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Anti Nociceptive, Anti Inflammatory and Anti Bacterial Properties of Leaf of Female *Borassus flabellifer* (Arecaceae)

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ABSTRACT: The *Borassus flabellifer* is a tall, erect palm, easy to recognize by its large, fan-shaped leaves. The different parts of the plant are used for ailments like secondary syphilis, antiperiodic, heart burns, liver, inflammation and spleen enlargement. The chemical constituents include gums, albuminoids, fats, steroidal glycosides and carbohydrates like sucrose. In the present work anti nociceptive, anti inflammatory, membrane stabilizing property, and anti microbial activities were carried out. The dried leaf powder was extracted with ethanol and submitted to chemical tests. It revealed the presence of saponins, flavonoids, steroids and tannins. In the anti nociceptive activity Eddy's hot plate method and tail immersion method were performed using tramadol as the standard. The leaf extract has shown a potent nociceptive activity for both the evaluation parameters. Carrageenan induced rat paw edema and HRBC membrane stabilizing property were performed for the anti inflammatory activity using diclofenac sodium and hydrocortisone as the standard drugs. The results showed that the ethanolic leaf extract exhibited potent anti inflammatory activity and membrane stabilizing property in a dose dependent manner. The antibacterial screening was performed on the organisms *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumonia* using gentamycin as the standard. In the antibacterial activity the leaf extract was found to be not effective in inhibiting the growth of bacterial strains.

Key words: *Borassus flabellifer*, Nociceptive, Plethysmometer, Alsevers solution, Hot plate, Tail immersion

Introduction

Nociception or the pain is part of a defensive reaction against dysfunction of the organism or imbalance in its functions, as well as against potentially dangerous stimulus[1]. Most of the nociceptors are polymodal, responding to noxious mechanical stimuli (painful pressure, squeezing or cutting of the tissue), noxious thermal stimuli (heat or cold), and chemical stimuli. Sensor molecules transduce mechanical, thermal and chemical stimuli into a sensor potential, to a sufficiently high level to trigger and conduct the axon to the dorsal horn of the spinal cord or the brainstem. Inflammation is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue[2] .The process of acute inflammation is initiated by cells already present in all tissues, mainly resident macrophages, dendrites cells, histiocytes, Kuppfer cells and mastocytes. At the onset of an infection, burn, or other injuries, these cells undergo activation and release inflammatory mediators responsible for the clinical signs of inflammation. [3].

The *Borassus flabellifer* is a tall, erect palm, easy to recognize by its large, fan-shaped leaves which are quite unlike the pinnate leaves of other palms. The different parts of the plant are used for ailments like secondary syphilis, antiperiodic, heart burns, liver, inflammation and spleen enlargement. The chemical constituents of *Borassus flabellifer* include gums, albuminoids, fats, steroidal glycosides and carbohydrates like sucrose. The fresh pulp is reportedly rich in vitamins A and C. The fresh sap is reportedly a good source of vitamin B-complex. The male inflorescence constitutes borassosides and dioscin, spirostane-type steroid saponins [4,5,6,7]. The analgesic, antipyretic and anti inflammatory activities of ethanolic extract of male flowers of the plant were established [8,9,10]. The current work is focused on the anti nociceptive, anti inflammatory, membrane stabilizing property and anti bacterial activities of the ethanolic leaf extracts of female *Borassus flabellifer*.

Materials and Methods

Plant material

The leaves of *Borassus flabellifer* L. (Arecaceae) were collected from Nalgonda district, Andhra pradesh during November to December. It was authenticated by Mr. A. Lakshma Reddy, retired Professor, Dept. of Botany, Nagarjuna Govt. College (Autonomous) Nalgonda, A.P, India. The specimen herbarium (voucher no: NCOP-NLG/ph'cog/2009-10/004) was prepared and deposited in the Department of Pharmacognosy for future reference. The selected parts of the plant were then dried in shade at temperature between 21-30°C for 20 to 30 days, after which these parts were chopped, ground and stored in air tight containers for further studies.

Equipments used

Rotary vacuum evaporator (Indosatt Scientific lab Equipments), Plethysmograph (Inco rat paw Plethysmograph mecury model), Hot plate (Inco), Incubator (Lab Husp), Laminar air flow(Toshiba S.No.0062).

Chemicals used

All the drugs and chemical used in this study were of analytical grade. Carrageenan sodium (Yarrow chem products, Mumbai), commercially available Diclofenac sodium (German remedy) Tramadol hydrochloride (Zydus Alidae), Gentamycin (Nicolas).

Experimental animals

Swiss Albino Mice (25-30g) and Wistar Albino Rats (150-200gm) of either sex were procured from National Institute of Nutrition, Hyderabad, A.P, India. The experimental protocol was initially approved from the Institutes animal ethics committee under the reference no. NCOP/IAEC/approval/08/2010 and then experimental studies were undergone according to their rules and regulations. The animals were housed under standard environmental conditions and had free access to standard pellet diet (Goldmohar brand, Lipton India Ltd.) and water *ad libitum.*

Bacterial culture used

Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia were procured from Department of Microbiology, Osmania University.

Preparation of the extract

The 500gm of powdered plant material was defatted with petroleum ether and extracted with ethanol in a soxhlet apparatus for 72 hrs at 20^0C. The extract was filtered through Whatmann filter paper and concentrated by rotary vacuum evaporator. The extract was subjected to preliminary chemical tests. The %yield of the extract was found to be 18.5%w/w.

Preliminary chemical tests

The chemical tests were performed as per procedures mentioned in standard reference books [11].

Evaluation of anti nociceptive activity

Eddy's hot-plate method [12,13,14,15]

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Swiss albino mice of either sex weighing about 25-30g were selected. All animals were fasted for 18 hrs. They were divided into four groups of six animals each. Animals of group I received water (1ml.); group II received tramadol hydrochloride (10 mg/kg) orally. Group III and IV received ethanolic leaf extracts at dose levels of 150 mg/kg and 300 mg/kg body weight, per oral. The time of reaction to pain stimulus of the mice placed on the hot plate heated at 55^{0}C was recorded at 30, 60, 90,120 and 180 minutes, after the administration of the test and the standard drug. The increase in reaction time against control group was compared and calculated.

Tail Immersion Method [12,14,15,16]

Healthy albino rats weighing about 150-200gm were taken. They were divided into four groups having six animals in each, numbered and placed into individual restraining cages leaving the tail hanging out freely. The animals were then allowed to adapt in the cages for 30 minutes before testing. The lower 5cm portion of the tail was marked and immersed in a water bath of freshly filled warm water of exactly 55^{o}C. Within a few seconds the rat reacted by withdrawing the tail. The reaction time was recorded by a stop watch. After each determination the tail was carefully dried. The reaction was determined before oral feeding of the drug and various extracts (150 mg/kg and 300 mg/kg body weight) which was recorded as zero minutes reading. The control, standard and test substances were given to the animals by gastric tube. After the drug was administered the reaction time was recorded at an interval of ½ h, 1h, 2h, 3h, and 4h. The cut off time of the immersion is 15 seconds. The mean reaction time was recorded for each group and compared with the value of standard drug.

Evaluation of anti-inflammatory activity

Invitro anti inflammatory activity by membrane stabilizing property[17]

The HRBC membrane stabilization has been used as a method to study the anti-inflammatory activity. Human blood was mixed with equal volume of sterilized Alsever solution. Alsever solution contains dextrose, sodium citrate, sodium chloride in water. The blood was centrifuged and the packed cells were washed with isosaline and 10% v/v suspension was made with Isosaline. The drug samples were prepared by suspending the residues in hot water. The assay mixture contained the drug, 1 ml phosphate buffer; 2 ml hypo saline, 0.5 ml HRBC suspension, hydrocortisone sodium was used as the reference drug. Instead of hypo saline 2 ml of distilled water used in the control. All the assay mixture were incubated at 37^{o}C for 30 minutes and centrifuged. The hemoglobin content in the supernated solution was estimated using spectrophotometer at 560nm. The percentage of hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100 %. The percentage of HRBC membrane stabilization was calculated using the formula.

$$\text{Percentage protection} = \frac{100 - \text{Optical density of drug treated sample}}{\text{Optical density of control}} \times 100$$

Invivo Anti-inflammatory activity by Carrageenan induced paw edema method [15,16]

Wistar albino rats of either sex weighing 150-200g were taken. They were divided into four groups. Group I received vehicle of 1 ml, group received II standard diclofenac sodium, group III and IV received 150 and 300 mg/kg body weight of the test drug by oral route. Edema was induced by subcutaneous administration of 1% of 0.05ml of aqueous solution of carrageenan into right hind paws .Paw volume were measured up to 24h after the carrageenan administration at an intervals of 1h, 3h, 6h, 12h and 24hr and paw volume was measured with the help of plethysmometer.

Statistical analysis

Results are expressed as Mean ± SEM The difference between experimental groups was compared by One-way Analysis of Variance (ANOVA) followed by Dunnett Multiple comparison test (control vs test) using the soft ware Graph Pad Instat.

Antibacterial screening[18]

All petridishes and graduated measuring pipettes were heat sterilized in an autoclave at 120ºC for 1hr. Media were steam sterilized at 121 ºc (15psi) for twenty minutes in an autoclave. All plates were prepared with an equal thickness of nutrient agar. Well plate technique was used to perform the antibacterial assay. Three test concentrations of 250, 500 and 750 µg/ml of the extract was made by dissolving in ethanol. The standard gentamycin at concentrations of 5 and 10 µg/ml was used. Ethanol(95%) was taken as the control. Bores of 5mm diameter were made by scooping out medium with a sterilized borer from petri dish which was inoculated with the organisms. The solutions of each test compound, control and reference standards were added separately and petri dishes were subsequently incubated at 37 ºC. After overnight incubation the zones of inhibition were measured.

Results

Table 1: Anti nociceptive activity of ethanolic extract of female *Borassus flabellifer* leaves on mice subjected to Eddy's hot plate method

Treatment	Dose (Mg/kg)	Reaction time in seconds				
		½ h	1h	2h	3h	4h
Control	1ml	3.6±0.036	3.4±0.036	3.2±0.036	3.3±0.036	3.13±0.021
Standard	10	5.6± 0.057**	8.22±0.007**	14.82±0.005**	17.58±0.005**	24.88±0.005**
Leaf	150	3.86±0.005**	5.97±0.006**	8.97±0.004**	10.81±0.003**	13.55±0.005**
Leaf	300	4.67±0.005**	6.92±0.011**	11.54±0.005**	13.02±0.003**	20.88±0.005**

**P < 0.01 considered significant, by Dunnett Multiple Comparison Test (One way analysis of variance, ANOVA)

Table 2: Anti nociceptive activity of ethanolic extract of female *Borassus flabellifer* leaves on rats subjected to the tail immersion method

Treatment	Dose	Reaction time in seconds				
	(mg/kg)	½ h	1h	2h	3h	4h
Control	1ml	2.3±0.057	2.04±0.005	2.3±0.057	2.1±0.040	2.4±0.057
Standard	10	4.38±0.005**	4.68±0.004**	5.48±0.005**	5.92±0.005**	7.93±0.016**
Leaf	150	3.48±0.005**	4.21±0.004**	4.53±0.005**	4.91±0.006**	5.52±0.009**
Leaf	300	3.05±0.005**	4.43±0.005**	4.66±0.005**	5.48±0.003**	5.93±0.009**

**P < 0.01 considered significant, by Dunnett Multiple Comparison Test (One way analysis of variance, ANOVA)

Table 3 Anti inflammatory activity of ethanolic extract of female *Borassus flabellifer* leaves on rats subjected to carrageenan-induced paw edema in rats

Treatment	Dose	Paw volume (ml)				
	(mg/kg)	1hr	3hr	6hr	12hr	24hr
Control	1ml	1.78±0.005	1.88±0.003	1.93±0.006	1.42±0.237	1.58±0.002
Standard	100	1.5±0.057**	1.41±0.006**	1.33±0.005**	1.14±0.005**	1.02±0.005**
Leaf	150	1.6±0.057**	1.54±0.005**	1.44±0.005**	1.39±0.003**	1.30±0.005**
Leaf	300	1.58±0.005**	1.43±0.003**	1.38±0.005**	1.20±0.003**	1.11±0.003**

**P < 0.01 considered significant, by Dunnett Multiple Comparison Test (One way analysis of variance, ANOVA)

Table 4: HRBC membrane stabilizing property of ethanolic extract of female *Borassus flabellifer* leaves

S.No	Conc (µg/ml)	Leaf extracts	Standard (Hydrocortisone)
		% protection	% protection
1	50	45.2	70.8
2	100	48.02	72.1
3	150	52.8	74.5
4	200	68.28	76.7
5	250	71.76	77.1

Figure 1: Anti bacterial activity of ethanolic extract of female *Borassus flabellifer* leaves

Klebseilla pneumoniae

E.coli

Staphylococcus aureus

Bacillus subtilis

The ethanol leaf extract has shown potent anti nociceptive activity at the dose of 300mg/kg with a reaction time of 20.88±0.005 seconds at 4th hour for Eddy's hot plate method and 5.93±0.009 seconds for the tail immersion method (Table 1 and Table 2).Results obtained were found to satisfactory and comparable to the standard tramadol. Leaf extract was found to be a potent anti inflammatory agent as it activity was persisting for 24 hours. The values were found to be significant statistically (Table 3). The extracts at a concentration from 50 µg/ml -250 µg/ml protects the human erythrocyte membrane against lysis induced by the hypotonic solution (Table 4). The leaf extract at 250 µg/ml has provided a protection of 71.76% as compared with 77.1% produced by the standard hydrocortisone at the same concentration (Table 4).Ethanol leaf extract has failed to exhibit anti bacterial property against all the four strains even at a concentration of 750µg/ml. The standard gentamycin at 5µg/ml and 10 µg/ml has shown a prominent activity with a zone of inhibition of 7.5 mm and 8mm(*E.coli*), 7.5 mm and 9mm(*Bacillus subtilis*), 7 mm and 8 mm(*Staphyllococcus aureus*), 7.5 mm and 9 mm (*Klebseilla pneumoniae*)(Figure 1).

Discussion

Pathological nociceptive input often causes central sensitization. This is an increase of excitability of spinal cord neurons. Hyper excitable spinal cord neurons are more susceptible to peripheral inputs and respond, therefore, more strongly to stimulation. Central sensitization amplifies the processing of nociceptive input and is thus an important mechanism that is involved in clinically relevant pain states [2]. The hot-plate and tail- immersion methods are useful in elucidating centrally mediated anti-nociceptive responses. This focuses mainly on changes above the spinal cord level [19]. The significant increase in pain threshold produced by the leaf extracts in these models suggests involvement of central pain pathways. The action of the anti inflammatory agents is the inhibition of the cyclooxygenase enzyme which is responsible for the conversion of arachidonic acid to prostaglandinsG2 to PGH_2 along with per oxidation which is associated with formation of long channels in membranes. The channel opening occurs due to release of chemical mediators and hence arachidonic acid is released from membrane and converted to prostaglandin. The extracellular activity of these enzymes is said to be related to acute and chronic inflammation. NSAIDS act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane [20]. The ethanol extract also significantly inhibited carrageenan induced rat paw edema. The inhibition at 3hrs was greater than 1hr after induction of edema (Table 3). This method was taken as a prototype of exudative phase of inflammation. The development of inflammation is biphasic. The initial phase is attributable to the release of histamine, serotonin and kinins in the first hour after injection of carrageenan. A more pronounced second phase is related to release of prostaglandin like substances in 2-3hrs [20]. Hence it can be concluded that the analgesic effect produced by the extracts may be via central mechanisms involving these receptor systems or via peripheral mechanisms involved in the inhibition of prostaglandins, leucotrienes and other endogenous substances that are key players in inflammation and arthritis. The possible anti inflammatory activity of

leaf extract may be due to inhibitory effect on release of inflammation mediators and /or by membrane stabilizing activity. The anti bacterial activity of the ethanolic leaf extracts have exhibited a false positive result which may be due to geographical variations or due to the development of resistance by the organisms.

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