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Bronchodilatory effect of *Acorus calamus* (Linn.) is mediated through multiple pathways

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**Abstract**

**Aim of the study:** This study was undertaken to provide a pharmacological basis for traditional use of *Acorus calamus* in airways disorders.

**Materials and methods:** Isolated guinea-pig trachea and atria were suspended in organ baths bubbled with carbogen and mechanisms were found using different parameters.

**Results:** In isolated guinea-pig tracheal segments, crude extract of *Acorus calamus* was more effective than carbachol in causing relaxation of high K\(^+\) (80 mM) precontractions, similar to verapamil, suggesting blockade of calcium channels. The n-hexane fraction was equipotent against both precontractions, similar to papaverine, while ethylacetate fraction was more potent against carbachol precontractions but had a negligible dilator effect against K\(^+\), similar to atropine and or rolipram. Pretreatment of tracheal preparations with n-hexane or ethylacetate fractions potentiated isoprenaline-induced inhibitory concentration–response curves, similar to papaverine or rolipram. Pretreatment of tracheal preparations with ethylacetate fraction caused a rightward parallel shift in carbachol response curve at lower concentration (0.003 mg/mL) similar to atropine and a non-parallel shift at higher concentrations (0.01 mg/mL), with reduction of maximum response, similar to rolipram. In isolated guinea-pig atrial preparations, crude extracts, its fractions and papaverine inhibited force and rate of contractions at higher concentrations than the smooth muscle while verapamil was equipotent.

**Conclusion:** These data indicate the presence of unique combination of airways relaxant constituents in crude extract of *Acorus calamus*, a papaverine-like dual inhibitor of calcium channels and phosphodiesterase in n-hexane fraction and a novel combination of anticholinergic, rolipram-like phosphodiesterase4 inhibitor in ethylacetate fraction and associated cardiac depressant effect, provide a pharmacological basis for traditional use of *Acorus calamus* in disorders of airways.

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1. Introduction

*Acorus calamus* Linn. (Araceae), a semi-aquatic perennial herb with creeping and much branched aromatic rhizome, has been known for at least 2000 years (Kapoor, 2000). The aromatic rhizome is commonly referred as calamus or sweet flage. It has been used over centuries as a herbal remedy for airways disorders, such as asthma, cough, throat irritation, bronchitis and as expectorant (Nadkarni, 1976).

The plant has been reported for the presence of glucoside, alkaloild and essential oil containing calamen, clamenol, calameon, asarone and sesquiterpenes (Baquar, 1989). It also contains a better glycoside named acorine along with eugenol, pinene and camphene (Kapoor, 2000).

Although *Acorus calamus* is a famous remedy for respiratory disorders but the current literature lacks pharmacological studies relevant to its potential therapeutic usefulness in these disorders. There are few studies available on its other biological activities, such as tranquilizing (Menon and Dandiya, 1967), antimicrobial (Daniilevskii and Antonishin, 1982), antiarrhythmoeal (Shoba and Thomas, 2001), antidysslipidemic (Parab and Mengi, 2002), neuroprotective (Shukla et al., 2002), antioxidant (Acuta et al., 2002), anticholinesterase (Oh et al., 2004), spasmylocotic (Gilani et al., 2006) and vascular modulator (Shah and Gilani, 2009). None of these studies have addressed the therapeutic potential of *Acorus calamus* in respiratory disorders. Therefore, this investigation was undertaken to have indirect approach to the underlying possible mechanisms responsible for the traditional use of *Acorus calamus* in airways disorders.
2. Materials and methods

2.1. Plant materials

Fresh rhizomes of *Acorus calamus* (3 kg) were collected in Swat District, N.W.F.P., Pakistan, in the month of November 2004 and authenticated by Assistant Professor Mehboob-ur-Rehman at the Department of Botany, Govt. PG Jehanzeb College Saidu Sharif Swat, Pakistan. A voucher specimen (A 200) was deposited at the herbarium of the same department.

2.2. Preparation of crude extract and fractionation

The plant material was cleaned off adulterant, which include; scales attached to the rhizome and adventitious roots and coarsely grounded. The powdered material (1.8 kg) was soaked in 70% aqueous-methanol at room temperature of 23–25 °C for 3 days with occasional shaking. It was filtered through a muslin cloth and then through a filter paper (Whatman qualitative grade 1). This procedure was repeated thrice and the combined filtrate was evaporated on rotary evaporator at 35–40 °C under reduced pressure (~760 mm Hg) to a thick, semi-solid mass of dark brown color; i.e. the crude extract (Ac.Cr), yielding approximately 10%.

Activity-directed fractionation of the crude extract was carried out by standard phytochemical procedures using different organic solvents (Williamson et al., 1998). A known quantity (50 g) of the extract was dissolved in distilled water. This was then introduced in a separating funnel, n-hexane (50–70 mL) was then added and the mixture was shaken vigorously with regular allowing the air to escape out. It was kept for about 30 min to let the two layers separate. The upper layer of the n-hexane was acquired and the same procedure was repeated twice and all the n-hexane layers were collected and concentrated on rotary evaporator to obtain the n-hexane (Ac.n-hexane) fraction. Ethylacetate (50 mL) was then added to the remaining layer and the same process was repeated as with the n-hexane, finally we got the ethylacetate (Ac.EtAc) fraction. The yield of both fractions was 23.6% and 30% respectively, while the remaining layer was discarded.

The crude extract and its fractions were stored and preserved at ~4 °C until use. The Ac.Cr and its fractions were solubilized in distilled water and 10% DMSO for use in the *in vitro* experiments, respectively, on the day of experiment.

2.3. Drugs and standards

The following reference chemicals were obtained from the sources specified: acetylcholine chloride, atropine phosphate, carbamyl choline chloride (carbachol), isoprenaline hydrochloride, papaverine hydrochloride, rolipram, potassium chloride and verapamil (Sigma–Aldridge Company, St. Louis, MO, USA). All chemicals were used were of the highest purity grade. Stock solutions of all the chemicals were made in distilled water/suitable solvent and the dilutions were made fresh on the day of experiment.

2.4. Animals

Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996) and approved by the Ethical Committee of Aga Khan University, Karachi. Guinea-pigs (500–550 g; n = 44) of local breed, either sex and 8–10 weeks old used in the study were bred and housed at the animal house of Aga Khan University under controlled environment (23–25 °C). Animals were given tap water ad libitum and a standard diet.

2.4.1. Isolated tissue preparations

2.4.1.1. Guinea-pig trachea. As described previously (Gilani et al., 2008), guinea-pigs were sacrificed by cervical dislocation and tracheal tube was carefully removed and placed in aerated carbogen gas (5% CO₂ in O₂). Tracheal tube was cut longitudinally on the ventral side into rings, which were then opened up into strips and mounted in a 20 mL tissue bath containing normal Kreb's solution, maintained at 37 °C and aerated with carbogen. The composition of Kreb's solution was (mM): KCl 4.7, NaCl 118.2, NaHCO₃ 25.0, CaCl₂ 2.5, KH₂PO₄ 1.3, MgSO₄ 1.2 and glucose 11.7 (pH 7.4). A tension of 1 g was applied to each tracheal strip and was kept constant throughout the experiment. After 1 h of equilibration period, tissues were tested for their contractile response to carbachol (CCh, 1 μM) and high K⁺ (80 mM) repeatedly. Following reproducible responses, sustained contractions were induced by addition of either of these agonists to the tissue bath. Test substance was then added in cumulative fashion to obtain concentration–response curves (CRCs), measured as percent of control with isometric force transducers coupled with a four-channel Grass model 7 Polygraph.

2.4.1.1.1. Determination of anticholinergic activity. Isolated guinea-pig tracheal preparations were incubated in normal Kreb's solution and control CRCs of the CCh were constructed. To test the possible anticholinergic activity, the tissues were pretreated with different concentrations of the test material for 30 min and CRCs of the CCh were repeated in the presence of the test material and the results were compared with atropine, as described previously (Gilani et al., 2008).

2.4.1.1.2. Determination of phosphodiesterase inhibitory activity. As described previously (Gilani et al., 2005; Khan and Gilani, 2009), guinea-pig tracheal strips were suspended in normal Kreb's solution in 20 mL organ baths and control isoprenaline (0.003–1 μM) inhibitory CRCs were constructed against the CCh-induced contractions. When the control CRCs of isoprenaline were found super-imposable (usually after two cycles), the tissue was pre-treated with the test substances for 30 min to test the possible potentiating effect. The CRCs of isoprenaline were reconstructed in the presence of different concentrations of the test material and compared with phosphodiesterase inhibitors (PDEIs).

2.4.1.2. Guinea-pig atria. Guinea-pigs were sacrificed by cervical dislocation; atria were removed carefully and fatty tissues cleaned off and mounted into 20 mL tissue baths filled with normal Kreb's solution at 32 °C aerated with carbogen, as previously described (Gilani et al., 2007). The composition of Kreb's solution was (mM): NaCl 118.2, NaHCO₃ 25.0, CaCl₂ 2.5, KCl 4.7, KH₂PO₄ 1.3, MgSO₄ 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 acetylcholine (ACh; 1 μM) and isoproterenol (1 μM) were obtained at least in duplicate. Tension changes in the tissue were recorded via a force-displacement transducer (model FT-03) using four-channel Grass model 7 Polygraph. Force of contraction was calculated from the amplitude of contraction while rate of spontaneous contraction was calculated by counting the number of contractions in a specified area of the chart by increasing the chart speed.

3. Statistics analysis

All the data expressed are mean ± standard error of the mean (SE), and the median effective concentrations (EC₅₀ values) are given with 95% confidence intervals (CI). The statistical parameter applied is the student t-test with *P*<0.05 noted as significantly different.
Fig. 1. Inhibitory effect of (A) the crude extract of Acorus calamus (Ac.Cr) and its (B) n-hexane (Ac.n-hexane) fraction and (C) verapamil and (D) papaverine on the high K⁺ and carbachol (CCh)-induced contractions in isolated guinea-pig tracheal preparations. Symbols represent ±SE, n = 4–6.

Fig. 2. Inhibitory effect of (A) the ethylacetate (Ac.EtAc) fraction of the crude extract of Acorus calamus, (B) atropine and (C) rolipram on the high K⁺ and carbachol (CCh)-induced contractions in isolated guinea-pig tracheal preparations. Symbols represent ±SE, n = 4–7.
4. Results

4.1. Effect on isolated guinea-pig trachea

When tested against high K⁺ (80 mM) and CCh (1 μM)-induced precontractions, the crude extract of Acorus calamus (Ac.Cr) caused a concentration-dependent inhibitory effect, possessing higher potency against K⁺ than CCh-induced precontractions, with EC₅₀ values of 0.06 (0.04–0.09; n = 5) and 0.27 mg/mL (0.20–0.34; n = 5) respectively, as shown in Fig. 1A. Similarly, verapamil was also found more potent against K⁺ than CCh-induced precontractions, with respective EC₅₀ values of 0.03 (0.02–0.05; n = 6) and 0.37 μM (0.25–0.54; n = 6), as shown in Fig. 1B. The n-hexane fraction was equipotent against both K⁺ and CCh-induced precontractions (Fig. 1C) with respective EC₅₀ values of 0.54 (0.27–1.10; n = 4) and 0.56 mg/mL (0.37–0.87; n = 4), similar to papaverine (Fig. 1D).

The ethylacetate fraction (Ac.EtAc) inhibited the CCh-induced precontractions at distinctly lower concentrations with EC₅₀ value of 4.01 μg/mL (2.70–5.80; n = 4) with negligible effect against high K⁺-induced precontractions (Fig. 2A), similar to the mixed pattern of atropine (Fig. 2B) and rolipram (Fig. 2C).

Pretreatment of the tracheal preparations with the Ac.n-hexane (0.01–0.03 mg/mL; n = 6) shifted, the isoprenaline-induced inhibitory CRCs, constructed on the CCh-induced precontractions, to the left (Fig. 3B), similar to that caused by papaverine (Fig. 3E). The Ac.EtAc (0.01–0.03 mg/mL; n = 6) also caused leftward shift of the isoprenaline-induced inhibitory CRCs (Fig. 3C), similar to rolipram (Fig. 3F) while the crude extract (Fig. 3A), similar to verapamil (Fig. 3D), did not alter the potency of isoprenaline for its inhibitory effect.

When tested for the possible anticholinergic effect, using carbachol as the agonist, the ethylacetate fraction caused a rightward parallel shift in the CCh curves at lower concentration (0.003 mg/mL; n = 6), with the maximum response to the CCh remained unaltered (P > 0.05) while a non-parallel shift at higher concentration (0.01 mg/mL; n = 6) with suppression of the maximum response (Fig. 4). The Ac.Cr and Ac.n-hexane were without such effect on the CCh curves, up to the concentration of 3 mg/mL (data not shown). Atropine caused a rightward parallel shift in the CCh curves; the shift was concentration-dependent without altering the maximum response even at the higher concentration used whereas rolipram caused a rightward parallel shift with suppres-
Fig. 4. Inhibitory effect of (A) the ethylacetate (Ac.EtAc) fraction of the crude extract of *Acorus calamus* (B), atropine and (C) rolipram on the carbachol (CCh) concentration–response curves in isolated guinea-pig tracheal preparations. Symbols represent mean ±SE, n = 4–6.

sion of maximum response, in a concentration-dependent fashion (Fig. 4).

4.2. Effect on isolated guinea-pig atria

The Ac.Cr concentration-dependently suppressed the force and rate of spontaneous atrial contractions at higher concentrations than in airway smooth muscle, with EC_{50} values of 5.71 (3.61–9.01; n = 4) and 5.10 mg/mL (3.21–8.25; n = 5) (Fig. 5A). The Ac.n-hexane exhibited cardiac suppressant effect at higher concentrations than in airway smooth muscle, with EC_{50} values of 2.73 (1.74–4.29; n = 5) and 2.67 mg/mL (1.91–3.73; n = 5) (Fig. 5B). The Ac.EtAc also suppressed the force and rate of atrial contractions with EC_{50} values of 9.10 (4.14–22.23; n = 6) and 7.36 mg/mL (3.81–14.25; n = 6) (Fig. 5C), showing less potency than observed in the airway smooth muscle preparations. Similarly, papaverine also induced cardiac suppression at higher concentrations than in airway smooth muscle, with EC_{50} values of 56.77 (30.82–104.60; n = 4) and 46.61 μM (36.60–60.59; n = 4) (Fig. 5D). However, verapamil caused cardiac suppression at similar concentrations as in the airway smooth muscle, with EC_{50} values of 0.29 (0.09–0.37; n = 5) and 0.35 μM (0.08–0.49; n = 5) (Fig. 5E).

5. Discussion

In view of the traditional use of *Acorus calamus* in hyperactive airways disorders, the plant extract was tested on tracheal preparations where it caused relaxation of high K+ and CCh-induced precontractions with higher potency against K+, similar to verapamil. The contractions induced by high K+ (>30 mM) are dependent on the entry of Ca++ into the cells through voltage-dependent calcium channels (VDCs) (Bolton, 1979) and a substance which can inhibit the high K+-induced contractions is therefore, considered to be a possible CCB (Godfraind et al., 1986). Thus, the inhibition of K+-induced precontractions by the Ac.Cr, at relatively low concentration, like verapamil, reflects the restricted Ca++ entry via VDCs, though additional mechanism cannot be ruled out. However, the Ac.n-hexane was equipotent against both high K+ and CCh-induced contractions, similar to papaverine, a dual inhibitor of PDE (Boswell-Smith et al., 2006) and calcium channels (Boselli et al., 1998), suggesting possible PDE inhibitory-like effect in addition to CCB. The PDE inhibitory effect of the Ac.n-hexane was further studied when its pretreatment shifted the isoprenaline-induced inhibitory CRCs to the left, similar to that caused by papaverine while the crude extract, like verapamil was without potentiating effect, as PDE inhibitors are known to potentiate the isoprenaline effect (Lorenz and Wells, 1983).

On the other hand, the Ac.EtAc behaved differently, it caused inhibition of the CCh-induced contractions at distinctly lower concentration with negligible effect on the high K+ precontractions, similar to atropine. This suggests the presence of atropine-like anticholinergic effect, in addition to other mechanism(s). The anticholinergic effect was further indirectly confirmed when pretreatment of the tracheal tissues with the Ac.EtAc, at lower concentration caused a parallel shift to the right without altering
maximum response, similar to that caused by atropine, a muscarinic receptor antagonist (Arunlakhshana and Schild, 1959) and non-parallel shift with suppression of the maximum response at higher concentration, suggestive of the presence of additional non-specific relaxant component(s).

In the trachea, PDE4, a cAMP-specific PDE, is the predominant isoenzyme in the majority of inflammatory cells, implicated in airways disease (Rabe et al., 1993), which contributes to about 50% of total PDE activity, an amount similar to that reported for human airways (Rabe et al., 1993; Torphy et al., 1993). There is evidence indicating that anticholinergic agents, particularly atropine is known to synergize the effect of rolipram, a selective PDE4 inhibitor (Iona et al., 1998), to produce a substantial relaxation of airways smooth muscle (Gonzalez et al., 2004). We hypothesized that the distinctly potent nature of the Ac.EtAc against the CCh-induced contractions may be due to the additional inhibition of rolipram-like PDE4 inhibitory constituent. When rolipram was tested against the CCh-induced contractions, it caused a partial inhibitory effect with maximum response reaching to about 50%, while it was found devoid of any effect on the high K+-induced contractions. The complete inhibition of the CCh-induced contractions may be due to the synergistic effect of the atropine-like anticholinergic and rolipram-like PDE4 inhibitory effect of the Ac.EtAc, while the partial inhibitory effect of rolipram is solely due to PDE4 inhibition, thus suggesting the presence of rolipram-like specific PDE4 inhibitor. The PDE inhibitory effect of the Ac.EtAc was further confirmed when pretreatment of the tracheal tissues with the Ac.EtAc caused potentiation of the isoprenaline-induced inhibitory responses, similar to that caused by rolipram. These data indicate that the Ac.EtAc possesses rolipram-like PDE4 inhibitory activity, which is also evident from the non-parallel shift with suppression in maximum response, a characteristic of a non-specific inhibitory response of the PDE inhibitors against different spasmodgens (Schmidt et al., 2000).

Fig. 5. Inhibitory effect of (A) the crude extract of Acorus calamus (Ac.Cr) (B); its n-hexane (Ac.n-hexane) (C) and ethylacetate (Ac.EtAc) fractions and (D) papaverine and (E) verapamil on the force and rate of spontaneous contractions in isolated guinea-pig atrial preparations. Symbols represents ±SE, n = 4–6.
Relaxation of airways is associated with a net increase in CAMP concentration as a consequence of inhibition of PDEs (Abdel-Latif, 2001) and Ca++ movements (Yan and Michael, 2006). Recent studies have helped to understand the important role of PDE4 in addition to non-specific PDEs in airways function (Rabe et al., 1993; Torphy et al., 1993) and their inhibitors are among the novel candidates for the treatment of asthma (Torphy, 1998). Similarly, the cholinergic innervations are the dominant neural bronchoconstrictors in humans and rodents (Barnes, 1992) associated with asthma. Anticholinergic agents are now considered important bronchodilators for the treatment of asthma (Nicholas, 2006). Thus the presence of papaverine-like non-specific, rolipram-like PDE4 specific inhibitor and anticholinergic constituents in the Acorus calamus provides possible pharmacological basis to its traditional use in airways disorders.

The PDE inhibitors alone are considered very effective bronchodilators but have limited therapeutic uses due to cardiac stimulation (tachycardia) as a side effect (Raeburn et al., 1993). The co-existence of CCB constituents with PDE inhibitor is likely to offset the cardiac stimulation associated with PDE inhibitors when used alone. When studied in cardiac preparations (isolated guinea-pig atria), interestingly, the extract had no stimulatory effect and the cardiac depression was observed at much higher concentrations than the inhibition seen in the airways, which is probably because of the interference of the known cardiac stimulant effect of PDE inhibition to the CCB effect. Because both the β-adrenergic agonists (isoprenaline) and PDE inhibitors increase intracellular level of cyclic 3′,5′-adenosine monophosphate through different mechanisms, considered relaxant in the smooth muscles and stimulant in the heart (Gilan et al., 2005; Brain and Hoffman, 2001).

In conclusion, the pharmacological study of the crude extract of Acorus calamus and its fractions in isolated guinea-pig tissues preparations revealed the presence of a unique combination of airways relaxant constituents. The non-specific papaverine-like PDE inhibitor, concentrated in the n-hexane fraction, anticholinergic and specific rolipram-like PDE4 inhibitor in the ethylacetate fraction and CCB in the crude extract, while the simultaneous cardiac and specific rolipram-like PDE4 specific inhibitor, concentrated in the n-hexane fraction, anticholinergic constituents in the Acorus calamus provides possible pharmacological basis to its traditional use in airways disorders.

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