
<td>0.75 ± 0.5ns</td>
<td>2 ± 0.62**</td>
</tr>
</tbody>
</table>

Each value shown represents mean ± SEM (n = 4–6).

* p < 0.05, ** p < 0.01, *** p < 0.001 and n.s. (non-significant) versus only acetic acid treated group (one-way ANOVA, followed by Dunnett’s test).

3.8. Formalin-induced nociception in mice

Table 4

Effect of *Areca catechu* extract and its aqueous fraction on histamine-induced paw edema in rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Percent inhibition of edema volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1h</td>
</tr>
<tr>
<td><em>Areca catechu</em> 100</td>
<td></td>
<td>4.3 ± 1.70**</td>
</tr>
<tr>
<td>Aqueous fraction 100</td>
<td></td>
<td>6.1 ± 2.11**</td>
</tr>
<tr>
<td>Chlorpheniramine 25</td>
<td></td>
<td>71.9 ± 2.99***</td>
</tr>
</tbody>
</table>

Each value shown represents mean ± SEM (n = 4–6).

*** p < 0.001 and n.s. (non-significant) versus only histamine treated group (one-way ANOVA, followed by Dunnett’s test).

Table 5

Effect of *Areca catechu* extract and its aqueous fraction on serotonin-induced paw edema in rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Percent inhibition of edema volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5h</td>
</tr>
<tr>
<td><em>Areca catechu</em> 100</td>
<td></td>
<td>2 ± 0.70**</td>
</tr>
<tr>
<td>Aqueous fraction 100</td>
<td></td>
<td>9.9 ± 2.88**</td>
</tr>
<tr>
<td>Methysergide 25</td>
<td></td>
<td>79.5 ± 2.63***</td>
</tr>
</tbody>
</table>

Each value shown represents mean ± SEM (n = 4–6).

*** p < 0.001 and n.s. (non-significant) versus only serotonin treated group (one-way ANOVA, followed by Dunnett’s test).

Table 6

Effect of *Areca catechu* extract and its aqueous fraction on formalin-induced paw nociception in mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Percent inhibition of nociception</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st phase</td>
</tr>
<tr>
<td><em>Areca catechu</em> 10</td>
<td>0</td>
<td>10.5 ± 1.37*</td>
</tr>
<tr>
<td>Aqueous fraction 10</td>
<td>0</td>
<td>5.2 ± 1.04**</td>
</tr>
<tr>
<td>Aspirin 10</td>
<td>0</td>
<td>8.4 ± 1.69**</td>
</tr>
<tr>
<td>Aqueous fraction 50</td>
<td>0</td>
<td>7.5 ± 2.88**</td>
</tr>
<tr>
<td>Aspirin 50</td>
<td>0</td>
<td>3.1 ± 2.79**</td>
</tr>
<tr>
<td><em>Areca catechu</em> 100</td>
<td>0</td>
<td>9.0 ± 2.99**</td>
</tr>
<tr>
<td>Aqueous fraction 100</td>
<td>0</td>
<td>8.6 ± 2.73**</td>
</tr>
<tr>
<td>Aspirin 100</td>
<td>0</td>
<td>10.2 ± 2.98**</td>
</tr>
</tbody>
</table>

Each value shown represents mean ± SEM (n = 4–6).

* p < 0.05, ** p < 0.01, *** p < 0.001 and n.s. (non-significant) versus only formalin treated group (one-way ANOVA, followed by Dunnett’s test).

Table 7

Effect of *Areca catechu* extract and its aqueous fraction on acetic acid-induced abdominal writhings in mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Percent inhibition of abdominal writhings</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Areca catechu</em> 10</td>
<td></td>
<td>12.4 ± 1.58*</td>
</tr>
<tr>
<td>Aqueous fraction 10</td>
<td></td>
<td>22.1 ± 1.19**</td>
</tr>
<tr>
<td>Aspirin 10</td>
<td></td>
<td>8.6 ± 0.74**</td>
</tr>
<tr>
<td><em>Areca catechu</em> 50</td>
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<td>50.1 ± 1.94**</td>
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<tr>
<td>Aqueous fraction 50</td>
<td></td>
<td>27.9 ± 1.83**</td>
</tr>
<tr>
<td>Aspirin 50</td>
<td></td>
<td>65.2 ± 1.41***</td>
</tr>
<tr>
<td><em>Areca catechu</em> 100</td>
<td></td>
<td>80.1 ± 1.07***</td>
</tr>
<tr>
<td>Aqueous fraction 100</td>
<td></td>
<td>49.3 ± 0.22***</td>
</tr>
<tr>
<td>Aspirin 100</td>
<td></td>
<td>65.2 ± 1.41***</td>
</tr>
</tbody>
</table>

Each value shown represents mean ± SEM (n = 4–6).

* p < 0.05, ** p < 0.01, *** p < 0.001 and n.s. (non-significant) versus only acetic acid treated group (one-way ANOVA, followed by Dunnett’s test).

3.8. Formalin-induced nociception in mice

In this inquisition, no effect was observed in the 1st phase of all doses of the test materials. However, in 2nd phase of the experiment, a marked dose-dependent inhibition in writhings was observed at all doses of *Areca catechu* extract and its aqueous fraction, similar to aspirin (Table 6).

3.9. Acetic acid-induced writhing in rats

In this assay *Areca catechu* extract and its aqueous fraction produced dose-dependent inhibition of acetic acid-induced abdominal writhing in rats, in a similar way to aspirin (Table 7).

3.10. Free radical scavenging activity

*Areca catechu* extract and its aqueous fraction showed concentration-dependent (0.3–30 μg/ml) free radical scavenging activity with respective IC50 values of the test materials. Our pre-

4. Discussion

This study reports the anti-inflammatory, antinociceptive and antioxidant activities of *Areca catechu* extract and its aqueous fraction using in vivo and in vitro assays. Carrageenan-induced paw edema test is a well-established animal model of inflammation to detect the anti-inflammatory activity of test materials. Our pre-

limentary studies in mice showed that *Areca catechu* extract, its hexane, ethyl acetate and aqueous fractions demonstrated a significant inhibition against carrageenan-induced edema, similar to aspirin, the aqueous fraction being distinctly the most effective. This might suggest additional anti-inflammatory mechanism(s) along with the involvement of cyclooxygenase (COX) inhibitory pathway. These findings are consistent with the earlier studies showing anti-inflammatory potential of betel nut extract (Lee and Choi, 1999; Chang et al., 2004; Huang et al., 2010). On the basis of these findings, plant extract and its fractions were further tested in acute inflammatory model of rat paw edema. In control animals, maximum edema induction by carrageenan was evident at the 2nd h of administration reaching a plateau at the 3rd h followed by a decline at the 4th h of observation, which is also in line with the previous study (Mazzanti and Braghieri, 1994). *Areca catechu* extract and its aqueous fraction reduced edema volume in a dose and time-related manner, indicating the anti-inflammatory effect of the plant extract and its aqueous fraction. The inhibition of edema observed was more pronounced in the later phase than in the earlier phase, which was similar to the effect of aspirin, a prototype of non-steroidal anti-inflammatory drugs (NSAIDs) (Burke et al., 2006), indicating that the anti-inflammatory effect of *Areca catechu* is possibly mediated through a cyclooxygenase enzyme inhibitory pathway. Moreover, hexane and ethyl acetate fractions of *Areca catechu* did not cause significant inhibition of edema in rats (data not shown), hence were not studied further. The difference in the anti-inflammatory effect of hexane and ethyl acetate fractions in mice and rats may be due to species selectivity, as species-specific biological activities are known to exist (Ghayur et al., 2005; Ghayur and Gilani, 2006).

Inflammation is characterized by the accumulation of a variety of mediators at the site of the injury or infection. Carrageenan induces paw edema biphasically: the initial phase extending from 0 to 2.5 h, predominantly results due to the release of histamine, serotonin and bradykinin, and the later phase begins due to the over-production of prostaglandins such as PGE₂ in tissue (Di Rosa, 1974). However, COX enzyme is known to play a key role in the development of the later phase of inflammation in the carrageenan-induced edema model by converting arachidonic acid into prostaglandins. This enzyme is considered to be a known target for a variety of NSAIDs such as aspirin, which inhibits rat paw edema at the later phase following carrageenan injection. When tested in rats, *Areca catechu* extract and its aqueous fraction demonstrated dose and phase-dependent inhibition of edema at the 2nd h with maximum respective inhibition of 59.5 ± 1.80 and 80.2 ± 0.76% in a pattern similar to that of aspirin (47.2 ± 1.91%).

The aqueous fraction was more potent than the crude extract and aspirin. Separate sets of experiments were conducted to determine the effect of *Areca catechu* extract and its aqueous fraction against histamine or serotonin-induced inflammation, where they failed to inhibit the edemogenic effect of histamine or serotonin. These data indicate that the betel nut extract and its aqueous fraction are unlikely to interfere with histamine or serotonin release at the initial phase of inflammation. Thus, it can be speculated that the inhibition of edema by the extract and its aqueous fraction at the later phase of inflammation-induced by carrageenan may be mediated possibly through the inhibition of prostaglandin synthesis similar to aspirin. However, the stronger anti-inflammatory response of the aqueous fraction than the parent extract and aspirin indicates the presence of some additional constituents, involving different mode(s) of action, in the aqueous fraction with relatively better efficacy. In our further attempt to explore the possible explanation, in addition to the COX-inhibitory effect of *Areca catechu*, we observed the prostaglandin degrading and/or inactivating actions of the crude extract and its aqueous fraction, which was evident by their inhibitory effect on PGE₂-induced rat paw edema, while aspirin was found to be ineffective in this model. Moreover, the crude extract and its aqueous fraction showed a lipooxygenase inhibitory property by exhibiting an anti-inflammatory effect on arachidonic acid-induced rat paw edema, similar to caffeic acid, a lipooxygenase inhibitor (Sadina et al., 1993), but aspirin was found inactive, as the arachidonic acid-induced paw edema is highly sensitive to inhibitors of the lipooxygenase pathway and is resistant to selective COX inhibitors (Di Martino et al., 1987). These data demonstrate the anti-inflammatory effect of *Areca catechu* extract and its aqueous fraction mediated through multiple pathways.

Chang et al. (2004) showed the role of *Areca catechu* extract and arecoline in the pathogenesis of oropharyngeal cancer by activation of MEK1/ERK/c-Fos pathway. However, there are controversies in the available literature on *Areca catechu* extract such as the fact that Chang et al. (2004) showed the dose-related pro and anti-inflammatory effects *Areca catechu* extract and arecoline in different epithelial cells, while at the same time, arecoline, an active constituent of *Areca catechu* was found to have a blunting effect on inflammatory mediators, such as prostaglandin E₂ and interleukin-6. Similarly, multiple studies (Sundqvist et al., 1989; Dave et al., 1992; Jeng et al., 2001) conducted in different cell lines, have also shown the cytotoxic and genotoxic effects of the crude extract and arecoline. On the other hand, Lee and Choi (1999) and Huang et al. (2010) have reported the anti-inflammatory (carrageenan-induced inflammation in rats) and anti-oxidant (biochemical assays) effects of *Areca catechu* extract, showing that despite harmful effects of *Areca catechu* extract, it still has sufficient potential to be studied. Our study, being in line with the previous reports (Lee and Choi, 1999; Huang et al., 2010), provided additional information in the following ways:

1. Species-specific anti-inflammatory effects of the crude extract and its respective fractions which were shown in mice and rats.
2. Anti-inflammatory effect of *Areca catechu* extract and its aqueous fraction mediated through multiple pathways i.e. cyclooxygenase and lipoxygenase inhibition of arachidonic acid metabolism (dual inhibitor property) and its additional effect causing degradation and/or inactivation of prostaglandins (PGE₂).
3. It has also been identified that the aqueous fraction was found to be most effective in its anti-inflammatory and analgesic effects as compared to the parent extract and rest of the tested fractions, hence, the aqueous fraction could be studied further for the isolation of some pure compound(s).

The NSAIDs are also used as analgesics because prostaglandins are pro-inflammatory mediators contributing to the signs and
symptoms of inflammation such as pain (Crofford, 2000). Areca catechu extract was thus assessed for the first time for its possible anti-nociceptive effect using two different in vivo models: the acetic acid (AA)-induced writhings in mice and the formalin-induced licking in rats.

When tested in AA-induced writhing assay, Areca catechu extract and its aqueous fraction exhibited dose-dependent analgesic effects, the aqueous fraction being more potent than the positive control, aspirin. AA induces inflammatory pain by causing capillary permeability and liberating endogenous substances that stimulate pain nerve endings (Raj, 1996). While, NSAIDs cause inhibition of COX enzyme in peripheral tissues and interfere with the transduction mechanism of primary afferent nociceptors (Fields, 1987).

In the formalin-induced licking (nociception) model, the irritant produces pain in two phases: the 1st phase extends from 0-5 min which is mediated by the central nervous system via direct activation of peripheral nociceptors, as reflected by a rise in levels of substance-P or bradykinin, whereas the 2nd phase (15-45 min) is mediated through the release of a variety of inflammatory mediators including bradykinin and prostaglandins at the peripheral site, causing pain perception (Shibata et al., 1989), which is known to be inhibited by aspirin. On the other hand, opioid receptor agonists, such as morphine exert an analgesic effect via inhibiting the initial phase of formalin-induced nociception (Souza et al., 1998). Pretreatment of animals with Areca catechu extract or its aqueous fraction inhibited the 2nd phase of formalin-induced nociception at maximum tested dose by 46.3 ± 0.18 and 78.8 ± 1.10% respectively, similarly to aspirin (52.9 ± 2.18%). The order of potency was: aqueous fraction > Areca catechu extract > aspirin. This suggests that Areca catechu extract and its aqueous fraction possess analgesic effect possibly mediated through the inhibition of prostaglandin synthesis.

These results show the anti-inflammatory and analgesic effects of Areca catechu involving different mode(s) of action with better efficacy than aspirin. Stronger effects of Areca catechu than aspirin are also in line with the proven concept that medicinal plants possess a combination of constituents, involving different mode(s) of actions, offering synergistic or side-effect nullifying effects (Gilani and Rahman, 2005).

Generation of reactive oxygen species (ROS) is associated with the pathogenesis of multiple diseases such as atherosclerosis, diabetes, cancer, arthritis and the aging process. Inflammation is also a complex process and ROS play an important role in the pathogenesis of inflammatory diseases (Conner and Grisham, 1996). Thus antioxidants, which can scavenge ROS, are expected to improve inflammatory disorders. The free radical scavenging activity of the extract and its aqueous fraction was evaluated on the basis of its ability to scavenge the synthetic DPPH. Areca catechu extract and its aqueous fraction exhibited significant antioxidant activity, similar to rutin. Free radicals, such as hydroxyl radicals have been demonstrated to be a contributing factor in the tissue injury and modulation of pain (Khalil et al., 1999). Thus, any antioxidant which either inhibits the generation of free radical enzymes or directly scavenges the reactive free radicals to remove excess free radicals effectively can also prevent inflammation through its antioxidative effect. The observed antioxidant effect of Areca catechu extract and its aqueous fraction is also consistent with the previous study (Lee and Choi, 1999). However, we found a stronger anti-oxidant activity of the extract as compared to earlier report of Lee and Choi (1999), which might be due to the effect of environmental and regional factors which are known to influence the expression of phytochemical constituents in the same plants grown in different areas (Harborne, 1993; Lee et al., 2007).

5. Conclusion

Areca catechu extract and its aqueous fraction possess anti-inflammatory and analgesic activities mediated possibly through cyclooxygenase and lipoygenase inhibitory pathways, and by degradation and/or inactivation of prostaglandin E2 (PGE2). These findings suggest that the areca extract and its aqueous fraction have good correlations with the medicinal use of Areca catechu in inflammatory disorders in the Unani (Greco-Arab) system of medicine. However, further bioassay-directed fractionation studies are required using a more potent aqueous fraction to identify the active compound(s) and their exact mode(s) of action.

Acknowledgement

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