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Studies on the Hypotensive, Cardio-suppressant, Vasodilator and Antiplatelet Activities of Betel Nut Crude Extract and its Constituents

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Abstract: This study reported the hypotensive, cardio-suppressant, endothelium-dependent vasodilator and antiplatelet activities of the crude extract of *Areca catechu* (Ac.Cr), commonly known as betel nut. Ac.Cr tested positive for the presence of terpenoids, flavonoids, amines, tannins, phenols, alkaloids and saponins and exhibited a dose-dependent (0.1 to 1 mg kg^{-1}) atropine-sensitive fall in the arterial blood pressure of normotensive rats under anaesthesia. In isolated guinea-pig atria, Ac.Cr (0.1 to 10 μg mL^{-1}) showed an atropine-sensitive inhibitory effect on the force and rate of spontaneous atrial contractions. In the endothelium-intact rat aorta, Ac.Cr showed inhibition of the phenylephrine-induced contractions. This relaxant effect was blocked by atropine and L-NAME and was found absent in endothelium-denuded preparations. Ac.Cr (1.0-1.75 mg mL^{-1}) also inhibited arachidonic acid (1.7 mM)-induced human platelet aggregation. Among all the known constituents of betel nut tested, only arecoline showed atropine-sensitive cardio-suppressant and vasodilator effects while catechin exhibited atropine-insensitive vasodilator and also antiplatelet activities. This study shows that betel nut exhibits blood pressure lowering and antiplatelet activities mediated at least partly through the presence of known constituents such as arecoline and catechin, respectively.

Key words: Betel nut, cholinergic, hypotensive, cardio-suppressant, endothelium-dependent vasodilator, antiplatelet

INTRODUCTION

Betel nut is the seed of the yellowish-red fruit of areca palm, *Areca catechu*, a handsome tree cultivated in all the warmer parts of Asia. The word 'Areca' has possibly originated from the Malay name of the tree while 'Catechu' is the name of the spice (*Acacia catechu*) that is mixed with the betel nut and some other ingredients to constitute the 'betel quid'. Betel nut is masticated to give the chewer physical and psychological effects ranging from enhanced ability to work, heightened alertness, euphoria, increased well being and effects on the cardiovascular, gastrointestinal and pancreatic systems[1]. Studies conducted on the nut report that, among others, it has anti HIV, antibacterial, hypoglycaemic, prohealing, antioxidant, antitumor, antidepressant, antischizophrenic, angiotensin-converting enzyme inhibitory[1] and gastric prokinetic activities[2].

Chewing of the nut is known to be associated with a drop in the blood pressure and heart rate[3] and it is traditionally used in the Sub-Continent to help control hypertension[4]. In this study, we tested the crude extract of betel nut on blood pressure in anaesthetized rats, in isolated cardiovascular tissue preparations and against arachidonic acid–induced platelet aggregation. The results were then compared with some of the known constituents of the nut such as arecoline, arecaidine, catechin, tannic acid, gallic acid and diosgenin[5,6], obtained from commercial sources, to determine the compounds possibly responsible for the observed activity in the crude extract.

MATERIALS AND METHODS

Animals: Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources,

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Commission on Life Sciences, National Research Council[7]. Balb-C mice (20-25 g), Sprague-Dawley rats (200-250 g) and guinea-pigs (500-700 g) of either sex used in the study were housed in the animal house of The Aga Khan University under a controlled environment (23-25°C). Animals were given tap water *ad libitum* and a standard diet consisting of (g kg^{-1}): flour 380, fibre 380, molasses 12, NaCl 5.8, nutrivet L 2.5, potassium metabisulphate 1.2, vegetable oil 38, fish meal 170 and powdered milk 150.

Chemicals: The following reference chemicals were obtained from the sources specified: acetylcholine chloride (ACh), arachidonic acid (AA), arecaidine hydrochloride, arecoline hydrobromide, atropine sulphate, carbamylcholine chloride (carbachol, CCh), (+)-catechin hydrate, diosgenin, gallic acid, isoprenaline hydrochloride, Nù-nitro-L-arginine methyl ester hydrochloride (L-NAME), norepinephrine hydrochloride (NE), physostigmine hemisulfate and tannic acid (Sigma Chemical Company, St. Louis, MO, USA). All chemicals used were of the highest purity grade. Stock solutions of all the chemicals were made in distilled water and the dilutions were made fresh in normal saline on the day of the experiment.

Plant material and extraction procedure: Betel nut (1 kg) was bought from a market in Karachi and a sample of the material was deposited at the Herbarium of Department of Biological and Biomedical Sciences, The Aga Khan University, Karachi with the voucher # AC-SE-05-99-18. The plant material was cleaned of adulterants, crushed and was 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 Grade-1 filter papers and the plant material re-soaked again twice. The combined filtrate was concentrated in a rotary evaporator under reduced pressure at 40°C to yield a viscous, dark brown crude extract (Ac.Cr) weighing 144 g. This extract was stored at -4°C until use and dissolved in distilled water on the day of the experiments to prepare the stock solution and different dilutions.

Preliminary phytochemical analysis: The betel nut crude extract was screened for the presence of different classes of compounds by thin layer chromatography using silica gel G (Merck) plates of 0.25 mm thickness[8]. The extract was dissolved in chloroform: methanol (1:1 v/v) while the development of plates was carried out with chloroform: methanol (4:1 v/v). After development, the plates were sprayed with the following solvents and reagents for detection of the respective classes of compounds: water (lipophilic compounds); sulphuric acid and heating at 105° for 5 min (organic compounds); 0.5% anisaldehyde in sulphuric acid, glacial acetic acid and methanol 5:10:85 v/v (terpenoids); 10% antimony trichloride in chloroform (flavonoids/terpenoids); 1% diphenylboric acid 2-aminoethyl ester in methanol followed by 5% polyethylene glycol 4000 in 96% ethanol (flavonoids); 0.5% ninhydrin in acetone (amino acids/peptides and secondary amines); 5% ethanolic sodium hydroxide (anthraquinones); 5% aqueous ferric chloride (tannins/phenols); 20% aqueous sodium carbonate followed by Folin-Ciocalteu reagent (phenols); 0.5% aqueous fast blue B salt followed by 0.1 M aqueous sodium hydroxide (phenols); Dragendorrf's reagent (alkaloids) and dilute sodium hydroxide (coumarins). Reagents were prepared according to Stahl[9]. Detection was carried out visually in visible light and under UV light (λ = 365 nm).

***In vivo* blood pressure (BP) in anaesthetized rats:** The experiment was performed as described previously[10]. Rats were anaesthetized with an intraperitoneal injection of sodium thiopental (Pentothal, 70-90 mg kg^{-1} body weight). When light anaesthesia was achieved, the right carotid artery was cannulated by polyethylene tubing PE-50, which was connected to a pressure transducer (P23 XL) coupled with a Grass (model 7) Polygraph. This connection was used for BP recording. The left jugular vein was cannulated with similar tubing to facilitate the intravenous injection of the drugs and the betel nut extract. The exposed surface of the cannulation was covered with cotton wool moistened in warm saline. After a 20 min period of equilibrium, the rats were injected intravenously with 0.1 mL saline (NaCl 0.9%) or with the same volume of the extract. Arterial BP was allowed to return to the resting level between injections. Standard drugs and the betel nut extract (all prepared in saline) were then administered by i.v. injections and flushed in with 0.1 mL saline. Control responses of standards as ACh (1 μg kg^{-1}) and NE (1 μg kg^{-1}) were obtained before testing the extracts. Changes in BP were recognized as the difference between the steady state values before and the lowest readings after injection. Mean arterial blood pressure (MABP) was calculated as the diastolic blood pressure plus one-third pulse width.

Isolated guinea-pig atria: Right and left atria from guinea-pigs were dissected out, cleaned of fatty tissues and mounted separately in 20 mL tissue baths containing Kreb's solution at 32°C and aerated with 5% carbon dioxide in oxygen (carbogen). The composition of Kreb's solution was (mM): NaCl 118.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5,

KCl 4.7, KH_2PO_4 1.3, $MgSO_4$ 1.2 and glucose 11.7 (pH 7.4). The tissues were allowed to beat spontaneously under the resting tension of 1 g. An equilibrium period of 30 min was given before the application of any drug. Control responses of ACh (0.1-0.3 μM) and isoprenaline (0.1 μM) were obtained at least in duplicate. Tension changes in the tissue were recorded via a Grass force-displacement transducer (model FT-03) using Grass (model 7) Polygraph. Ac.Cr along with some of its known constituents (Fig. 1) such as arecoline, arecaidine, catechin, tannic acid, gallic acid and diosgenin, were tested on the atrial contractility for determining their activity.

Isolated endothelium-intact rat aorta: The procedure of Furchgott and Zawadski[11] was followed with some modifications. Thoracic aorta was isolated from male rats. Care was observed in isolating the tissue to avoid any damaging of endothelium. The isolated aorta was then transferred into carbogen aerated Kreb's solution. It was then carefully cleaned of fats and then made into rings 3 mm wide. Individual rings were hung in 5 mL tissue baths, at 37°C. A preload of 1 g was applied to the preparation and the tissue incubated for 30 min. Changes in tension were recorded via World Precision Instrument's (WPI) Isometric Force transducers (Fort 100) connected to Transbridge 4 M and displayed on to a Personal Computer via CVMS Data Acquisition System. Following an equilibrium period of 30 min, contractions were taken with PE (1 μM). The tissue was stabilized with repeated doses of PE and considered stabilized only when the similar responses were obtained. Later a contraction was induced with PE (1 μM) and once a plateau was achieved, ACh (0.1 μM) was administered to confirm endothelium-dependent relaxation. The endothelium lining of the tissues was removed by gentle rubbing, which resulted in the disappearance of this relaxation. To study whether or not the vasodilator effect of the test substance is mediated through an intact-endothelium, the tissues were preincubated with the respective antagonists such as atropine (1 μM) or L-NAME (0.1 mM) for 20 and 60 min, respectively to explore the possible mode of action. Ac.Cr along with its known constituents (Fig. 1) such as arecoline, arecaidine, catechin, tannic acid, gallic acid and diosgenin, were tested on the rat aorta preparation for determining their activity.

Preparation of human platelets and measurement of platelet aggregation: Blood was taken by vein puncture from normal human volunteers reported to be free of

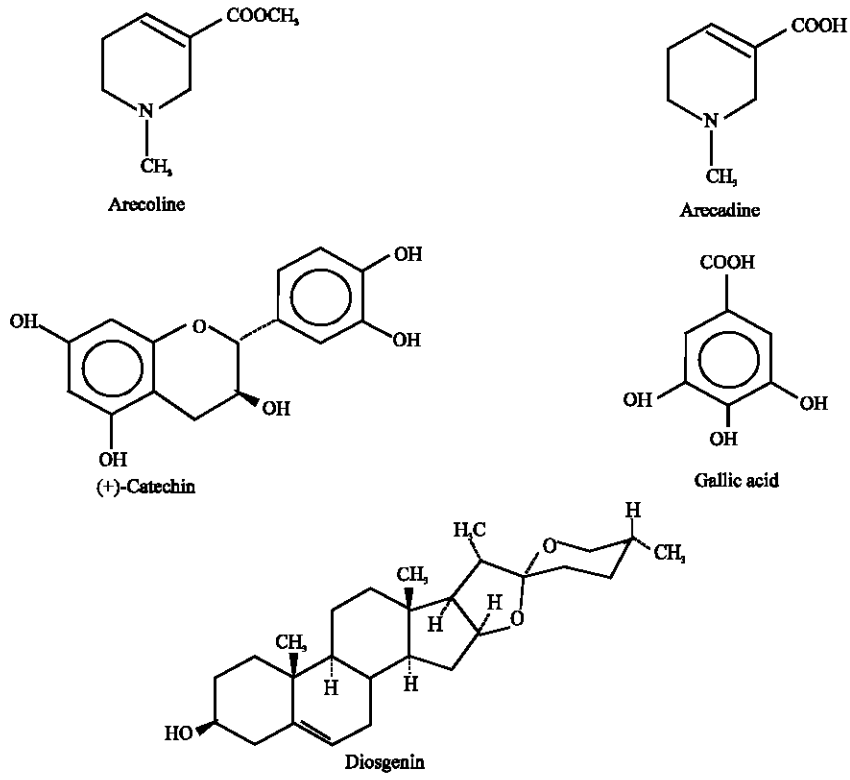

Fig. 1: Chemical structures of the betel nut constituents, arecoline, arecaidine, (+) -catechin, gallic acid and diosgenin

medication for one week. Blood samples were mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 1500 rpm for 15 min at 20°C to obtain Platelet Rich Plasma (PRP). The remaining blood samples were centrifuged at 4000 rpm for 5 min to obtain the platelet poor-plasma (PPP). Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37°C with PRP having platelet counts between 2.5 and 3.0 $x10^8$ platelets/ml of plasma [12]. Experiments were performed within 2 h of PRP preparation. Aggregation was monitored using Dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA) using 0.45 mL aliquots of PRP[12]. The final volume was made up to 0.5 mL with the test drug dissolved either in normal saline or appropriate vehicle known to be devoid of any effect on aggregation. The platelet suspension was stirred at 1000 rpm. The light transmission was adjusted to 0 and 100% with PRP and PPP, respectively. Aggregation was induced with AA (1.7 mM) and the sub-threshold concentration was determined. The antiplatelet effects of the crude extract and the different betel nut constituents were studied by pretreatment of PRP with these potential inhibitors for one min followed by addition of AA. The resulting aggregation was recorded for 5 min after challenge, by the change in light transmission as a function of time. Once the antiplatelet activity of these test substances was established, inhibitory EC_{50} values were calculated.

Acute toxicity in mice: Animals were divided in groups of 10 mice each. The test was performed using increasing doses (0.5, 1 and 2.5 g kg^{-1}) of Ac.Cr, given orally, in 10 mL kg^{-1} volume to different groups serving as test groups. Another group of mice was administered saline (10 mL kg^{-1}, p.o.) as negative control. The mice were allowed food *ad libitum* during a 24 h test and kept under regular observation for mortality and behavioural changes.

Statistical analysis: All the data expressed are as mean±SEM (SEM, n=number of experiments) and the median effective concentrations (EC_{50} values) with 95% confidence intervals (CI). The statistical parameter applied is the Student's t-test (paired or unpaired) with $p< 0.05$ noted as significantly different (GraphPAD program, GraphPAD, San Diego, CA, USA). Concentration-response curves were analysed by non-linear regression (GraphPAD program).

RESULTS

Preliminary phytochemical analysis: Ac.Cr tested positive for the presence of organic, coloured, double-bonded and fluorescent compounds, while terpenoids, flavonoids, amino acids/peptides, secondary amines, tannins, phenols, alkaloids and saponins were also found present. The rest of the compounds did not test positive. Critical evaluation of the TLC chromatograms revealed that the extracts had most extensive spots for amines and phenols followed by terpenoids, flavonoids and alkaloids.

Effect on blood pressure in anaesthetized rats: Intravenous administration of Ac.Cr produced a dose-dependent (0.1-1.0 mg kg^{-1}) fall in arterial BP in normotensive rats under anaesthesia (Fig. 2 and 3) with an EC_{50} of 0.16 mg kg^{-1} (0.08-0.33, 95% CI, n=3). Pretreatment of animals with atropine (1 mg kg^{-1}) blocked the BP lowering effect of the extract similar to that of ACh (Fig. 2).

Effect on guinea-pig atria: Ac.Cr exhibited a dose-dependent (0.1 to 10 μg mL^{-1}) suppression of the force and rate of spontaneous atrial contractions (Fig. 4 and 5A) with EC_{50} values of 0.95 μg mL^{-1} (0.58-1.50, n=5) and 2.51 μg mL^{-1} (1.24-5.08, n=5), respectively. ACh also produced a dose-dependent (0.002-0.2 μg mL^{-1}, n=4) inhibitory effect on the atrial force of contraction (Fig. 4, 5A) with an EC_{50} of 0.06 μg mL^{-1} (0.04-0.09, n=4). The cardio-suppressant effects of Ac.Cr and ACh were completely blocked in the presence of atropine (Fig. 4).

The constituents of betel nut namely arecoline, arecaidine, catechin, tannic acid, gallic acid and diosgenin were also tested on the atrial tissues. Arecoline produced a dose-dependent atropine-sensitive (0.002-0.240 μg mL^{-1}) inhibitory effect on the force of atrial contractions as expected (Fig. 5A) with an EC_{50} value of 0.03 μg mL^{-1} (0.02-0.05, n=4). None of the other compounds tested (up to 10 mg mL^{-1}) showed any effect on the cardiac preparation.

Effect on endothelium-intact rat aorta: When tested on the PE (1 μM)-induced contractions, Ac.Cr dose-dependently (1.0 to 30.0 μg mL^{-1}) depressed the induced contractions (Fig. 5B) with an EC_{50} value of 2.78 μg mL^{-1} (1.57-4.94, n=5) similar to an effect shown by the positive control ACh, which exhibited its relaxation with an EC_{50} value of 0.02 μg mL^{-1} (0.01-0.03, n=4, Fig. 5B). Vasodilation, observed from both the extract and ACh, was sensitive to atropine (1 μM) and L-NAME (0.1 mM) pretreatment and was also abolished in the endothelium-denuded preparations.

The known commercially available compounds of betel nut namely arecoline, arecaidine, catechin, tannic acid, gallic acid and diosgenin were also tested on phenylephrine (1 μM)-induced contractions in the endothelium-intact rat aorta preparation. Arecoline and

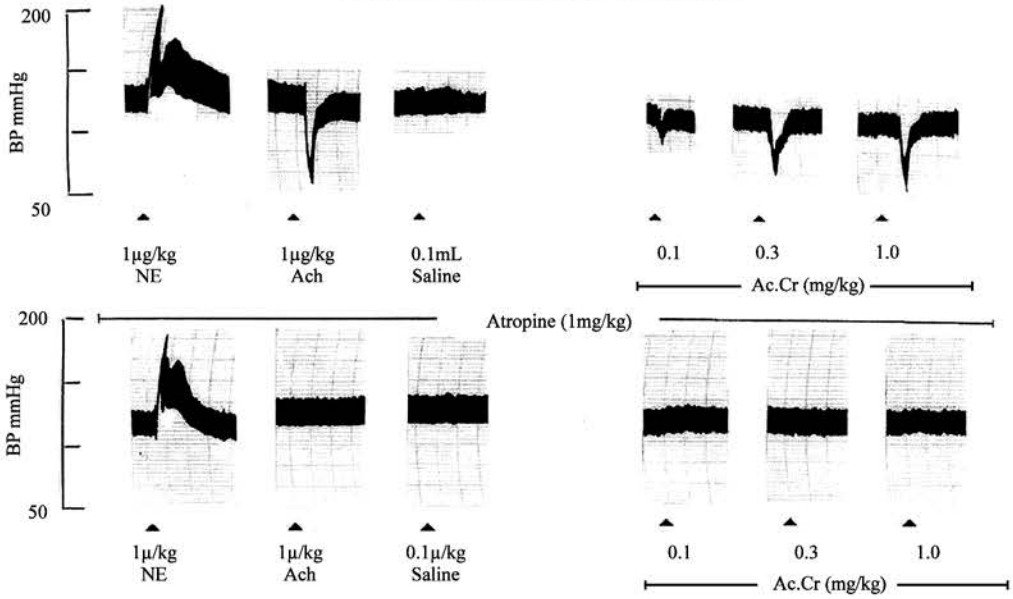

Fig. 2: Typical tracing showing the dose-dependent hypotensive effect of betel nut crude extract (Ac.Cr) in comparison to norepinephrine (NE) and acetylcholine (ACh) in the absence and presence of atropine on blood pressure (BP) in anesthetized rat

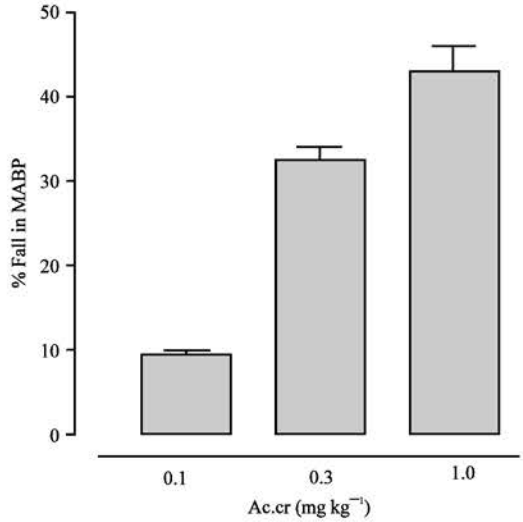

Fig. 3: Bar diagram showing the effect of betel nut crude extract (Ac.Cr) on mean arterial blood pressure (MABP) in anesthetized rat (values shown are mean ± SEM, n=3)

catechin were the only compounds which produced a dose-dependent vasodilator effect (Fig. 5B) with EC_{50} values of 0.17 µg mL^{-1} (0.13-0.22, n=4) and 396.90 µg mL^{-1} (306.31-514.40, n=11), respectively. This inhibitory response of arecoline was absent when tested in the presence of atropine (1 µM), L-NAME (0.1 mM) or when using the endothelium-denuded preparations. Catechin mediated relaxation was resistant to atropine or by denuding the endothelium. None of the other compounds tested (up to 10 mg mL^{-1}) showed any activity on the PE-contracted rat aorta preparation.

Effect on arachidonic acid-induced platelet aggregation: Ac.Cr dose-dependently (1.0-1.75 mg mL^{-1}) inhibited the AA-induced platelet aggregation (Fig. 6A) with an EC_{50} value of 1.57 mg mL^{-1} (1.46-1.68, n=6). The extract at the dose of 1.75 mg mL^{-1} inhibited the AA-induced aggregation (Fig. 6B). When the pure compounds were tested up to 10 mg mL^{-1} against AA-induced platelet aggregation, only catechin was found active (Fig. 6B). Catechin showed the inhibitory effect at a dose range of 3.0-4.5 mg mL^{-1} with an EC_{50} value of 3.59 mg mL^{-1} (3.53-3.65, n=6).

Acute toxicity in mice: The extract was found safe up to the oral dose of 2.5 g kg^{-1} for 24 h without any apparent behavioural side effects.

DISCUSSION

The BP lowering effect of betel nut crude extract goes in parallel with its traditional use in hypertension[4]. The hypotensive effect of ACh and that of the extract were completely blocked by atropine, a competitive blocker of acetylcholine at muscarinic receptors[13,14] thus indicating that the betel nut extract mediates its BP lowering effect through a mechanism similar to that of ACh.

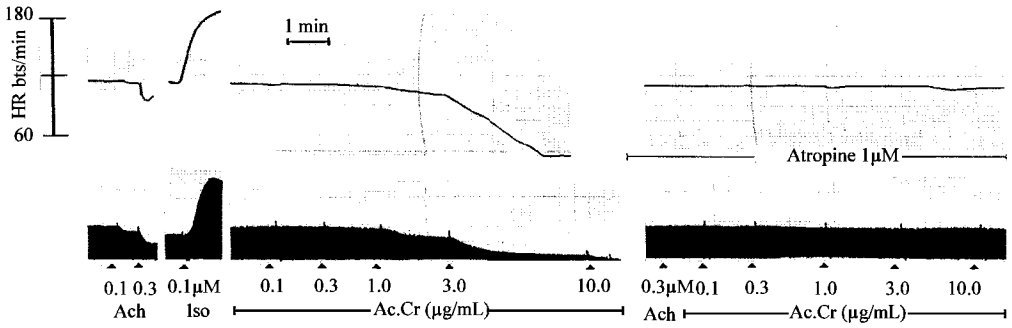

Fig. 4: Typical tracing showing the dose-dependent negative inotropic and negative chronotropic effect of betel nut crude extract (Ac.Cr) in comparison to isoprenaline (Iso) and acetylcholine (ACh) in the absence and presence of atropine in isolated guinea-pig atrium

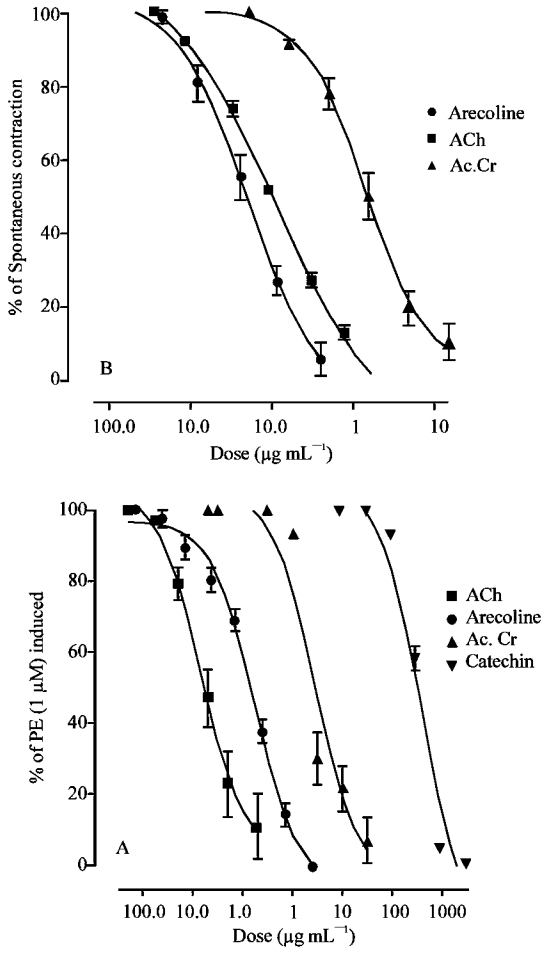

Fig. 5: Dose-response curves showing the effect of: [A] betel nut crude extract (Ac.Cr), arecoline and acetylcholine (ACh) on the force of guinea-pig atrial contractions and [B] betel nut crude extract (Ac.Cr), arecoline, acetylcholine (ACh) and catechin on phenylephrine (PE) induced-contractions in endothelium-intact rat aorta (values shown are mean±SEM, n=4-11)

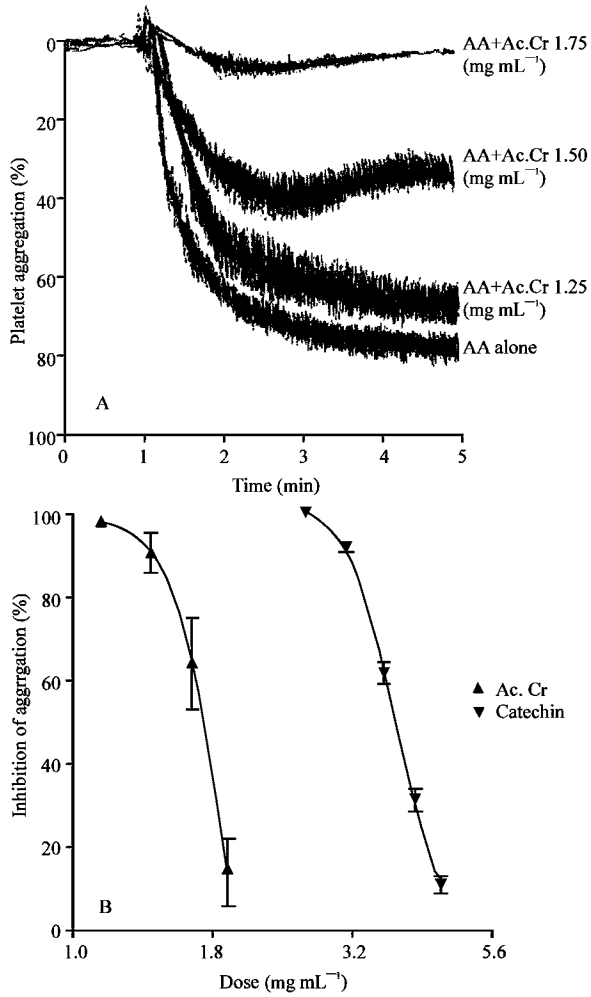

Fig. 6: [A] Typical tracing showing the antiplatelet effect of increasing doses of betel nut crude extract (Ac.Cr) against arachidonic acid (AA, 1.7 mM) - induced human platelet aggregation in human blood. Lower panel [B] shows the concentration responses curves for the inhibitory effect of Ac.Cr and catechin on AA-induced aggregation (values shown are mean±SEM, n=6)

To further investigate the BP lowering effect of Ac.Cr, experiments were performed in isolated cardiovascular tissues namely the guinea-pig atria and endothelium-intact rat aorta. Cholinergic activity of Ac.Cr was also observed in the guinea-pig atria preparations. Out of all the betel nut constituents tested, only arecoline, a known cholinergic agonist[15], exhibited an inhibitory effect on the atrial contractions. Arecoline has been shown previously to exhibit such a negative effect on the rate and force of cardiac contractions[16,17].

Blood pressure is a product of cardiac output and vascular resistance[18]. To see the effect of Ac.Cr and its pure compounds on vascular tissues, rat aorta was selected which is considered a prototype tissue used for evaluating potential endothelium modulating vasodilators[19,20]. Ac.Cr, when tested on the PE-induced contractions, showed a dose-dependent relaxant activity which was completely blocked in the presence of atropine, as seen earlier in the *in vivo* blood pressure test and atrial tissues. ACh produces dilation of essentially all vascular beds[21]. This dilation is due to the presence of muscarinic M_3 receptor subtypes which are located on the endothelial cells of the vasculature[22]. Stimulation of these receptors leads to release of nitric oxide (NO) from the endothelium which diffuses to the cells in the vicinity to show relaxation[23]. The vascular endothelium plays a pivotal role in modulating the contractility of the vascular tone through the release of vasodilating factors[24]. This release of NO is mediated by an increase of cGMP contents in the vascular smooth muscle in response to activation of guanylyl cyclase[25]. Thus to determine the involvement of NO, Ac.Cr initiated vasorelaxation was tested in the presence of L-NAME, a standard inactivator of NO synthesis[26]. The vasodilator effect of Ac.Cr was blocked in the presence of L-NAME, similar to that of ACh. To further confirm the endothelium-dependent vasorelaxant effect of the extract, Ac.Cr was tested in an endothelium-denuded preparation. Both Ac.Cr and ACh were found devoid of any vasodilator activity in the denuded preparation.

Phytochemical analysis of the extract showed, among others, the presence of alkaloids and phenols in the extract. Betel nut is known to contain nine major alkaloids[1,27] and the commonly known are arecoline (7.5 mg g^{-1} weight), arecaidine (1.5 mg g^{-1}), guvacoline (2.0 mg g^{-1}), guvacine (2.9 mg g^{-1}), isoguvacine, arecolidine and choline. It also has phenolic compounds such as hydroxychavicol and safrole[28]. Tannins, gallic acid, catechin, oily matter, gum and amino acids are also present in the areca nut[3]. When the constituent pure compounds of betel nut were tested in the rat aorta, only arecoline and catechin showed a dose-dependent vasodilator activity. Arecoline is known to be a vasodilator[29] and this work has shown that it is around 15 times more potent than the crude extract in its vasodilator activity. Although catechin was more than hundred times less potent than Ac.Cr, this is the first evidence of vasodilator activity of catechin. This is important particularly since arecoline is unlikely to mediate its effects when chewed in the form of the betel quid along with the other ingredients, such as betel leaf, lime and catechu, when it is readily hydrolysed by lime and converted to arecaidine[15,30,31] which has virtually no parasympathetic effect but is a GABA uptake inhibitor[32,33]. Thus the finding that catechin, an active ingredient of betel nut[1], has a vasodilator effect might play an important role in the vasodilator effect mediated by betel nut. The vasorelaxation shown by catechin was found to be resistant to atropine pretreatment thus implying that some other pathway is involved in its vasodilator effect.

The extract, when tested against AA-induced platelet aggregation in human blood, exhibited a dose-dependent inhibitory effect. AA is a potent mediator of platelet aggregation and is released endogenously by phospholipase A_2 resulting in the formation of thromboxane A_2, which in turn exhibits a vasoconstrictor and pro-aggregant effect[34]. Cardiovascular diseases such as arterial hypertension, thrombosis and atherosclerosis result in many other secondary problems, including blood coagulation and platelet aggregation disorders[35]. This antiplatelet activity of the extract reiterates the possible preventive or therapeutic use of betel nut in the related diseases. When the available pure compounds of betel nut were tested for a possible antiplatelet effect, none of them was found active, except for catechin, which exhibited its effect at a dose much higher than that of Ac.Cr. This indicates that catechin might be partially responsible for the antiplatelet effects of the extract, the role of other known compounds such as phenols, known to possess antiplatelet activities, cannot be ruled out[36,37]. The vasodilator and antiplatelet activities of catechin indicate that, apart from arecoline, other medicinally active compounds also exists in betel nut, negating a general misconception that arecoline is the only medicinally active compound in betel nut[38,39]. In the acute toxicity test, Ac.Cr was found to be safe in mice when administered up to a fairly large oral dose of 2.5 g kg^{-1}.

This study has shown the cardiovascular inhibitory effects of betel nut crude extract and some of its known constituents, predominantly through cholinergic action and may justify the cardiovascular depressant effects associated with betel nut chewing. The antiplatelet activity might be contributing factor to a potential use of betel nut in cardiovascular disorders.

ACKNOWLEDGMENTS

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