

IN VITRO ANTICHOLINERGIC AND ANTIHISTAMINIC ACTIVITIES OF ACORUS CALAMUS
LINN. LEAVES EXTRACTS

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Abstract

The present investigation was aimed at determining the effects of hexane, acetone, methanol and aqueous extracts of *Acorus calamus* leaves (ACHE, ACAE, ACME and ACAQE) on cholinergic and histaminic system using isolated frog rectus abdominis muscle and guinea pig ileum. A dose dependent potentiation of Ach response (anticholinesterase like effect) was found with ACAE and ACME at 0.25, 0.5, 0.75 and 1 mg/ml, but at higher dose of ACAE, ACME, ACAQE and ACHE (5, 20 mg/ml) inhibit the Ach response (antinicotinic effect). These results revealed biphasic effect of *Acorus calamus* leaves extracts on acetylcholine induced contractile response in isolated frog rectus abdominis muscle preparation (i.e. potentiation effect at lower dose and inhibitory effect at higher dose). Studies on isolated guinea pig ileum demonstrated antihistaminic effect in a dose dependent manner (100-1000 µg/ml) with ACAE, ACME and ACAQE. In addition, the dose dependent inhibition of Ach response (antimuscarinic effect) was observed with ACAE and ACME. In conclusion, *Acorus calamus* leaves extracts exerts antinicotinic, anticholinesterase like activities in isolated frog rectus abdominis muscle and antihistaminic, antimuscarinic effect in guinea pig ileum. It has been suggested that these observed activities can be further studied for therapeutic potential of *Acorus calamus* leaves in the treatment of cognitive disorders and asthma.

Keywords- Sweet flag leaves, anticholinesterase, antimuscarinic, antinicotinic, cumulative dose response, biphasic effect

Introduction

Acorus calamus Linn. (Family- Araceae) commonly known as “sweet flag” or Waan-Nam, is a well known medicinal plant used in ayurvedic medicine for hundreds of years. The rhizomes were utilized extensively by the Chinese, Indians and American Indians as well as by other cultures (Motley, 1994). Its roots and rhizomes are used in various ailments including many mental disorders, such as hysteria, insanity, insomnia, melancholia, neurasthenia, epilepsy, diarrhoea and asthma (Hazra et al., 2007; Mukherjee et al., 2007). The various pharmacological activities of *Acorus calamus* such as analgesic (Mukherjee et al., 2007), anticonvulsant (Achliya et al., 2005), antispasmodic (Gilani et al., 2006), anti-inflammatory (Vohora et al., 1990), antibacterial (Aqil and Ahmad, 2007), antiulcer and cytoprotective activity (Mukherjee et al., 2007) anti-schizophrenia (Singh et al., 1991), anti-anxiety (Date and Kulkarni, 1995a; 1995b), tranquilizer and CNS depressant activity (Pandi et al., 2009), neuromodulatory effect in dopaminergic system (VengadeshPrabu et al., 2009) have been reported. Traditionally, the roots and rhizomes of *Acorus calamus* L have been used in the Indian and Chinese systems of the medicine for hundreds of years for their beneficial role in improving learning performance, and for their anti-aging effect (Bagchi et al., 1991; Zhang et al., 1994). *In vitro* antioxidant and anticholinesterase activity of *Acorus calamus* reported earlier with roots and rhizomes methanolic extracts using rat brain homogenate Faiyaz et al., 2009). The formulated syrup containing aqueous ethanol extracts of various traditional herbs like *Adhatoda vasica*, *Acorus calamus*, *Glycyrrhiza glabra*, *Ocimum sanctum*, *Tylophora asthmatica*, *Piper longum* and *Solanum xanthocarpum* was evaluated for its antihistaminic activity by the inhibition of histamine induced contractions on the guinea pig ileum. The results showed that the formulated cough syrup inhibited histamine induced contractions of guinea pig ileum at 2.5 to 25 µg/ml concentrations in a dose dependent manner (AnbuJebaSunilson et al., 2010). Anti-asthmatic activity of an ayurvedic recipe namely madhuyashtyadi syrup in which *Acorus calamus* as an ingredient, after administration showed a significant relief in bronchospasm without any side effect (ElayaRaja et al., 2009).

Most of the reported literature on *Acorus calamus* for its pharmacological activities was done mainly by using roots and rhizomes extracts. However the effects of leaves extracts of *Acorus calamus* on cholinergic and histaminic system hitherto not been reported in the literature. So, the present study is designed to evaluate muscarinic, nicotinic and histamine receptors modulating effects of various extracts of *Acorus calamus* leaves such as hexane, acetone, methanol, and aqueous extracts (ACHE, ACAE, ACME, and ACAQE) using isolated frog rectus abdominis muscle and guinea pig ileum.

Materials and Methods

Plant material

Acorus calamus is an aromatic plant which is mainly found in wet and marshy places. The fresh and matured leaves of *Acorus calamus* were collected from well-grown plants at Kollimalai hills of Tamilnadu, India and authenticated by Dr. S M. Khasim, MSc., PhD., Assistant Professor, Department of Botany, Acharya Nagarjuna University, Guntur, Andhra Pradesh, INDIA. A voucher specimen (ECP/ACL/01/2010) has been deposited in the museum of Department of Pharmacognosy, The Erode College of Pharmacy, Erode for future reference. The collected leaves were cleaned thoroughly with distilled water and dried under shade. The shade dried leaves were pulverized in a mechanical grinder to obtain coarse powder.

Preparation of *Acorus calamus* leaves extracts

The coarse powder of dried leaves was soaked in hexane, acetone, methanol and water respectively at room temperature. It was soaked in a particular solvent for 3 days, whereby the treated solvent being recovered and replaced each day with fresh solvents were then pooled together. The extracts were finally obtained by steam distillation followed by evaporation at 37° C of the remaining solvent. The samples were uniquely coded and stored at 10° C till further use. The extracts were weighed and the percentages of different extractive values were calculated in terms of air dried weight of the plant material. The percentage yield of ACHE, ACAE, ACME, and ACAQE were found to be 1.2, 10.2, 12.4 and 9.8 % w/w g respectively.

Phytochemical analysis

Phytochemical investigations of *Acorus calamus* leaves extracts for identification of active principles such as carbohydrates, alkaloids, proteins, volatile oils, triterpenes, flavonoids, saponins, phenols, resins and tannins were carried out using the methods previously described by Trease and Evans (2002). The presence of all of the active principles except proteins was ascertained for the different extracts of *Acorus calamus* leaves.

Animals

Frogs (*Rana tigrina*), male non-albino guinea pigs (*Cavia porcellus*, 400-500 g) procured from disease free animal house of The Erode College of Pharmacy, Erode, Tamilnadu, India. Institutional Animal Ethics Committee (IAEC) approved the experimental protocol (ECP/IAEC-A/2010/06/01) and care of the animals was taken as per guidelines of CPCSEA, India.

Drugs and Chemicals

Acetylcholine chloride, Atropine sulphate (Lobachemie Pvt. Ltd., India), Histamine dihydrochloride (Himedia laboratories Pvt. Ltd., India), Pheneramine maleate injection (Avil®), Dimethyl sulphoxide (Merck Laboratories, India), Hexane and Acetone (Nice Chemical Co., India), Methanol (Cheme Pure Laboratories, India), Distilled water (Leo Scientific Supplier, Erode, India), and ingredients of physiological salt solution (Nice Chemical Co., India) were used.

Effect of *Acorus calamus* leaves extracts (ACHE, ACAE, ACME and ACAQE) on acetylcholine -induced contractile response in isolated frog rectus abdominis muscle

Cumulative dose response curve was obtained by increasing the concentration of drug in the bath fluid step by step without washing out the preceding doses (Ghosh, 2008; Barlow, 1974). Standard procedure for setting up isolated tissue was used. The contractile response with increasing doses of acetylcholine in a sequence 10, +10, +20, +40, +80, +160, +320 µg/ml was recorded, so that the concentration in the bath increases step wise 10, 20, 40, 80, 160, 320 and 640 µg/ml respectively. The same set of study in the presence of test compounds (ACHE, ACAE, ACME, and ACAQE) instead of vehicle was repeated. The concentrations of test extracts used were 0.25, 0.50, 0.75, 1, 5 and 20 mg/ml. The concentration response curves of acetylcholine that is one in the absence and other in the presence of test compounds was plotted. The potentiating or inhibiting effect of test compounds on acetylcholine response was noted and the relative EC50 or EC80 values were calculated. The antagonism or agonism effect of the extract was measured in terms of the dose ratio by finding out the equiactive doses of agonist in the presence and in the absence of the test compounds. Higher the dose-ratio (>1) represents more specific antagonist effect of the extract (Ghosh, 2008).

$$\text{Dose ratio} = \frac{\text{EC}_{50} \text{ in the presence of test compounds}}{\text{EC}_{50} \text{ in the absence of test compounds}}$$

Effect of ACHE, ACAE, ACME and ACAQE on histamine or acetylcholine -induced contractile response in isolated guinea pig ileum

The *Acorus calamus* leaves extract was evaluated for their antihistaminic and antimuscarinic activity using guinea pig ileum. Standard procedure for setting up isolated tissue was used (Ortiz-De-Urbina et al., 1990). The non-cumulative dose dependent response due to histamine was recorded. The different histamine doses (0.1, 0.2, 0.4, 0.8 and 1.6 ml of 10 µg/ml) were used. In another set of experiments, non-cumulative dose dependent response due to acetylcholine was recorded. The different acetylcholine (Ach) doses (0.1, 0.2, 0.4, 0.8 and 1.6 ml of 10 µg/ml) were used. From the dose response curve due to histamine or Ach, two submaximal doses were selected (A, 2A). i.e. 2A is double the dose of A. 2A response was considered as 100% response. In presence of various concentrations of *Acorus calamus* leaves extracts (100-1000 µg/ml, at least 10 min incubation) in ascending order, 2A response of agonists (histamine or Ach) was recorded. Decrease in 2A response with respect to vehicle control represents inhibitory effect of extracts on corresponding agonist. By using the given formula % histamine/acetylcholine inhibition was calculated (Ortiz-De-Urbina et al., 1990).

$$\% \text{ Inhibition} = \frac{a - b}{a} \times 100$$

Where a= height of the histamine/Ach response in presence of vehicle, DMSO (in cm)
b= height of histamine/Ach response in presence of test/standard (in cm).

Statistical analysis-

All the data expressed are mean ± standard error of the mean (SE). The data were analyzed by one way ANOVA followed by student t-test with p < 0.05 noted as significantly different.

Results

Effect of *Acorus calamus* leaves extracts (ACAE, ACME, ACHE and ACAQE) on acetylcholine -induced contractile response in frog rectus abdominis muscle

Effect of *Acorus calamus* leaves extracts (ACAE, ACME, ACHE and ACAQE) on Cumulative Dose Response Curve (CDRC) of Acetylcholine (Ach, 100 µg/ml) using frog rectus abdominis muscle is as shown in Table 1. In present study, a significant (p<0.001) dose dependent inhibition of AchE (i.e., potentiation of Ach response) was found with ACAE (0.25, 0.5, 0.75 and 1 mg/ml) and ACME (0.25, 0.5, 0.75 and 1 mg/ml) but interestingly at higher dose of ACAE and ACME (5, 20 mg/ml) significantly (p<0.001) inhibit the Ach induced contractile response. ACAQE and ACHE at higher dose of 5, 20 mg/ml, also showed significant (p<0.001) inhibitory effect on Ach induced contractile response is as shown in Table 1. These results revealed biphasic effects of *Acorus calamus* leaves extracts on acetylcholine-induced contractile response in isolated frog rectus abdominis muscle (i.e. potentiation effect at lower dose and inhibitory effect at higher dose). All the *Acorus calamus* leaves extracts *per se* could not produce any contractile response in frog rectus abdominis muscle. These results revealed none of the extracts could produce direct agonist like activity on nicotinic receptors at neuromuscular junction.

Effect of ACHE, ACAE, ACME and ACAQE on histamine or acetylcholine -induced contractile response in isolated guinea pig ileum

The inhibitory effect of *Acorus calamus* leaves extracts (ACAE, ACME, ACHE and ACAQE) on histamine -induced contractile response in isolated guinea pig ileum is as shown in Fig 1. In present study, a dose dependent significant inhibition (p<0.001) of histamine response was observed with all the extracts of *Acorus calamus* used except with hexane extract (ACHE). The effect of these extracts on Ach -induced contractile response is as shown in Fig 2. The dose dependent inhibition (p<0.001) of Ach response was observed only with ACAE and ACME.

Table 1: Effect of *Acorus calamus* leaves extracts (ACAE, ACME, ACHE and ACAQE) on cumulative dose response curve of acetylcholine using frog rectus abdominis muscle

S.NO	TREATMENT(mg/ml)	ED ₅₀ (µg/ml)	ED ₈₀ (µg/ml)	DOSE RATIO	RESPONSE
1.	DMSO- 1ml	---	89.13±7.28	---	--
2.	ACAE (0.25)	---	6.92±0.72***	0.08	Potentialiation
3.	ACAE (0.50)	---	25.70±1.24***	0.29	Potentialiation
4.	ACAE (0.75)	---	13.80±0.98***	0.15	Potentialiation
5.	DMSO- 1ml	---	18.04±2.02	---	--
6.	ACAE (1)	---	7.94±0.60***	0.44	Potentialiation
7.	ACAE (5)	---	u.d	u.d	Inhibition
8.	ACAE (20)	---	u.d	u.d	Inhibition
9.	DMSO- 1ml	---	51.29±3.65	---	----
10.	ACME (0.25)	---	u.d	u.d	Potentialiation
11.	ACME (0.5)	---	u.d	u.d	Potentialiation
12.	ACME (0.75)	---	u.d	u.d	Potentialiation
13.	DMSO- 1ml	---	72.52±4.29	---	---
14.	ACME (1)	---	22.38±1.80***	0.31	Potentialiation
15.	ACME (5)	---	134.89±9.65***	1.86	Inhibition
16.	ACME (20)	---	u.d	u.d	Inhibition
17.	DMSO- 1ml	14.79±0.84	---	---	---
18.	ACAQE (1)	15.12±1.18 ^{n.s}	---	1.00	No change
19.	ACAQE (5)	38.90±1.62***	---	2.63	Inhibition
20.	ACAQE (20)	u.d	---	u.d	Inhibition
21.	DMSO- 1ml	14.38±1.14	---	---	---
22.	ACHE (1)	u.d	---	u.d	Inhibition
23.	ACHE (5)	u.d	---	u.d	Inhibition
24.	ACHE (20)	u.d	---	u.d	Inhibition

Values are the mean ± SE, n=5. ***p<0.001 when compared to vehicle control. n.s- not significant. u.d- undetermined (interpolating points are away from the dose response curve).

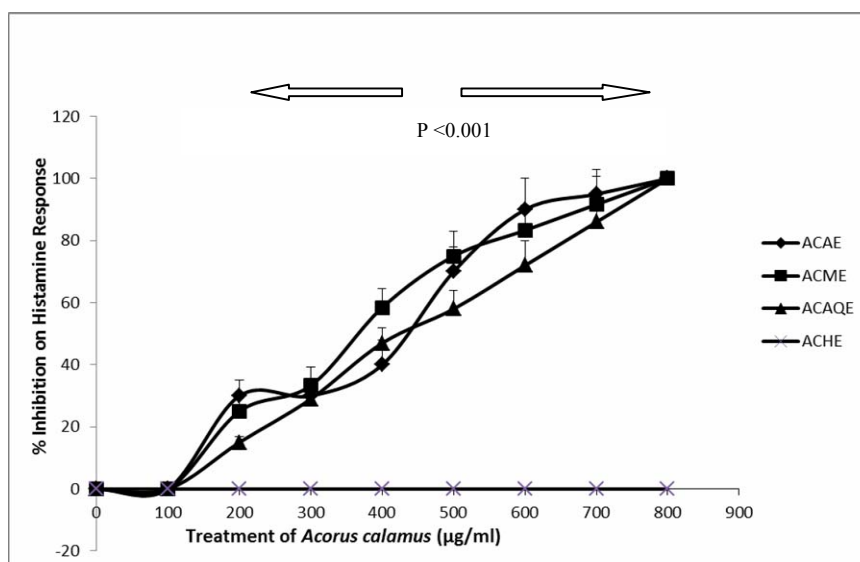


Figure 1: Effect of *Acorus calamus* leaves extracts (ACAE, ACME, ACHE and ACAQE) on contractile response of histamine using guinea pig ileum

Values are the mean $\pm$ SE, $n=5-6$. $p<0.001$, when compared ACAE, ACME and ACAQE to vehicle control. Pheneramine maleate (50µg/ml) showed 100% inhibition on histamine response (data not shown).

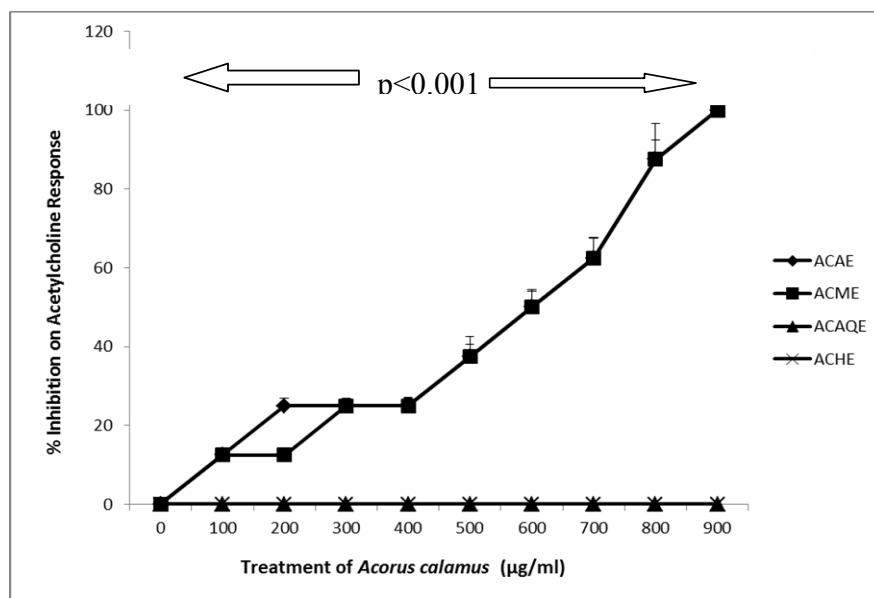


Figure 2: Effect of *Acorus calamus* leaves extracts (ACAE, ACME, ACHE and ACAQE) on contractile response of acetylcholine using guinea pig ileum

Values are the mean $\pm$ SE, $n= 5-6$. $p<0.001$, when compared ACAE and ACME to vehicle control. Atropine sulphate (50µg/ml) showed 100% inhibition on acetylcholine response (data not shown).

Discussion

Acetylcholine is believed to affect the memory, sleep, and concentration abilities, and also to be involved in some severe diseases such as Alzheimer, Parkinson and epilepsy (Nishizaki et al., 1999; Rammsayer, 2000). The symptoms of all types of dementia are presumed to be related to impaired neurotransmission and degeneration of neuronal circuits in the brain areas affected (Poirier, 2002). Cognitive deterioration occurring in patients with probable Alzheimer's disease (AD) is associated with a progressive loss of cholinergic neurons and a consequent decline in the levels of acetylcholine (ACh) in the brain particularly in the temporal and parietal neocortex and hippocampus (Whitehouse, 1982). Acetylcholine Esterase enzyme (AChE) is found among neurofibrillary tangles and neuritic plaques and its inhibition is an effective tool for the treatment of Alzheimer's disease and related dementia (Faiyaz and Urooj, 2010). Tacrine, a standard drug exerts its pharmacological effect by increasing the acetylcholine level in the mouse brain. Hence the AChE inhibitory effects of plant extracts indicate their potential in the development of natural therapeutics for Alzheimer's disease and related problems (Faiyaz and Urooj, 2010). There are several researches focused on the search of new AChE inhibitors from the herbal resources (Giacobini, 2004). It has been previously reported, the methanolic extracts of *Acorus calamus* roots showed strong inhibitory effect on AChE using the Ellman colorimetric method. Significant inhibition of the enzyme at 200 µg/ml was observed for extracts from *Acorus calamus* (Oh et al., 2004). From the results of the present study, it is observed that acetone and methanol extract of *Acorus calamus* leaves extracts at lower doses exert potentiating effect on acetylcholine response (i.e.) AChE inhibiting effect at 250-1000 µg/ml. These results are consistent with the earlier findings on anticholinesterase activities of methanol extracts of *Acorus calamus* rhizomes (Oh et al., 2004; Faiyaz et al., 2009). Most of the acetyl cholinesterase inhibitors are known to contain nitrogen (SatheshKumar et al., 2010) and the higher activity of these extracts might be due to their rich alkaloid content. The phytochemical results of the present study are consistent with the earlier reports on roots and rhizomes extracts of *Acorus calamus*. Phytochemically, it has been reported for the presence of terpenoids, steroids, xanthenes, lignans, flavones, glycosides, flavonoids, saponins, tannins, polyphenolic compounds, mucilage, volatile oil and bitter principle. The plant has also been reported for the presence of glucoside, alkaloids and essential oil containing calamen, clamenol, calameon, asarone and sesquiterpenes (ElayaRaja et al., 2009; Paithankar et al., 2011). Surprisingly, at higher concentrations (5, 20 mg/ml) of *Acorus calamus* leaves extracts (ACAE, ACME, ACHE and ACAQE) showed opposing inhibitory effect on acetylcholine response (i.e. antinicotinic effect). These results warrant further study of neuromuscular blocking effects of *Acorus calamus* at higher doses.

Histamine is one of the important mediators of allergy, inflammation and bronchoconstriction, which were released after degranulation of mast cell by an antigen exposure. Targeting histamine, either prevention of its release from mast cell or use of histaminergic receptor antagonist becomes part of antihistaminic therapy in allergic diseases. Various antihistamines are used in the treatment of allergic disorders due to their H₁-antagonism. In spite of the availability of abundant antihistamines in the market, the search continues for the development of novel antihistaminic agents with reduced sedation, anticholinergic and cardiovascular effects (Shireesha et al. 2008). The present study, *in vitro* antihistaminic activity of *Acorus calamus* leaves extracts (ACAE, ACME and ACAQE) using isolated guinea pig ileum revealed a dose dependent inhibition of histamine was observed for these extracts (Fig 1). In addition, ACAE and ACME showed antimuscarinic activity in the guinea pig ileum (Fig 2). It is already reported that the crude extract of *Acorus calamus* rhizomes caused a concentration-dependent inhibitory effect against carbachol, a cholinergic agonist –induced precontractions (Shah and Gilani, 2010). The present results are consistent with these earlier findings. The cholinergic innervations are the dominant neural bronchoconstrictors in humans and rodents (Barnes, 1992) associated with asthma. Anti-muscarinic agents are now considered important bronchodilators for the treatment of asthma (Nicholas, 2006). Thus the presence antimuscarinic and antihistaminic constituents in the *Acorus calamus* provides possible pharmacological basis to its traditional use in airways disorders. The present study scientifically validated the traditional usage of *Acorus calamus* for asthmatic ailments.

From results of the present study, it is concluded that *Acorus calamus* leaves extracts exerted anticholinesterase activity (at doses, <1000 µg/ml), antinicotinic neuromuscular blocking effect (at higher doses, >1000 µg/ml), antihistaminic and antimuscarinic activities (at lower doses, <1000 µg/ml). These receptor activities of *Acorus calamus* might be responsible for the traditional claim for the effective treatment of cognitive disorders and asthma. However, there is a need to isolate and characterize these compounds for their effective utilization in the treatment of Asthma, cognitive disorders and Alzheimer's diseases. Studies in this direction are currently underway in our laboratory.

Acknowledgement

We are grateful to Dr. V. Ganesan, M.Pharm., PhD., Principal and Mr. A. Nataraajan, B.A., H.D.C., Secretary & Correspondent, The Erode College of Pharmacy and Research Institute, Erode, Tamilnadu, India for providing necessary infrastructure to carry out this research and Prof. Datin Dr. Zahurin Mohamed, The Head, Department of Pharmacology, University of Malaya, Kuala Lumpur, Malaysia for providing facility to write this manuscript.

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