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Open Access Research Article

Screening and evaluation of ethanolic extract from *Casuarina equisetifolia* inflorescence on isolated chick rectum, frog rectum and frog rectus abdominus muscle for identification of muscarinic and nicotinic receptor's action

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ABSTRACT

The present experiments were undertaken to justify the use of an ethanolic extract from inflorescence of *Casuarina equisetifolia,* Family: Casuarinaceae, influencing the nicotine responses on isolated chick rectum and frog rectum (Smooth Muscles) and frog rectus abdominus muscle (Skeletal Muscle). The isolated tissues were mounted in organ bath filled with physiological solution and was suitably aerated. After equilibration, responses were taken to different doses of nicotine (log doses) till a ceiling response was obtained. A sub-maximal dose of nicotine was selected and responses to this dose was taken and ensured that there is reproducibility of response. The drum was allowed to move for 1min., different concentrations of extracts into the organ baths were added and allowed to act for 1min without flushing the baths, then the sub-maximal dose of nicotine was added and allowed to act for 1min. this procedure was repeated (without extract) till the original response was obtained. The inference drawn from these experiments, the ethanolic extract of inflorescence of *Casuarina equisetifolia* antagonised the action of nicotine on isolated chick rectum, relaxed the effect of nicotine on frog rectum and it potentiated the effect of nicotine on frog rectus abdominus muscle. The nicotine receptors of rectus abdominus is activated, perhaps by the prevention of hydrolysis of acetylcholine by the extract. Based on the results obtained from the isolated chick rectum the ethanolic extract is having antinicotinic activity and it may act on the nicotinic acetylcholine receptors (nAChRs**)** as well as muscarinic acetylcholine receptors (mAChRs) on other isolated tissues. The extract might contain ganglionic blocking activity or non-specific activity or membrane stabilising activity also.

Keywords: Nicotine, Ethanolic extract, Inflorescence of *Casuarina equisetifolia,* Isolated Chick Rectum, Isolated Frog Rectum and Isolated Frog Rectus Abdominus Muscle.

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INTRODUCTION

The discipline of pharmacology has used the isolated tissue bath system for over 150 years[1]. The isolated tissue preparation and method of bioassay may will be considered the trademark of pharmacologist. These experiments provide an evidence for the determination of drug potency in biological tissues which can be directly translated to human studies, 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[2]. The primary advantage of this technique is that the tissue is living and functions as a whole tissue, with a physiological outcome (contraction or relaxation) that is relevant to the body. It is a synthesis of steps (drug-receptor interaction, signal transduction, second messenger generation, change in smooth muscle excitability, and change in tissue function). While other techniques allow the study of each of these steps (*e.g.* radioligand binding for drug affinity, measurement of second messengers), the isolated tissue bath technique allows for integration of all these steps, it comes closer to how the drugs examined would work in the body as a whole[1].

Nicotine is a naturally occurring alkaloid found in tobacco plants. Nicotine has a physiologic effect because it binds to receptors on the neuromuscular system. Nicotine affects the skeletal, smooth and cardiac muscles, which can result in acute and chronic muscular dysfunctions such as a

decrease in appetite, paralysis and asphyxiation. Nicotine acts on nicotinic receptors in the brain and muscles. Nicotine travels through the bloodstream and reaches the brain within 10 to 19 seconds[3]. However, nicotine cannot be used as a drug because it is well known for habit forming nature. Structurally acetylcholine and nicotine are unrelated. So, in alternative that work on the same receptors or improve acetylcholine activity at the same site selectively have to be developed[4]. Nicotinic acetylcholine receptors (nAChRs**)** mediate the physiological effects of exogenous nicotine. They also play critical physiological roles throughout the brain and body by mediating cholinergic excitatory neurotransmission, modulating the release of neurotransmitters, and having long-term effects, for example, gene expression and cellular connections. Mammalian nAChR subunits are derived from a family of sixteen different genes (α1-α7, α9-α10, β1-β4, γ, δ, and ε) which have distinctive distributions, and assemble into pentameric receptors. Importantly, the combinations and orders of nAChR subunits within functional pentamers dictate nAChR subtype properties[5].

India is the largest producer of the medicinal plants and is rightly called the "Botanical Garden of the world"[6]. *Casuarina equisetifolia*, family Casuarinaceae is an ornamental plant grown in India, tropical Africa and Sri Lanka. Due to the presence of amino acids, taraxerol, lupenone, lupeol, alicyclic acid, gallic acid and sitosterol, this plant is reported as antibacterial, antifungal, anti-inflammatory, anticancer, antioxidant and analgesic. The bark of *C. equisetifolia* is used as an astringent. A polyherbal gel containing *C. equisetifolia* has shown anti-acne activity in rats[7]. The present screening and evaluation of experiments were investigated the use of an ethanolic extract from inflorescence of *Casuarina equisetifolia* influencing the Nicotine responses on isolated chick rectum, isolated frog rectum (Smooth Muscles) and isolated frog rectus abdominus muscle (Skeletal Muscle) using various *ex vivo* methods.

MATERIALS AND METHODS

MATERIALS

Collection and preparation of ethanolic extract from inflorescence of *Casuarina equisetifolia*

The dried inflorescence was collected in bulk from Padmabhushan Dr. B.V. Raju foundation, Vishnupur, Bhimavaram, West Godavari (District), A.P., India. Then the debris and leaves were removed from the collected material and dried in shade. The pollen grains were separated from inflorescence. It was coarsely grounded in a mixer. The powder was extracted by continuous hot extraction using the Soxhlet apparatus at a temperature of 78ºC for 48 h using 95% ethanol and then evaporating to dryness under shade (chloroform was used as a preservative while drying). The extract was collected and preserved in a desiccator for further studies.

The plant material was identified and authenticated on 18/01/2013 by Taxonomist, Prof. Dr. K. Madhavachetty, S.V. University, Tirupathi, India, Voucher reference number is 2983.

The extract was prepared with the physiological solution (Tyrode's solution and 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: Preparation of Tyrode's Solution and Ringer Solution

S. No.	Ingredients	Tyrode's solution (Chick Rectum & Frog Rectum)	Ringer solution (Frog Rectus Abdominus Muscle)
1.	NaCl	8.0gm	9.0gm
2.	KCl	0.20gm	0.42gm
3.	$MgCl_2$	0.10gm	---
4.	$CaCl_2$	0.20gm	0.24gm
5.	$NaHCO_3$	1.0gm	0.50gm
6.	Dextrose	1.0gm	1.0gm
7.	Distilled water	Up to 1lit.	Up to 1lit. (1 lit. of this solution diluted to 1.4 lit. with distilled water forms the frog Ringer solution).
Either CaCl2 or NaHCO3 should be added at the end, in order to prevent the formation of CaCO3 which forms a precipitate.			

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

Drugs: Approximately 3 mL of Liquid form pure Nicotine was collected from the Andhra University, Visakhapatnam, Andhra Pradesh India.

METHODS

Investigation on Isolated Chick Rectum

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 ± 5^0 C; 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 rectum was isolated from the animal. Separate the rectum. Then immediately transferred to the warm Tyrode's solution respectively maintained at 37±1^0C. Frontal writing lever was used to record the responses. The lumen of the tissue was flushed with warm physiological solution to remove the debris in the tissue. Then the chick rectum was mounted in organ

bath 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 bath was flushed periodically with physiological solution and equilibrated for 20 min. lever-holder was readjusted to a horizontal position. This tissue was invariable having spontaneous motility, which subside after some time. After equilibration of chick rectum responses were taken to different doses of nicotine (log doses) till a ceiling response obtained. A sub-maximal dose of nicotine was selected and responses to this dose was taken and ensured that there is reproducibility of response. The drum was allowed to move for 1min., added different concentrations of extracts into the organ baths and allowed it to act for 1min without flushing the baths; added the sub-maximal dose of 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 was obtained[8].

Investigation on Isolated Frog Rectum

Method 2: Frog (*Rana tigrina*) was stunned by head-blow using a steel road and pithed. The skin and abdomen were cut and opened to locate the rectum (which is huge sac-like structure) and the last portion of the GI tract. Separate the rectum from the intestine. Then immediately transferred to a china dish containing frog Ringer solution at room temperature. Frontal writing lever was used to record the responses. The lumen of the rectum was flushed with physiological solution to remove the debris in it. Then the frog rectum was mounted in 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 500 mg. Flushed the bath periodically with physiological solution and equilibrated for 20 min. readjusted the lever-holder to a horizontal position. This tissue was invariable having spontaneous motility, which is likely to subside after some time. After equilibration of frog rectum in physiological solution, responses were taken to different doses of nicotine (log doses) till a ceiling response. A sub-maximal dose of nicotine was selected and responses to this dose was taken and ensured that there is reproducibility of response. The drum was allowed to move for 1min., added different concentrations of extract into the organ bath and allowed to act for 1min. without flushing the bath, added the sub-maximal dose of nicotine and allowed it to act for 1min. with gentle tapping of drum (to prevent friction, if any). Repeated this procedure (without extract) till the original response is obtained[9].

Investigation on Isolated Frog Rectus Abdominus Muscle

Method 3: 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 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, 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, responses were taken to different doses of nicotine (log doses) till a ceiling response was obtained. A sub-maximal dose of nicotine was selected and responses to this dose was taken and ensured that there is reproducibility of response. The drum was allowed 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 nicotine and allowed it to act for 1min. with gentle tapping of drum (to prevent friction, if any). Repeated this procedure (without extract) till the original response is obtained[9].

RESULTS AND DISCUSSION

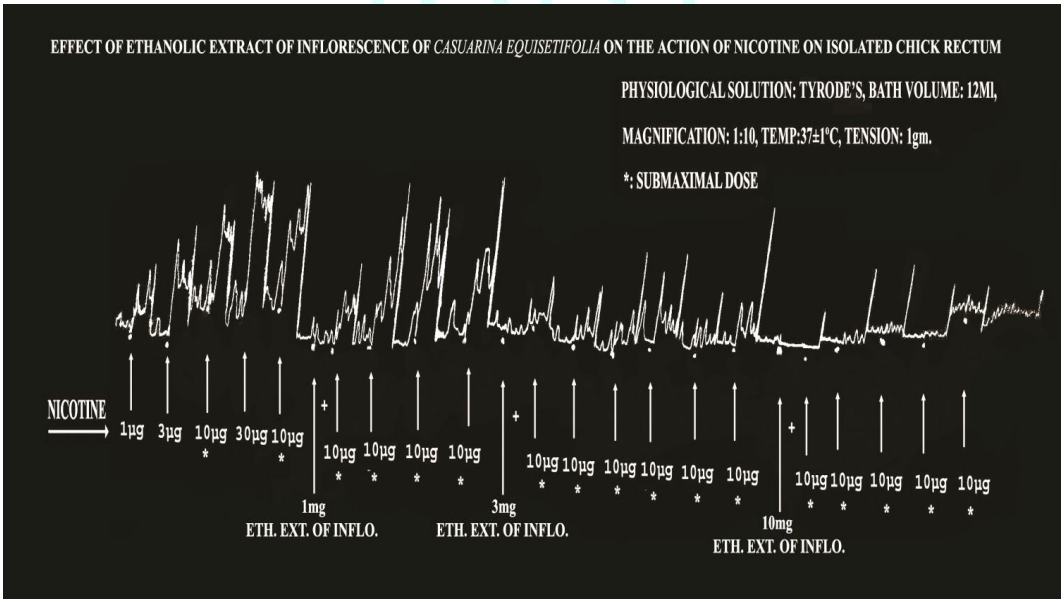

Figure 1: Investigation on Isolated Chick Rectum

Figure 1, shows the effect of ethanolic extract of inflorescence of *Casuarina equisetifolia* on the action of nicotine on isolated chick rectum. The responses to different doses of nicotine from 1µg to 30µg (log doses) till a ceiling response were obtained. A sub-maximal dose of nicotine (10µg*) was selected and the response to this dose was taken and ensured that there is reproducibility of response. Allowed the drum to move for 1min., added 1mg

of extract into the organ bath contained Tyrode's solution and allowed it to act for 1min without flushing the bath, added the sub-maximal dose of nicotine 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 reduced the sub-maximal dose of nicotine and it was reversible. The drum was allowed to move for 1min., added 3mg extract into the organ bath contained Tyrode's solution and then the above procedure was repeated. The inference drawn from this experiment is that the extract reduced the sub-maximal dose of nicotine and it was not reversible. Finally added 10mg of extract into the organ bath and then the above procedure was repeated. The inference drawn from this experiment is that the extract completely blocked the effect of nicotine and it was not reversible.

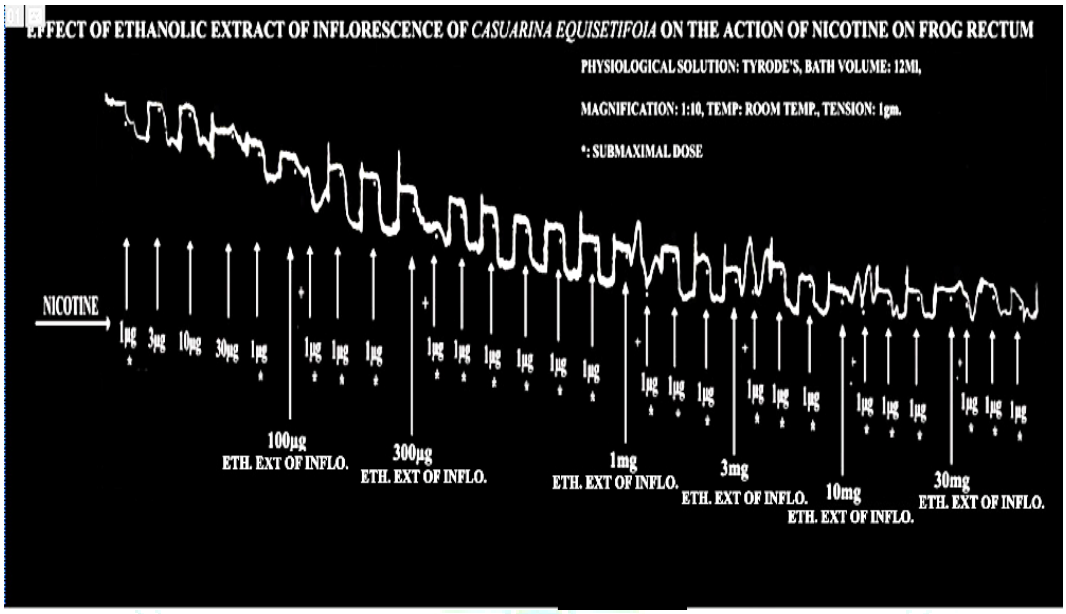

Figure 2: Investigation on Isolated Frog Rectum

Figure 2, shows the effect of ethanolic extract of inflorescence of *Casuarina equisetifolia*on the action of nicotine on isolated frog rectum. The relaxations to different doses of nicotine from 1µg to 30µg (log doses) till a ceiling response ware taken. A sub-maximal dose of nicotine (1µg*) was selected and the response to this dose was taken and ensured that there is reproducibility of response. The drum was allowed to move for 1min., added 100µg of ethanolic extract of inflorescence which itself produced relaxation, following relaxant effect by nicotine. The following two responses were potentiated. 300µg of extract produced greater relaxation followed by relaxation caused by 1µg* of nicotine. 1mg of extract produced initial short relaxation followed by contraction followed by relaxation with 1µg* of nicotine. 3mg, of extract produced similar effect as before but with more prominent relaxant-contractile. Relaxant effect *per se* and nicotine produced permanent contraction. 10mg of extract produced a response 'M' shaped and 30mg of the extract produced predominantly relaxant effect.

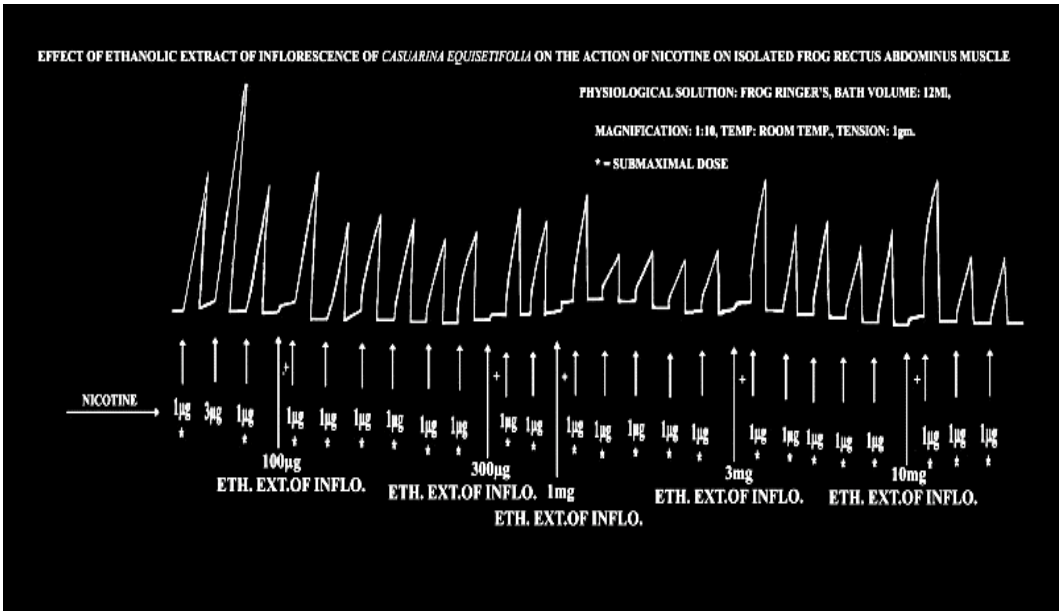

Figure 3: Investigation on Isolated Frog Rectus Abdominus Muscle

Figure 3; shows the effect of ethanolic extract of inflorescence of *Casuarina equisetifolia* on the action of nicotine on isolated frog rectus abdominus muscle. The contractions to two different doses of nicotine *i.e.* 1µg* and 3µg (log doses) were recorded. A sub-maximal dose of nicotine (1µg*) was selected and the response to this dose was taken and ensured that there is reproducibility. The drum was allowed to move for 1min., added 100µg, 300µg, 1mg, 3mg and 10mg of extract into the organ bath as shown in figure and allowed it to act for 1min without flushing the bath, added the sub-maximal dose of nicotine and allowed it to act for 1min. Repeated this procedure (without extract) till the two consecutive doses were same. The inference drawn from this graph is that the extract potentiated the effect of nicotine and it was reversible.

DISCUSSION

The literature review revealed that various pharmacological investigations were carried out with *Casuarina equisetifolia* (beach she-oak). Pharmacological investigations found out from the bark of the tree still have important uses for traditional medicine, especially for treating digestive tract ailments[2]. Phytosterol from the leaves of the plant shows antibacterial activity, hypoglycemic, antifungal, molluscicidal, cytotoxic, bark and wood showed significant anticancer and anthelmintic activities[10]. 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[2]. The present screening and evaluation of the plant revealed the use of an ethanolic extract from inflorescence of *Casuarina equisetifolia,* influencing the Nicotine responses on isolated chick rectum, isolated frog rectum (Smooth Muscles) and isolated frog rectus abdominus muscle (Skeletal Muscle) using various *ex vivo* methods.

The inference drawn from these experiments, the ethanolic extract of inflorescence of *Casuarina equisetifolia* (Eth. ext. Inflo. *C. equisetifolia*) has produced dose-dependent contractions with nicotine on isolated chick rectum. It antagonised the action of nicotine. The extract might contain ganglionic blocking activity or non-specific activity or membrane stabilising activity as this blocked is irreversible (Refer Figure 1).

Eth. ext. Inflo. *C. equisetifolia* relaxated the effect of nicotine on frog rectum (not strictly dose-dependent). Increased doses of the extract produced multiple responses, the characterisation of which requires the use of different antagonists to arrive at different conclusion (Refer Figure 2).

Eth. ext. Inflo. *C. equisetifolia* Potentiated the effect of nicotine on frog rectus abdominus muscle. The nicotine receptors of rectus abdominus is activated, perhaps by the prevention of hydrolysis of acetylcholine by the extract. The responses of the tissue gradually reduced (Refer Figure 3).

CONCLUSION

Based on the results obtained from the isolated chick rectum the ethanolic extract is having antinicotinic activity and it may act on the nicotinic acetylcholine receptors (nAChRs**)** as well as muscarinic acetylcholine receptors (mAChRs) on isolated frog rectus abdominus muscle and isolated frog rectum. The extract might contain ganglionic blocking activity or non-specific activity or membrane stabilising activity also. 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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CONFLICT OF INTEREST

Authors have declared that no conflict of interests exists.

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