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# Cardio-selective inhibitory effect of the betel nut extract: possible explanation

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*Received April 25, 2006, accepted June 6, 2006*

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*Pharmazie 62: 67–71 (2007)* *doi: 10.1691/ph.2007.1.6583*

Chewing of betel nut, the seed of *Areca catechu*, is associated with a host of physical and psychological effects while it is also traditionally used in constipation and hypertension. In this study, we report the cardio-selective cholinomimetic activity of the betel nut crude extract (Ac.Cr). Ac.Cr, that tested positive for saponins, tannins, phenols, alkaloids and terpenes, exhibited dose-dependent atropine-sensitive inhibition of isolated guinea-pig atrial contractility with an $EC_{50}$ value of 0.93 μg/ml (0.57–1.51, 95% CI). In rabbit jejunum, Ac.Cr showed atropine-sensitive spasmogenicity with an $EC_{50}$ of 7.31 μg/ml (5.41–9.88, 95% CI) showing that it is around 8 times more potent in the cardiac than the intestinal preparation. Both carbachol and physostigmine exhibited acetylcholine-like stimulant activity in jejunum with the latter being more potent in jejunum than in atrial tissues. Activity-directed fractionation of Ac.Cr yielded fractions with similar cholinergic activity in atria and jejunum except the aqueous fraction being 6 times more potent in the atria. Arecoline, the known betel nut compound with cholinergic activity showed similar potency in both tissues while catechin and tannic acid exhibited intestinal spasmolytic effect but were inactive in atria. The results show the cardio-selective inhibitory effect of Ac.Cr which might possibly be due to selective gut-spasmolytic behaviour of catechin and tannic acid thus reducing the cholinomimetic activity of Ac.Cr in the gut though the preferential binding of the constituents of betel nut extract at muscarinic receptor subtypes in heart cannot be ignored.

## 1. Introduction

Betel nut is the seed of *Areca catechu* (Arecaceae); a tree abundantly cultivated in South and South-East Asian countries. The nut is chewed for its masticatory properties consequently imparting a variety of physical and psychological effects such as heightened alertness, euphoria, increased well being and effects on the cardiovascular, gastrointestinal and pancreatic systems (Gilani and Ghayur 2005). The history of betel chewing dates back to the Pacific region around 3600 years ago while its influx into the Indian Sub-Continent can be traced back to 320-499 AD. Now, betel nut is not only chewed in Asia but all over the world as this plant product is the world's most widely consumed drug after nicotine, ethanol and caffeine (Marshall 1987).

Betel nut is used traditionally in a number of ailments for its laxative, digestant, carminative, antidiarrhoeal, antiheartburn, antiulcer, anthelmintic, antimalarial, astringent, memory enhancing, blood pressure lowering and diuretic properties (Kapoor 1990; Gilani and Ghayur 2005). Biological studies conducted on the nut report that it has anti HIV (Kusumoto et al. 1995); antibacterial (Chen et al. 1987); hypoglycaemic (Chempakam 1993); prohealing (Padmaja et al. 1993); antioxidant (Ohsugi et al. 1999); antitumor (Iwamoto et al. 1988); antidepressant (Dar et al. 1997); antischizophrenic (Sullivan et al. 2000); angiotensin-converting enzyme inhibitory (Inokuchi et al. 1986) and prokinetic and acetylcholinesterase inhibitory activities (Gilani et al. 2004). Recently we reported cardiovascular inhibitory and antiplatelet activities of the betel nut extract (Gilani et al. 2006).

In this investigation, we report the cardio-selective inhibitory cholinergic effect of the crude extract of betel nut (Ac.Cr), and this selective behaviour was found to be concentrated in the aqueous fraction. Apart from the standard cholinomimetics, carbachol (CCh) and physostigmine, different commercially available known compounds of betel nut such as arecoline, arecaidine, catechin, tannic acid, gallic acid and diosgenin (Chu 2001; Gilani and Ghayur 2005) were also tested for bioactivity and were found with diverse pharmacological activities.

## 2. Investigations and results

### *2.1. Phytochemical analysis*

The crude extract and all the fractions showed the presence of saponins, tannins, phenols, alkaloids and terpenes.

### *2.2. Effect on guinea-pig atria*

Ac.Cr dose-dependently (0.1 to 10 μg/ml) suppressed the rate and force of spontaneous atrial contractions (Figs. 1

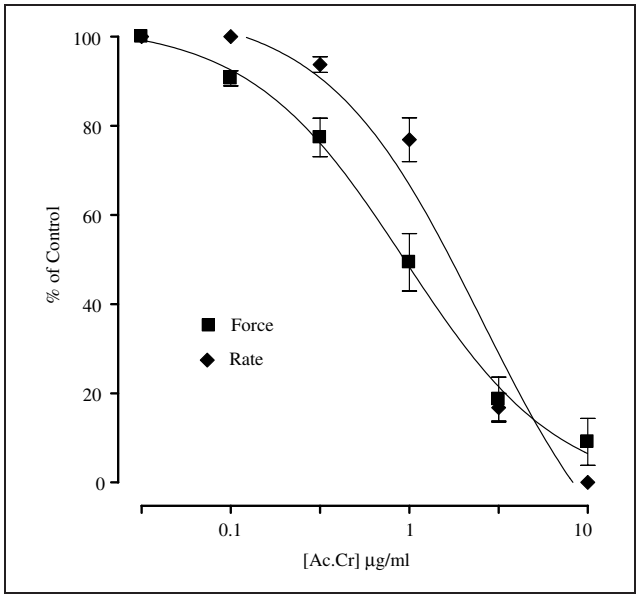


Fig. 1: Dose-response curves showing the inhibitory effect of betel nut crude extract (Ac.Cr) on the rate and force of guinea-pig atrial contractility (values shown are mean ± SEM, n = 5)

and 2A) with respective $EC_{50}$ values of 2.51 µg/ml (1.23–5.08, 95% CI, n = 5) and 0.93 µg/ml (0.57–1.51, n = 5). All the betel nut fractions, namely: petroleum spirit (Ac.Pt), chloroform (Ac.Cl), ethyl acetate (Ac.EtAc) and aqueous (Ac.Aq), when tested on the force of spontaneous atrial contractions, showed an inhibitory effect (0.3–1000 µg/ml, Fig. 3) with $EC_{50}$ values of 7.68 µg/ml (6.26–9.42, n = 4), 9.14 µg/ml (6.45–12.94, n = 4), 35.88 µg/ml (26.60–48.40, n = 4) and 1.98 µg/ml (1.57–2.50, n = 4) respectively. The cardio-suppressant effect of Ac.Cr and its fractions was compared with that of standards, carbachol which mediated its negative inotropic effect (Fig. 2B) with an $EC_{50}$ value of 0.013 µg/ml (0.007–0.022, n = 4) and physostigmine which also dose-dependently (0.3–10 µg/ml) suppressed the artial force of contraction (Fig. 2C) with an $EC_{50}$ value of 4.44 µg/ml (3.27–6.03, n = 4). The cardio-relaxant effects of the extract, its fractions, carbachol and physostigmine, were completely blocked by atropine (1 µM) pretreatment.
Some of the constituents of betel nut namely: arecoline, arecaidine, catechin, tannic acid, gallic acid and diosgenin were also tested on the atrial tissues. None, except arecoline, produced a cardio-suppressant effect up till the highest dose of 10 mg/ml. The cardio-suppressant effect of arecoline was atropine-sensitive and mediated at the $EC_{50}$ value of 0.03 µg/ml (0.02–0.05, n = 4, Fig. 2D).

### *2.3. Effect on rabbit jejunum*

Ac.Cr exhibited a dose-dependent spasmogenic effect in the spontaneously beating rabbit jejunum (Fig. 2A) with an $EC_{50}$ of 8.83 µg/ml (6.73–11.59, n = 10). The effect was reproducible, washable and was completely blocked when challenged with atropine (0.1 µM) while it was insensitive to hexamethonium (0.3 mM) pretreatment. The betel nut fractions; Ac.Pt, Ac.Cl, Ac.EtAc and Ac.Aq showed a dose-dependent (1.0–1000 µg/ml), atropine-sensitive, stimulant effect (Fig. 3) with $EC_{50}$ values of 11.97 µg/ml (8.07–17.76, n = 8), 15.33 µg/ml (8.76–26.81, n = 6), 53.29 µg/ml (32.04–88.65, n = 10) and 10.58 µg/ml (7.57–14.78, n = 6) respectively. Likewise, CCh (0.002–2.0 µg/ml) and physostigmine (0.03–0.83 µg/ml) also exhibited dose-dependent spasmogenic effects with $EC_{50}$ values of 0.036 µg/ml (0.012–0.066, n = 4, Fig. 2B) and 0.15 µg/ml (0.08–0.29, n = 4, Fig. 2C) respectively. This spasmogenic effect

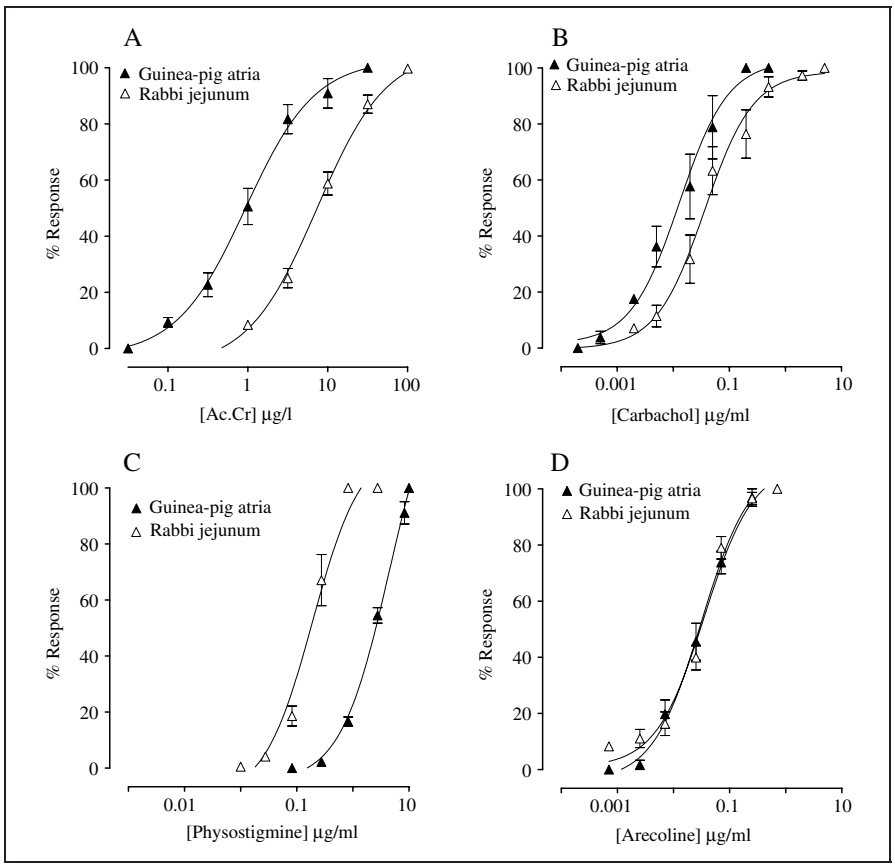


Fig. 2:
Dose-response curves showing the comparative cholinomimetic activity of [A] betel nut crude extract (Ac.Cr), [B] carbachol, [C] physostigmine and [D] arecoline on isolated guinea-pig atrial force of contraction (n = 4) and spontaneously contracting rabbit jejunum (n = 4–6) preparations (values shown are mean ± SEM)

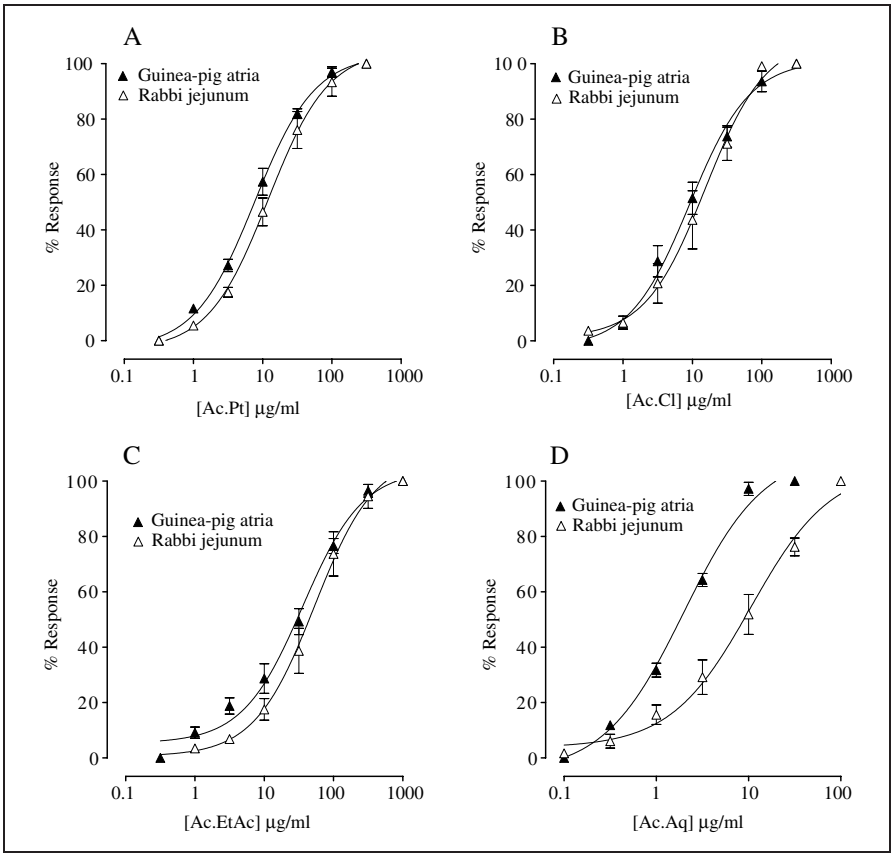


Fig. 3:
Dose-response curves showing the comparative cholinomimetic activity of betel nut fractions: [A] petroleum spirit, [B] chloroform, [C] ethyl acetate and [D] aqueous on isolated guinea-pig atrial force of contraction (n = 4–5) and spontaneously contracting rabbit jejunum (n = 6–10) preparations (values shown are mean ± SEM)

of carbachol and physostigmine was blocked in the presence of atropine (1 µM) pretreatment as expected.

The commercially available constituents of betel nut namely: arecoline, arecaidine, catechin, tannic acid, gallic acid and diosgenin were also tested on spontaneous contractions of rabbit jejunum. Out of all these pure compounds, only arecoline showed an atropine-sensitive spasmogenic effect (Fig. 2D) with an $EC_{50}$ value of 0.04 µg/ml (0.02–0.05, n = 6) while catechin and tannic acid exhibited spasmolytic activity (Fig. 4) with respective $EC_{50}$ values of 2.39 mg/ml (1.55–3.70, n = 5) and 1.46 mg/ml (0.85–2.48, n = 6).

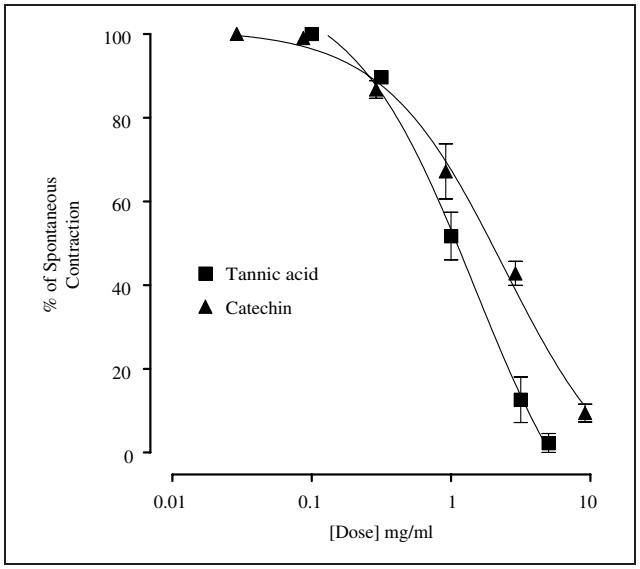


Fig. 4: Dose-response curves showing the spasmolytic activity of catechin (n = 5) and tannic acid (n = 6) on spontaneously contracting isolated rabbit jejunum (values shown are mean ± SEM)

## 3. Discussion

The extract exhibited dose-dependent inhibitory effects on the force and rate of atrial contractions. These inhibitory effects were completely abolished in the presence of atropine, a competitive blocker of acetylcholine at muscarinic receptors (Arunlakhshana and Schild 1959; Gilani et al. 1997). Likewise, two standard cholinergic agonists were also tested in atria, namely carbachol, a direct muscarinic agonist (Brown and Taylor 1995) and physostigmine, an indirect agonist acting via inhibition of acetylcholinesterase (AChE) enzyme (Robinson 1968).

Apart from the standards, Ac.Cr fractions were also tested along with some of the commercially available known constituents of betel nut. All the betel nut fractions exhibited a dose-dependent suppression of atrial contractions while, out of all betel nut compounds, only arecoline, a known muscarinic agonist (Bowman and Rand 1980), was able to depress the atrial contractility. Betel nut contains a number of major alkaloids present (Chu 2001), such as arecoline, arecaidine, arecolidine, guvacoline, guvacine, isoguvacine and choline. Besides, it also contains phenolic compounds as hydroxychavicol and safrole, while tannins, gallic acid, catechin, oily matter, gum and amino acids are also present (Gilani and Ghayur 2005). The phytochemical analysis performed on Ac.Cr showed the presence of saponins, phenols, alkaloids, flavonoids and terpenes and thus we were able to screen some alkaloids, flavonoids and tannins for justification of the results obtained from the crude extract.

When tested in isolated rabbit jejunum, Ac.Cr dose-dependently stimulated the spontaneously contracting prepara-

tion via an atropine-sensitive pathway indicating muscarinic receptor activity. The spasmogenic effect of Ac.Cr in jejunum was mediated at a dose around 8 times higher than required in the atrial tissues ($P < 0.0001$). A simple explanation for such a selective cholinergic effect of Ac.Cr in atria could be that the extract is specifically binding to the $M_2$ receptors compared with the $M_3$ receptor subtypes in the intestine, or its specific action is probably due to its binding with a heterogeneous group of acetylcholinesterases in different tissues. Physostigmine was tested to compare it with the results of betel nut extract, as the latter has been reported to possess AChE inhibitory in addition to direct cholinomimetic activity (Gilani et al. 2004). Both of the standard agonists, carbachol and physostigmine, expectedly exhibited an atropine-sensitive inhibition of artial contractions. Interestingly, physostigmine was found around 30 times more potent for its effect in the jejunum. The activity of physostigmine, being an enzyme inhibitor, depends on the availability of the enzyme (in this case AChE) in the tissue under study (Silver 1978; Taxi and Rieger 1986), showing that probably the jejunum has higher levels of the AChE enzyme, thus the scope of activity for physostigmine is more in the jejunum than in the atria. Had Ac.Cr been acting courtesy of its AChE inhibitory potential, it would have shown selectivity for jejunum rather than atrium, similar to physostigmine, but this is not the case! Carbachol, a cholinergic agonist known to be resistant to AChE (Brown and Taylor 1995), did not show any specific effect on the tissues as expected. However, considering the fact that a single plant contains as many as hundreds of chemicals (Williamson et al. 1996), other options for selective action cannot be ignored. It is possible that different compounds with different specificities exist in betel nut, which interact in a way to give a final resultant specific effect as seen in atria. Alternatively, it is possible that the plant constituents interact preferentially with subtypes of muscarinic receptors in atria ($M_2$) and active compounds responsible for this specific effect remain to be identified.

When the different betel nut fractions were screened in jejunum, only Ac.Aq was able to differentiate between the cardiac and smooth muscle tissues and was around 6 times more potent in atria ($P < 0.0001$) for its atropine sensitive inhibitory effect. This not only reiterated the finding with the parent extract, but also suggested that the responsible component(s) for cardio-selectivity is/are concentrated in the aqueous portion of the extract. These results might also shed more significance on the findings of Vinoy et al. (2002) who found that users of betel nut had a significantly lower heart rate at rest and during exercise. Another study also reported cardiac slowing as an initial effect while gastrointestinal stimulation was shown as a subsequent effect of betel chewing (Chu and Chang 1994).

When some of the known compounds of betel nut were screened in jejunum, only arecoline showed muscarinic activity at doses like those used in the atrial tissues, thus ruling out the possibility of arecoline being responsible for the cardio-specific effect of Ac.Cr. On the contrary, interestingly catechin and tannic acid both exhibited dose-dependent spasmolytic activity in the gut, which was not observed from these compounds in the atrial preparation. This not only suggests that catechin and tannic acid are specific intestinal smooth muscle relaxants, with no activity on the atrial contractility, but also that their presence in betel nut ensures an unabated cholinergic-mediated cardiosuppressant effect, owing to their inertness in the atrial muscles, while rendering the cholinergic-mediated stimulant effect less active in jejunum with their spasmolytic potential. Interestingly, the prototype cardio-selective antimuscarinic drug, himbacine, was reported by our group over a couple of decades ago (Gilani and Cobbin 1984, 1986) and here we provide the first evidence for the cardio-selective muscarinic agonist activity of betel nut extract which might explain some of the medicinal uses of betel nut chewing.

Our study has for the first time reported gut relaxant effect of some constituents of betel nut, justifying the additional folk use of betel nut in diarrhoea and dysentery (Said 1996). The results of this study along with our earlier findings (Gilani et al. 2004) have also negated the general idea, as also shared by a previous report (Vinoy et al. 2002), that only arecoline is the medicinally active ingredient in betel nut (Chu 1994; Evans 1996). Tannic acid and catechin, with their specific relaxant effects on the gut, may be the main antidiarrhoeal components in betel nut but their presence might not necessarily be wholly responsible for the specific cardio-suppressant effect of the extract until or unless muscarinic $M_2$ subtype-specific activity of the extract or its constituent(s) is completely ruled out.

## 4. Experimental

### *4.1. Drugs and standards*

The following reference chemicals were obtained from the sources specified: arecaidine hydrochloride, arecoline hydrobromide, atropine sulphate, carbamylcholine chloride (carbachol), (+)-catechin hydrate, hexamethonium chloride, diosgenin, gallic acid, isoprenaline hydrochloride, nicotine, physostigmine and tannic acid (Sigma Chemical Company, St. Louis, MO, USA). The following chemicals were used to make the physiological salt solutions: potassium chloride (Sigma Chemical Company), calcium chloride, glucose, magnesium chloride, magnesium sulphate, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate (E. Merck, Darmstadt, Germany). 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.

### *4.2. Animals*

Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, and were approved by the Aga Khan University's Ethics Committee for Research on Animals. Rabbits (1 kg) and guinea-pigs (500–600 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): 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.

### *4.3. Plant material and extraction procedure*

Betel nut (1 kg) was bought from a market in Karachi, and a sample of the material was deposited in the Herbarium of Natural Products Research Group, 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 a filter paper and the plant material was resoaked twice. The combined filtrate was concentrated in a rotary evaporator under reduced pressure at 40 °C to yield a viscous and dark brown coloured crude extract (Ac.Cr, 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. A part of this crude extract was kept for the preliminary pharmacological studies (23 g) and the rest was used for fractionation (121 g). Activity-directed fractionation of the extract was carried out by standard phytochemical procedures using different organic solvents (Williamson et al. 1996). A known quantity of Ac.Cr was dissolved in distilled water and then separation was done with petroleum spirit (40–60 mL) in a separating funnel. This mixture was shaken vigorously with regularly allowing the air to escape out and kept for about 30 min to let the two layers separate. The layer of petroleum spirit was acquired and concentrated in a rotary evaporator to obtain the petroleum spirit fraction (Ac.Pt, 17 g). Likewise, chloroform (Ac.Cl, 18 g) and

ethyl acetate fractions (Ac.EtAc, 7 g) were also obtained while the remaining layer was considered as the aqueous fraction (Ac.Aq, 77 g).

### *4.4. Preliminary phytochemical analysis*

Ac.Cr, along with all its fractions, was screened qualitatively by use of different organic solvents and reagents for the presence of some phyto-constituents such as saponins, flavonoids, tannins, phenols, coumarins, sterols, terpenes, alkaloids and anthraquinones (Wagner et al. 1984). Briefly, saponins were detected on observance of any froth formation following rigorous shaking of the extract dissolved in distilled water. Testing for flavonoids required mixing the extract with $AlCl_3$ and appearance of yellow colouration indicated a positive test. Presence of phenols and tannins was determined after appearing of any green or dark green colour after dissolution of extract in aqueous $FeCl_3$. For detecting coumarins, a piece of filter paper was moistened in NaOH and then kept over a test tube with boiling plant extract solution. If the filter paper later showed any yellow fluorescence under UV light, this was considered to be a positive test for coumarins. Detection for any sterols and terpenes in the extract involved treating of the extract with petroleum ether and then extracting with $CHCl_3$. The subsequently acquired $CHCl_3$ layer was treated with acetic anhydride and concentrated HCl. The appearance of pink to purple and green to pink colours suggested the presence of terpenes or sterols, respectively. Alkaloids were screened by mixing with Dragendorff's reagent. Lastly for detecting anthraquinones, the extract was dissolved in 1% HCl, then in benzene and later if the extract showed pink, violet or red colour with $NH_4OH$, that indicated a positive test for the presence of anthraquinones.

### *4.5. Isolated guinea-pig atria*

The isolated tissue experiments were carried out as described previously (Gilani et al. 2004, 2006). 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 (composition in 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) at 32 °C and aerated with a mixture of 5% carbon dioxide in oxygen (carbogen). The right atria, due to the presence of pace making cells contract naturally, but the left atria were stimulated with rectangular pulses of 3 ms duration, at 2.5 Hz from a Grass model SD 7 stimulator with the voltage maintained at 10–20% above the threshold. 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 CCh (0.1–0.3 μM) and isoprenaline (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 a Grass (model 7) polygraph. The crude extract, its fractions, physostigmine and all the betel nut pure compounds were tested on the atrial contractility for any activity.

### *4.6. Isolated rabbit jejunum*

Rabbits had free access to water but were fasted for 24 h before the experiment. The animals were sacrificed by cervical dislocation, the abdomen was cut open and the jejunal portion isolated out. Preparations, 2 cm long, were mounted separately in 10 mL tissue baths containing Tyrode's solution (composition of in mM: KCl 2.7, NaCl 136.9, $MgCl_2$ 1.1, $NaHCO_3$ 11.9, $NaH_2PO_4$ 0.4, glucose 5.6 and $CaCl_2$ 1.8; pH 7.4) maintained at 37°C and aerated with carbogen. A preload of 1 g was applied and the tissues kept undisturbed for an equilibrium period of 30 min after which control responses to a sub-maximal dose of CCh (0.3 μM) were obtained and the tissue presumed stable only after the reproducibility of these responses. The plant extract and its fractions were examined later for any spasmogenic activity. Some of the standards, such as carbachol and physostigmine while known constituents of betel nut namely: arecoline, arecaidine, catechin, tannic acid, diosgenin and gallic acid were also screened for any activity.

### *4.7. Statistical analysis*

All the data expressed are as mean ± standard error of mean (SEM, n = number of experiments) and the median effective concentrations ($EC_{50}$ values) with 95% confidence intervals (CI). Statistical parameter applied is two-way analysis of variance (ANOVA) with $P < 0.05$ noted as significantly different (GraphPAD program, GraphPAD, San Diego, CA, USA). Dose-response curves were analysed by non-linear regression (GraphPAD program).

Part of this work was presented at 'Joint Meeting of the Italian and Dutch Pharmacological Societies', $12^{th}$–$14^{th}$ Feb 2003, Lunteren, The Netherlands and the abstract was published in Naunyn Schmiedebergs Arch Pharmacol 2004; 369: R167.

Acknowledgements: This study was partially supported by funds made available by the Higher Education Commission, The Government of Pakistan under the scheme of Distinguished National Professor Research Allowance.

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