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PRELIMINARY SCREENING OF AQUEOUS EXTRACT OF *CASUARINA EQUISETIFOLIA* INFLORESCENCE ON ISOLATED CHICK INTESTINE AND FROG RECTUS ABDOMINUS MUSCLE FOR IDENTIFICATION OF MUSCARINIC RECEPTORS ACTIVITY

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ABSTRACT

Isolated tissue bath experiments are a classical pharmacological tool for evaluating concentration-response relationships in a myriad of contractile tissues. While this technique has been implemented for over 100 years, the versatility, simplicity and reproducibility of these experiments help it to remain an indispensable tool for pharmacologists and physiologists alike. The present experiments were undertaken to justify the use of an aqueous extract of inflorescence of *Casuarina equisetifolia*, Family: Casuarinaceae, on Acetylcholine (Ach) induced contraction on isolated frog rectus abdominus muscle and isolated chick Intestines. The log dose of Acetylcholine contractile responses were recorded on kymograph paper until the ceiling response was obtained. In both the tissues, the extract potentiates the sub-maximal dose of Ach contractions. The sub-maximal dose of Ach (without extract) was repeatedly added till the original response was obtained. Also, in the intestine preparation, 1mg concentration of extract was heated at 80^0C in the water bath and cooled, then immediately added into the organ bath contained a physiological solution and allow it to act for 1min, then added the sub-maximal dose of Ach. The potentiating responses were observed after heating of the extract. Repeated the same for reproducibility of the results. The inference drawn from these experiments, the aqueous extract of inflorescence of *Casuarina equisetifolia* potentiate the Ach induced contractions and it was reversible and the extract is thermostable. It indicates that the extract may act on the nicotinic acetylcholine receptors (nAChRs) as well as muscarinic acetylcholine receptors (mAChRs).

KEYWORDS: Acetylcholine, Aqueous Extract of Inflorescence, *Casuarina Equisetifolia*, Intestinal Smooth Muscle and Skeletal Muscle.

INTRODUCTION

Pharmacology is the study of drugs and their actions on target sites in biological systems.[1] The discipline of pharmacology commonly relies on experiments conducted on biological cells or tissues primarily derived from laboratory animals.[2] The most pharmacological investigations are to determine how drugs work in biological systems (e.g. binding receptors, ion channels, carrier systems or enzymes). Research in pharmacology usually requires excised isolated organs or tissues that are surgically prepared to allow for the study of drugs and their interactions with targets within tissues (e.g. smooth muscle preparations such as isolated uterus and ileum or skeletal muscle preparations such phrenic nerve diaphragm and frog rectus abdominus muscle).[1] The isolated tissue preparation and method of bioassay may well be considered the trademark of pharmacologist.[2] These experiments provide an evidence for the determination of drug potency in biological tissues which can be directly translated to human studies[1], characterization of specific receptor or its subtypes, to determine concentration response curve of an agonist, to study antagonism of drug and in new drug discovery. The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) is an authority which monitors animal experiments conducted in institutions through ethics committee and is mainly concerned with promoting the humane care of animals used in biomedical and behavioral research. For this purpose, the Government has made "Breeding and Experiment on Animals (Control and supervision) Rule 1998" as amended during 2001 and 2006, to regulate the experimentation on animal. So the use of an alternative source definitely serves the purpose of decreasing, if not completely eliminating, the killing of laboratory animals just for a strip of tissue.[2]

The basic contractile activity that occurs due to the occurrence of slow waves and spikes is modulated by the enteric nervous system (ENS). In the autonomic nervous system, including sympathetic and parasympathetic nerves modulates contractile activity indirectly through the ENS. ACh has been shown to increase the amplitude of spontaneous contractions at cholinergic receptors. There are two broad classes of cholinergic receptors: nicotinic and muscarinic. This classification is based on two chemical agents that mimic the effects of ACh at the receptor site nicotine and muscarine. ACh is the major excitatory neurotransmitter at all autonomic ganglia, at many autonomically innervated organs, at the neuromuscular junction and at many synapses in the CNS. In the peripheral nervous system, ACh is the neurotransmitter at the neuromuscular junction between the motor nerve and skeletal muscle.[3]

The present preliminary screening of experiments is to investigate the pharmacological activities of aqueous extracts of the inflorescence of *Casuarina equisetifolia* on the action of Acetylcholine upon the tone and contractility of the chick intestine and frog rectus abdominus muscle by using various *ex vivo* methods.

MATERIALS AND METHODS

MATERIALS

Collection and preparation of extract of C*asuarina equisetifolia* inflorescence: The dried inflorescence was collected in bulk from Padmabhushan Dr. B.V. Raju foundation, Vishnupur, Bhimavaram, West Godavari (District). The plant material was identified and authenticated on 18/01/2013 by Taxonomist Prof. K. Madhava Shetty, S.V. University, Tirupathi, voucher reference number is 2983.

The debris and leaves were removed from the collected material and dried in shade. It was coarsely grounded in a mixer. This coarse powder was successively extracted using macerated with distilled water at room temperature (chloroform was used as a preservative while drying) for 24 hrs to obtain the aqueous extract. The extract was concentrated by distilling off the solvent and then evaporating to dryness under shade. This dried extract was collected and was stored in a desiccator for further use.

The extract was prepared with the physiological solution *i.e.* Ringer solution without any suspending agent. The extract was added to the physiological solution and then vortexed on a vortex mixer followed by centrifugation. The supernatant solution was taken to investigate the activity of the tissues.

Preparation of Physiological Solutions

Table 1.

S.No.	Ingredients	Kreb's solutions (Chick Intestine)	Ringer solution (Frog Rectus Abdominus Muscle)
1			9.0gm
2	NaCl	5.5gm	0.42gm
3	KCl	0.35gm	0.24gm
4	$CaCl_2$	0.28gm	0.50gm
5	$NaHCO_3$	2.1gm	---
6	$MgSO_4$	0.11gm	---
7	NaH_2PO_4	0.16gm	---
8	KH_2PO_4	0.16gm	1.0gm
9	Dextrose	1.0gm	Up to 1lit.
10	Distilled water	Up to 1lit.	(1 lit. of this solution diluted to 1.4 lit. with distilled water forms the frog Ringer solution).
Either $CaCl_2$ or $NaHCO_3$ should be added at the end, in order to prevent the formation of $CaCO_3$ which forms a precipitate.			

All analytical grade chemical agents used for the experiments were purchased from Sigma Chemicals Co. (St. Louis, USA).

METHODS

Investigation on Isolated Chick Intestine

Method-1: Healthy adult chicks of either sex weighing between 1- 2 kg were procured from the local market for experimental purpose. All the animals were acclimatized and were maintained under standard husbandry conditions *i.e.* room temperature of 24 ± 5^0C; relative humidity 45-55%. The animals had free access to standard poultry feed obtained from the local market, with water provided *ad libitum* under strict hygienic conditions. The chicks were killed by euthanasia *i.e.* by giving air through the femoral vein. Then they were dissected from the tail region towards the top and the intestine was isolated from the animal. Separate the intestine. Then immediately transferred to the warm Kreb's solutions respectively maintained at 37±1^0C. Frontal writing lever was used to record the responses. The lumens of the tissues were flushed with a warm physiological solution to remove the debris in the tissue. Then the chick intestine (ileum) was mounted in different organ baths filled with physiological solution and was suitably aerated. A bath volume of 12 ml was

maintained in the organ bath with a tension of 1gm. The baths were flushed periodically with physiological solution and equilibrated for 20 min. lever-holder was readjusted to a horizontal position. This tissue invariably will have spontaneous motility, which subsides after some time.

After equilibration of chick intestine, responses were taken to different doses of Ach. (Log doses) till a ceiling response obtained. Selected a sub-maximal dose of Ach and took the response to this dose and ensured that there is reproducibility of response. Allow the drum to move for 1min., added different concentrations of extracts into the organ baths and allow it to act for 1min without flushing the baths; added the sub-maximal dose of Ach and allowed it to act for 1min with gentle tapping of the drum (to prevent friction, if any). Repeat this procedure (without extract) till the original response was obtained.

Method-2: Testing of the effect of temperature on the stability of active principle tested *per se* on chick intestine. 1mg aqueous extract of inflorescence of *casuarinas equisetifolia* was heated for 1minute at 80^0C in the water bath and cooled. Next same procedure was followed with increased temperature. Added the sub-maximal dose of Ach and allow it to act for 1min. Repeat this procedure (without extract) till the original response is obtained.

Investigation on Isolated Frog Rectus Abdominus Muscle

Frog (Rana tigrina) was stunned by head-blow using a steel road and pithed. The skin was removed and the rectus abdominus muscle was cut longitudinally. Then immediately transferred to a china dish containing frog Ringer solution at room temperature. The tissue after tying on either end is gently stretched 3-4 times. The tissue was mounted in an organ bath filled with physiological solution and is suitably aerated. A bath volume of 12 ml was maintained in the organ bath with a tension of 1gram. Flushed the bath periodically with physiological solution and equilibrated for 20 min. readjusted the lever-holder to a horizontal position. In addition to this, the extra weight of 1gm. was added closer to the writing point. This helps in further stretching of the tissue and in making it more sensitive to drugs. After equilibration of frog rectus abdominus muscle in physiological solution, take responses were taken to different doses of Ach and nicotine (log doses) till a ceiling response was obtained. Selected a sub-maximal dose of Ach and nicotine and took the response to this dose ensuring reproducibility of response. Allowed the drum to move for 1min., added different concentrations of extract into the organ bath and allow it to act for 1min. without flushing the bath, add the sub-maximal dose of Ach and nicotine and allowed it to act for 1min. with gentle tapping of the drum (to prevent friction, if any). Repeated this procedure (without extract) till the original response is obtained.[4]

RESULTS AND DISCUSSION

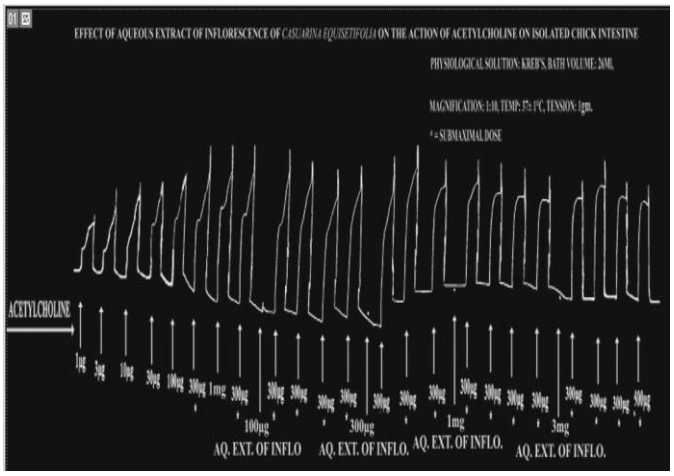

Figure: 1.

Figure: 1 shows the effect of aqueous extract of inflorescence of *Casuarina equisetifolia* on the action of Ach on isolated chick intestine. The contractions to different doses of Ach from 100µg to 1mg (log doses) till ceiling response was recorded. Selected a sub-maximal dose of Ach (300µg*) and took the response to this dose and ensured that there is reproducibility of response. Allowed the drum to move for 1min., added 100µg, 300µg, 1mg and 3mg of extract into the organ bath of Kreb's solution and allowed it to act for 1min without flushing the bath, added the sub-maximal dose of Ach and allowed it to act for 1min. Repeat this procedure (without extract) till the original response was obtained. The inference drawn from this experiment is that the extract potentiated the effect of Ach and it was reversible.

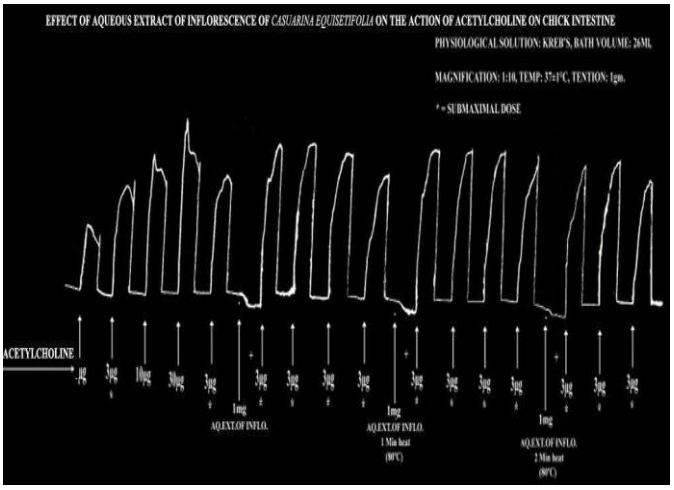

Figure: 2.

Figure: 2 shows the effect of aqueous extract of inflorescence of *Casuarina equisetifolia* on the action of Ach on isolated chick intestine. The contractions to different doses of Ach from 1µg to 30µg (log doses) till a ceiling response were obtained. Selected a sub-maximal dose of Ach (3µg*) and took the response to this dose and ensured that there is reproducibility of response.

Allowed the drum to move for 1min., added 1mg of extract into the organ bath contained Kreb's solution and allowed it to act for 1min without flushing the bath, added the sub-maximal dose of Ach and allowed it to act for 1min. Repeated this procedure (without extract) till the original response was obtained. The inference drawn from this experiment is that the extract potentiated the effect of Ach and it was reversible.

Allowed the drum to move for 1min. Then the 1mg concentration of extract was heated for 1min at 80^0C in the water bath and cooled, then immediately added to the organ bath contained Kreb's solution and allow it to act for 1min. without flushing, the bath added the sub-maximal dose of Ach and allowed it to act for 1min. Repeat this procedure (without extract) till the original response was obtained. The inference drawn from this experiment is that the extract potentiated the effect of Ach and it was reversible. Again the 1mg concentration of extract was heated for 2min at the same temperature in the water bath repeated the same procedure. The inference drawn is that the extract potentiated the effect of Ach and it was reversible. Finally, the aqueous extract of inflorescence of *Casuarina equisetifolia* is thermostable.

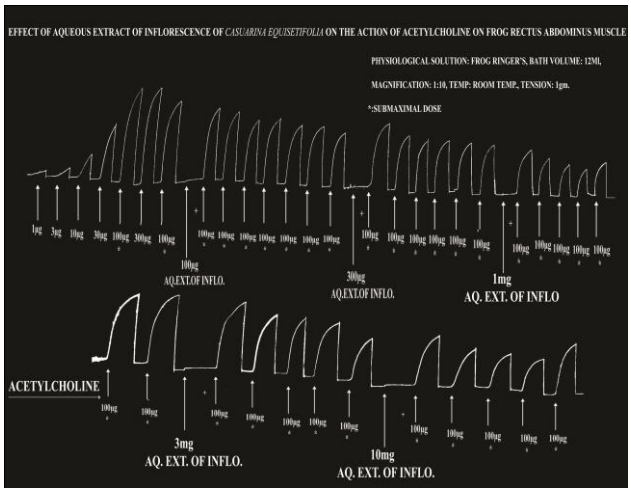

Figure: 3.

Figure: 3 shows the effect of aqueous extract of inflorescence of *Casuarinas equisetifolia* on the action of Ach on isolated frog rectus abdominus muscle. The contractions to different doses of Ach from 1µg to 300µg (log doses) till a ceiling response were obtained. Selected a sub-maximal dose of Ach (100µg*) and took the response to this dose and ensured that there is reproducibility of response. Allowed the drum to move for 1min., added 100µg, 300µg, 1mg, 3mg and 10mg of extract into the organ bath contained frog Ringer's solution and allowed it to act for 1min without flushing the bath, added the sub-maximal dose of Ach and allowed it to act for 1min. Repeated this procedure (without extract) till the original response was obtained. The inference drawn was that the extract potentiated the effect of Ach and it was reversible.

DISCUSSION

The literature review revealed that various chemicals and pharmacological investigations were carried out with *Casuarina equisetifolia* (beach she-oak). The chemical constituents isolated from different plant parts shows the presence of flavanoids glycosides of kaempferol and quercetin in leaves[5] alicyclic acids (shikimic and quinic acid), polyols (dextrose, fructose and sucrose) and amino acids in fruit, bark and wood[6] and pharmacological investigations found out from the bark of the tree still has important uses for traditional medicine, especially for treating digestive tract ailments. The plant found minor use as a dye plant and is still used for this purpose to a small degree. The needles in decoction form used as a lotion for swelling. The fruits mixed with powdered nutmeg to treat a toothache. It is also used in the treatment of a cough and ulcers.[7] The tannin content in different plant parts enables to use it as an astringent and in the treatment of diarrhea and dysentery.[8] In Tonga, an infusion of the bark is commonly taken as a potion or squeezed into the mouth of infants with mouth infections. In the present study, preliminary screening of the plant revealed the presence of aqueous extracts of the inflorescence of *Casuarina equisetifolia*.

The observational assessment indicates that the aqueous extract of inflorescence might contain active principles that exhibit muscarinic activity. This is evident because of *Casuarinas equisetifolia* potentiated the action of Acetylcholine on chick intestine (Smooth muscle-mAChRs). So, this extract might have the anticholinesterase like activity. The extract when heated it retained the property of anticholinesterase indicating that it is thermostable. Invariably these extract had a tendency to produce a reduction in that tone of the tissue. The extract had a similar effect of anticholinesterase like activity on frog rectus abdominous muscle (Skeletal muscle-nAChRs).

CONCLUSION

It is clearly evident from the results of preliminary screening that the aqueous extracts of the inflorescence of *Casuarina equisetifolia* have some active pharmacological principles. The extract potentiate the Ach induced contractions on both skeletal and smooth muscle preparations and it is thermostable property also, indicates it may act on the nicotinic acetylcholine receptors (nAChRs**)** as well as muscarinic acetylcholine receptors (mAChRs) respectively. These activities were proved continuously, it indicates this plant material might be used for anticholinesterase activities. Further evaluation is warranted to explore the possibility of finding some more pharmacological actions for the therapeutic gain of *Casuarina equisetifolia* in future.

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