Identification of antiplatelet and acetylcholinesterase inhibitory constituents in betel nut

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Objective: To investigate the possible mechanism and the compound(s) responsible for the antiplatelet and acetylcholinesterase (AChE) inhibitory effects of Areca catechu crude extract (Ac.Cr).

Methods: Aqueous-methanol (70%) was used for extraction of plant material (betel nut). Antiplatelet activity was measured in human platelet-rich plasma by using a Lumi-aggregometer while anti-AChE activity was measured spectrophotometrically in vitro. In an attempt to find the responsible compound(s) in betel nut for antiplatelet and anti-AChE activities, different commercially available betel nut compounds were tested.

Results: Ac. Cr inhibited platelet aggregation induced by arachidonic acid (AA), adenosine diphosphate (ADP), platelet-activating factor (PAF), epinephrine and Ca2+-ionophore. Ac. Cr was the most potent in inhibiting ADP- and Ca2+-ionophore-induced aggregation. In the AChE assay, Ac. Cr showed significant AChE inhibitory activity with almost complete inhibition of the enzyme. Out of the tested compounds, none of the compounds in betel nut showed any antiplatelet effect except for catechin that was the most potent against epinephrine-induced aggregation. Catechin was significantly less potent than Ac. Cr, indicating a presence of additional compound(s) with antiplatelet activity. For the AChE inhibitory effect, only tannic acid, gallic acid, diosgenin and isoguvacine were found to be active, whereby tannic acid was more potent than Ac. Cr.

Conclusion: This study shows the possible antiplatelet and AChE inhibitory potential of betel nut while further studies are needed to confirm and identify more compounds in betel nut for these actions.

Keywords: Areca catechu; plant extracts; platelet aggregation inhibitors; cholinesterase inhibitors; catechin; tannic acid
Betel nut is the seed of *Areca catechu* Linn. (Arecaceae family), a tree found abundantly in South and Southeast Asia. Betel nut is chewed by people in these areas of Asia, and other parts of the world, to give a variety of physical and psychological effects such as an enhanced ability to work, heightened alertness, euphoria, a feeling of well-being and physiological effects on the cardiovascular, gastrointestinal, and pancreatic systems. Scientific studies conducted on the nut have shown that it possesses anti-human immunodeficiency virus, hypoglycaemic, antioxidant, antitumor, antischizophrenic, angiotensin-converting enzyme inhibitory, gastric prokinetic, vasodilator, hypotensive, cardio-suppressant, analgesic, anti-inflammatory and hepatoprotective properties. Apart from some of these beneficial effects, chewing betel nut has also been shown to induce oral submucous fibrosis, oral cancers, dependency syndrome, and anti-voluntary and abortifacient effects.

Among some of the beneficial effects of betel nut, it is known to cause a drop in blood pressure and heart rate, and known to help in memory retention, the reason why it is used by traditional healers in South Asia for hypertension and dementia. In light of these medicinal uses, the authors have previously reported the cardiovascular and memory-protective activities of betel nut. In order to continue this research, the authors report here the antiplatelet and acetylcholinesterase (AChE) inhibitory effects of betel nut. The antiplatelet potential was investigated in human blood against platelet aggregation induced by different agonists such as arachidonic acid (AA), adenosine diphosphate (ADP), platelet-activating factor (PAF), epinephrine and Ca\(^{2+}\)-ionophore. This study also attempts to explore the possible mechanisms of antiplatelet effects and screen some commercially-available constituents of betel nut, such as arecoline, arecaidine, catechin, tannic acid, gallic acid, diosgenin, isoguvacine and guvacine, in order to discover the compound(s) responsible for the antiplatelet and AChE inhibitory effects of betel nut.

1 Materials and methods

1.1 Chemicals AA, ADP, arecaidine, arecoline, acetylthiocholine (ATCh), Ca\(^{2+}\)-ionophore (A23187), (−)-catechin, diosgenin, 5,5-dithiobis (2-nitro) benzoic acid (DTNB), electric eel AChE (type VI-S), epinephrine, gallic acid, guvacine, isoguvacine, PAF, and tannic acid, were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of all the chemicals were prepared and the dilutions were made freshly in a suitable vehicle on the day of experiment.

1.2 Plant materials and extraction procedure

Betel nut (dried variety, 1 kg) was bought from a market in Karachi, Pakistan and a sample of the material was identified and deposited at the Herbarium of the Department of Biological and Bio-medical Sciences, Aga Khan University, Karachi, Pakistan (voucher No. AC-SE-05-99-18). The plant material was cleaned of adulterants, crushed, and soaked in 2 L of 70% aqueous methanol at room temperature for a total of 3 days after which the filtrate was collected through Whatman qualitative filter papers (Grade-1) and the plant material was re-soaked twice. The combined filtrate was concentrated in a rotary evaporator under reduced pressure at 40 °C to yield a viscous, dark brown *Areca catechu* crude extract (Ac. Cr) weighing 144 g (yield 14.4% w/w). This extract was stored at −4 °C until use and dissolved in distilled water on the day of the experiment to prepare the stock solution and different dilutions.

1.3 Preparation of human platelets and measurement of platelet aggregation

All the experiments were performed in accordance with the ethical guidelines of the Ethical Review Committee of the Aga Khan University and by standards formulated in the Helsinki Declaration of 1964 (revised in 2004). Experiments were carried out as previously described. Blood was drawn by vein puncture from human volunteers with a mean age of 21.1 years and of both genders, 8 (58%) men and 6 (42%) women. The volunteers were healthy, with no known disease or sickness and free from medication for at least 7 days. Blood samples were mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260 × g for 15 min at 20 °C to obtain platelet-rich plasma (PRP). The remaining portion of the blood sample was centrifuged at 1790 × g for 5 min to obtain the platelet poor-plasma (PPP). Experiments were performed within 2 h of PRP preparation. Platelet aggregation was monitored by using a dual-channel lumi-aggregometer (Model 400 Chronlog Corporation, Chicago, IL, USA) using 0.45 mL aliquots of PRP. The light transmission was adjusted to 0 and 100% with PRP and PPP, respectively. Aggregation was induced by the addition of aggregatory agents such as AA (1.7 mmol/L), ADP (4.3 μmol/L), PAF (8 mmol/L), epinephrine (20 μmol/L) and Ca\(^{2+}\)-ionophore (10 μmol/L) to the PRP. The antiplatelet effect of the test substance (betel nut extract/betel nut pure compounds) was studied by pretreating the PRP with the test substance for 1 min followed by addition of the aggregatory agonist. The resulting aggregation was recorded for 5 min via change in light transmission as a function of time. The antiaggregatory effects of the test substances were compared to acetylsalicylic acid, a standard antiplatelet agent, which showed its antiplatelet activity against AA-induced aggregation with an inhibitory concentration of 50% (IC\(_{50}\)) value of 0.03 mg/mL (95% confidence interval (CI): 0.03–0.03, n=5).
1.4 Enzyme assay for cholinesterase inhibition
The modified spectrophotometric method of Ellman et al.\(^{[20]}\) was followed. Electric eel AChE was used, while ATCh was used as the substrate of the reaction. DTNB was used for the measurement of the AChE activity. In this procedure, 140 μL of 0.1 mmol/L sodium phosphate buffer (pH 8.0) along with 10 μL of DTNB and 20 μL of the test substance solution were mixed and incubated for 15 min at 25 °C.

The reaction was then initiated with the addition of 10 μL of ATCh. The hydrolysis of ATCh was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion formed as a result of the reaction of DTNB with acetylthiocholine iodide (ATCI), released by the enzymatic hydrolysis of ATCI at a wavelength of 412 nm. The test substance and the control were dissolved in 5% ethanol. All the reactions were in triplicate, and the initial rates were measured as the rate of change in mean optical density per minute (mOD/min) and used in subsequent calculation. Physostigmine was used as a standard AChE inhibitor and it demonstrated an inhibitory effect on AChE at an IC\(_{50}\) value of 0.04 μg/mL (95% CI: 0.04–0.04; n = 3).

1.5 Statistical analysis
All the data were expressed as \( \bar{x} \pm s_x \) and the IC\(_{50}\) with 95% CI concentration-response curves were analyzed by non-linear regression (GraphPad program, GraphPad, San Diego, CA, USA).

2 Results

2.1 Effects of Ac. Cr on human platelet aggregation
Ac. Cr inhibited platelet aggregation induced by different agonists in a concentration-dependent manner (0.07 to 2.00 mg/mL) (Figure 1A). The agonists used were AA, ADP, PAF, epinephrine and Ca\(^{2+}\)-ionophore while the IC\(_{50}\) values for the inhibitory effect of Ac. Cr were: 1.590 mg/mL (95% CI: 1.490–1.695, n = 6), 0.628 mg/mL (95% CI: 0.299–1.318, n = 4), 1.902 mg/mL (95% CI: 1.673–2.163, n = 5), 1.677 mg/mL (95% CI: 1.631–1.725, n = 5) and 0.987 mg/mL (95% CI: 0.521–1.402, n = 5), respectively.

2.2 Effects of betel nut pure compounds on human platelet aggregation
Different constituents of betel nut, such as arecoline, arecaidine, catechin, tannic acid, gallic acid, diosgenin, isoguavine and guavacine were tested for their antiplatelet effect against the different aggregatory agonists. Except for catechin, none of these compounds showed any antiplatelet effect up to the tested concentration of 10 mg/mL. Catechin demonstrated an antiplatelet effect (Figure 1B) on all the aggregatory agonists such as AA, ADP, PAF, epinephrine and Ca\(^{2+}\)-ionophore at a concentration range of 1 to 5 mg/mL and with IC\(_{50}\) values of 3.630 mg/mL (95% CI: 3.560–3.701, n = 5), 1.876 mg/mL (95% CI: 1.676–2.099, n = 4), 2.551 mg/mL (95% CI: 2.501–2.603, n = 4), 1.541 mg/mL (95% CI: 1.485–1.598, n = 5) and 2.640 mg/mL (95% CI: 2.568–2.714, n = 5), respectively.

2.3 Effects of Ac. Cr on AChE activity
The extract exhibited a strong inhibition of AChE activity in vitro (see Table 1 for IC\(_{50}\) value) in the AChE assay. This is in accordance with the authors’ earlier findings\(^{[9]}\). Ac. Cr showed a maximum of (90.1 ± 0.4)% inhibition of the enzyme.

2.4 Effects of betel nut pure compounds on AChE activity
Different pure compounds of betel nut were tested for their AChE inhibitory effect. The compounds tested were: arecoline, arecaidine, catechin, tannic acid, gallic acid, diosgenin, isoguavine and guavacine. The study found that all of these, except for arecoline, arecaidine, catechin and guavacine, showed considerable AChE inhibitory activity (see Table 1 for IC\(_{50}\) values). The efficacy shown by these compounds, in terms of maximum inhibition of AChE activity, was: (55.7 ± 5.1)% (tannic acid), (69.8 ± 0.8)% (gallic acid), (78.3 ± 4.0)% (diosgenin) and (85.5 ± 2.0)% (isoguavine), respectively.

![Figure 1](image-url)

**Figure 1** Effects of betel nut crude and pure compounds on human platelet aggregation
Concentration-response curves show the inhibitory effect of (A) betel nut 70% aqueous-methanolic extract and (B) (+)-catechin, against platelet aggregation induced by agonists such as arachidonic acid (AA, 1.7 mmol/L), adenosine diphosphate (ADP, 4.3 μmol/L), platelet-activating factor (PAF, 8 nmol/L), epinephrine (20 μmol/L) and Ca\(^{2+}\)-ionophore (10 μmol/L) in human venous blood. The symbols represent \( \bar{x} \pm s_x \) from 4 to 6 determinations and expressed as a percentage of agonist-induced aggregation.
Table 1  Comparative IC_{50} values for the inhibitory effect of betel nut extract and its constituents on acetylcholinesterase

<table>
<thead>
<tr>
<th>Test substance</th>
<th>n</th>
<th>IC_{50} value (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac. Cr</td>
<td>3</td>
<td>2.80 (1.80−3.80)</td>
</tr>
<tr>
<td>Arecoline</td>
<td>3</td>
<td>(not active)</td>
</tr>
<tr>
<td>Arecaidine</td>
<td>3</td>
<td>(not active)</td>
</tr>
<tr>
<td>Catechin</td>
<td>3</td>
<td>(not active)</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>3</td>
<td>0.10 (0.01−0.19)</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>3</td>
<td>47.10 (38.10−56.10)</td>
</tr>
<tr>
<td>Diosgenin</td>
<td>3</td>
<td>103.60 (97.60−109.60)</td>
</tr>
<tr>
<td>Isoguvacine</td>
<td>3</td>
<td>17.20 (16.30−18.10)</td>
</tr>
<tr>
<td>Guvacine</td>
<td>3</td>
<td>(not active)</td>
</tr>
</tbody>
</table>

Values represent means along with 95% confidence intervals in parenthesis. IC_{50}: effective concentration causing 50% inhibition; Ac. Cr: Areca catechu crude extract.

3 Discussion

Ac. Cr showed concentration-dependent inhibition of platelet aggregation induced in human platelets by different agonists such as AA, ADP, PAF, epinephrine and Ca^{2+}-ionophore. The extract was more potent in its antiplatelet effect against aggregation induced by ADP and Ca^{2+}-ionophore when compared with other agonists. Contrary to the findings here, Jeng et al.\(^{[50]}\) have shown that betel nut induces, rather than inhibits, platelet aggregation in rabbit blood. This discrepancy can be attributed to the difference in species, as has been shown in the past; the same plant components can have variable response in different species\(^{[31, 32]}\).

In human platelet membrane, AA serves as a precursor for thromboxane A_{2} (TXA_{2}) synthesis\(^{[32]}\) while PAF is also a potent stimulator of TXA_{2} production\(^{[27]}\). When synthesized or stimulated, TXA_{2} exerts its proaggregatory effect by binding to specific receptors, leading to aggregation by Ca^{2+} influx\(^{[44]}\). Epinephrine on the other hand interacts with a_{2}-receptors in platelets which correlate with an increase in free cytosolic Ca^{2+}\(^{[30]}\). ADP is also known to elicit platelet aggregation via an increase in Ca^{2+} influx\(^{[30]}\). The extract not only blocked ADP-mediated platelet aggregation but also inhibited aggregation induced by Ca^{2+}-ionophore, possibly indicating interference with Ca^{2+} signaling. However, these are preliminary findings and more detailed studies using specific receptor and channel blockers are imperative for a definitive conclusion detailing the mechanism of antiplatelet action of this extract.

To discover the compound(s) in betel nut that could be responsible for the antiplatelet effect of the parent extract, a number of commercially available betel nut compounds were tested. The compounds tested were arecoline, arecaidine, catechin, tannic acid, gallic acid, diosgenin, isoguvacine and guvacine. None of these compounds showed any antiplatelet effects against the different aggregatory agonists except for catechin. This result reiterates the earlier finding of antiplatelet effect of this compound on AA-induced aggregation\(^{[30]}\). Catechin, a flavonoid, inhibited the agonist-induced aggregation in the following order of potency: epinephrine > ADP > PAF = Ca^{2+}-ionophore > AA. It was generally less potent than the parent extract for its antiplatelet effect. This indicates that catechin might be only partially responsible for the antiplatelet effect of the extract. The antiplatelet role of other compounds in betel nut cannot be ruled out. An alternative explanation could be that the effect of catechin, when being presented in its natural composition, gets potentiated due to the presence of other possible chemical(s). The presence of synergistic combinations in the crude herbal products has already been documented\(^{[27]}\). The authors have earlier shown Ca^{2+} inhibitory activity of catechin in gut, airway and vascular smooth muscle preparations\(^{[48]}\). This reported antiplatelet activity of betel nut, together with earlier reports of the cardio-suppressant, vasodilator and hypotensive effects of betel nut\(^{[10, 11]}\) and vasodilator activity of its constituent catechin\(^{[38]}\) reiterates the possible presence of therapeutic constituents in this plant. However, this study is rather preliminary in scope, further pharmacological, chemical and clinical studies are required on betel nut to investigate these findings in detail. This is recommended due to the fact that there are other studies done on betel quid chewing (which involves chewing of betel nut along with lime and Piper betle leaf) which have shown this mixture to have detrimental effects on cardiovascular health\(^{[10-14]}\).

Ac. Cr was then evaluated for possible AChE inhibitory effects. The extract showed a robust AChE inhibitory effect. This AChE inhibitory effect, along with the known presence of cholinergic constituents in betel nut\(^{[10-11]}\), possibly indicates its usefulness in memory disorders. AChE inhibitors and muscarinic agonists are the drugs of choice in such conditions\(^{[33, 41]}\). However, these are conclusions drawn upon enzymatic assays, which demand further investigations in detail.

In order to determine the probable compound(s) in betel nut for this AChE inhibitory effect, the effects of different commercially-available compounds of betel nut on AChE were tested, namely: arecoline, arecaidine, catechin, tannic acid, gallic acid, diosgenin, isoguvacine and guvacine. Tannic acid was found to be more potent compared with the parent extract and all the other compounds (see Table 1 for comparison of IC_{50} values), indicating that it may be the principal compound with this activity in betel nut. The four compounds were found active on the AChE inhibition assay here (tannic acid, gallic acid, diosgenin and isoguvacine) and are being reported for their anti-AChE activity for the first time in the literature. Although arec-
oline was found inactive in this AChE assay, it has been shown in the past to improve memory in patients with Alzheimer disease\cite{44} and increase cognitive performance in primates\cite{45}. Recently in another study, arecoline was shown to have neurotoxic effects in rats through enhancement of oxidative stress\cite{46}. These contradictory findings again show the need for more comprehensive studies in this area.

In conclusion, this study shows the antiplatelet and anti-AChE effect of betel nut and some of its known compounds. The extract showed inhibition of platelet aggregation induced by all of the aggregatory agonist used in this study. More detailed studies are needed to ascertain the mechanism of this antiplatelet effect. Catechin was found to be, at least, partially responsible for the antiplatelet effect while tannic acid, gallic acid, diosgenin and isouguavine were possibly responsible for the anti-AChE activity. The results indicate the presence of therapeutic compounds in betel nut while further studies are needed to investigate the new pharmacological and therapeutic questions that have arisen from this study.

4 Acknowledgements

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5 Competing interests

The authors declare that they have no competing interests.

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槟榔子中抗血小板聚集及抑制乙酰胆碱酯酶活性的有效成分

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目的：研究槟榔子（Areca catechu）粗提取物中所含的抗血小板聚集及抑制乙酰胆碱酯酶活性的有效成分及其作用机制。

方法：使用70％甲醇水溶液对槟榔子进行粗提取。使用生物发光血小板凝集仪在富血小板血浆中测定槟榔子粗提取物的抗血小板聚集作用。使用分光光度计在试管内测定槟榔子粗提取物对乙酰胆碱酯酶活性的抑制作用。检测槟榔子中的多种化合物以测定槟榔子中抗血小板聚集及抑制乙酰胆碱酯酶活性的有效成分。

结果：槟榔子粗提取物能够抑制花生四烯酸、二磷酸腺苷、血小板活化因子、肾上腺素及钙离子载体引起的血小板聚集，尤其对二磷酸腺苷及钙离子载体引起的血小板聚集的抑制最为明显；槟榔子粗提取物能够显著抑制乙酰胆碱酯酶的活性。在所检测的槟榔子所含化合物中，只有儿茶素对肾上腺素引起的血小板聚集有显著的抑制作用，而这种抑制作用显著弱于槟榔子粗提取物；提示槟榔子中的其他成分参与了这种抑制作用；鞣酸、没食子酸、鞣质苷元和异去甲槟榔次碱能够抑制乙酰胆碱酯酶的活性，其中鞣酸的抑制作用强于槟榔子粗提取物。

结论：槟榔子中含有抗血小板聚集及抑制乙酰胆碱酯酶活性的有效成分，而发挥这些功效确切成分有待进一步的研究证实。

关键词：槟榔；植物提取物；血小板聚集抑制剂；胆碱酯酶抑制剂；儿茶素；鞣酸