Asian Journal of Chemistry Vol. 22, No. 5 (2010), 3525-3530

Evaluation of Analgesic Activity of *Casuarina equisetifolia* Frost (Casuarinaceae)

A.N. AHER*, S.C. PAL, S.K. YADAV†, U.K. PATIL† and S. BHATTACHARYA‡
Department of Pharmacognosy, Natural Product Laboratory,
N.D.M.V.P.S. Samaj's College of Pharmacy, Shivajinagar Gangapur Road, Nashik-422 002, India
Fax: (91)(253)2580250; Tel: (91)(235)2375663; E-mail: anilkumar_nasik@yahoo.co.in

The formalin induced paw licking time in the early and late phases of mice were used to assess analgesic activity of methanolic extracts of wood, fruit, leaf and bark of *Casuarina equisetifolia*. The extracts of wood, leaf, fruit and bark have shown potent activity (3.40 ± 0.45, 4.60 ± 0.45, 6.40 ± 1.45 and 8.00 ± 1.45 phase II at the dose of 100 mg/kg). The wood extract (50 and 100 mg/kg) significantly attenuated the writhing responses induced by an intraperitoneal injection of acetic acid and delayed the time of reaction of mice to thermal stimulation produced by the hot plate. The profound activity of wood may be contributed by high quantity of tannic acid (22.97 % w/w), estimated by Folin-Ciocalteu reagent. The results confirmed central and peripheral analgesic activities of wood extract and affirm the claim by Fijian traditional medicine practitioners.

Key Words: *Casuarina equisetifolia*, Analgesic activity, Acetic acid, Formalin, Writhing, Total phenolics, Folin-Ciocalteu method, Tannic acid.

INTRODUCTION

Casuarina equisetifolia (Casuarinaceae) is beautiful tree with droping branches, 10-50 m high[1], found in dry hill sides and open forests of India, Sri Lanka and Australia[2]. The phytoconstituents have been isolated from the plant so far; kaempferol, quercetin[3], alicyclic acids (shikimic acid and quinic acid), amino acids[4], taraxarol, lupenone, lupeol, gallic acid, β-sitosterol[5], catechin and gallocatechin[6,7]. The plant is used as astringent[1], diarrhoea[8], dysentery, cough, ulcers, toothache, lotion for swelling[9] and diabetes[10]. The biological activities, *viz.* anticancer, antibacterial[9], hypoglycemic, antifungal[2] of the leaf has been reported.

Literature review suggests that the analgesic response of the plant has not been pharmacologically studied, attempts were made for the investigations. The per cent phenolic content in various parts of plant was estimated spectrophotometrically by Folin-Ciocalteu method at λ_{max} 765 nm.

†Department of Pharmacognosy, V.N.S. Institute of Pharmacy, Neelbud, Bhopal-462 044, India.
‡Division of Pharmaceutical Sciences, Shri Guru Ram Rai Institute of Technology & Science, Dehradun-248 001, India.

EXPERIMENTAL

Swiss Albino mice of either sex weighing around 18-25 g were procured from National Institute of Toxicology, Pune, India. They were acclimatized to the standard laboratory conditions at the temperature of 25 ± 1 ºC for 5 days. The animals had free access to food and water, maintained under light and dark cycles of 12 h each. All experiments were carried out during day time from 9.00 am to 2.00 pm. The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol and cares of animals were taken as per guidelines of CPCSEA, Department of Animal Welfare, Government of India.

Authentification of plant material: The plant specimen was collected from Gangapur dam locality, Nashik, India authenticated as *Casuarina equisetifolia* Linn family Casuarinaceae, Voucher no. ANA1, Ref. No. BSI /WC/ Tech./2005/867 dated 22.12.2005 by P.S.N. Rao, Joint Director, Botanical Survey of India, Pune, India. The plant materials were dried in vacuum oven to 40 ºC at 160 mm of Hg.

Preparation of plant extracts: Coarsely powdered materials of leaf, bark, wood and fruits (50 g each) were subjected to reflux with 200 mL of methanol for 4 h coded as MEL, MEB, MEW and MEF followed by subsequent filtration and evaporation to yield extracts.

Estimation of phenolic content: *Standard for calibration curve:* Tannic acid (Research Lab., Bombay, India) was purified by the method reported[11]. *Folin-Ciocalteu reagent:* Qualigens phenol reagent (Folin & Ciocateus) Prod. No. 35953, B. No. 47066511-5, Glaxo-Smith Kline Pharmaceuticals Limited, Mumbai-30, India.

Sodium carbonate solution: Sodium carbonate (200 g) was dissolved at 70-80 ºC in and volume was made with distilled water up to 1 L, filtered through glass wool and allowed to stand overnight.

Calibration curve: The stock solutions of extracts 1000 µg/mL and tannic acid (25-200 µg/mL) were prepared in distilled water. The total phenolic content of the extract was measured[12,13] as follows: 1 mL of standard aliquot was taken in 25 mL of volumetric flask, added with 10 mL of water, 1.5 mL of Folin-Ciocalteu reagent, allowed to stand for 10 min. 4 mL of sodium carbonate was added in each volumetric flask, volume was adjusted with distilled water. The mixture was kept for 1 h and the absorbance was recorded at 765 nm by UV spectrophotometer 160 A (Shimadzu, Japan) against reagent blank. Percentage of total phenolics was calculated from the calibration curve of tannic acid and total phenolics were expressed as percentage tannic acid (Fig. 1).

Formalin-induced pain in mice: The analgesic activity of the extracts were quantified[14,15]. Mice (overnight fasted with access to water) were divided into 10 groups (n = 5). Group 1 control, received water (0.1 mL), groups 2-9 received extract (MEL, MEW, MEB and MEF (50 and 100 mg/kg) while group 10 received pentazocine (10 mg/kg) intraperitoneally. After 0.5 h, each animal was injected with 0.1 mL of formalin solution (1 % v/v in distilled water) subcutaneously into the subplanator region of left hind paw. Mouse was placed in open observation

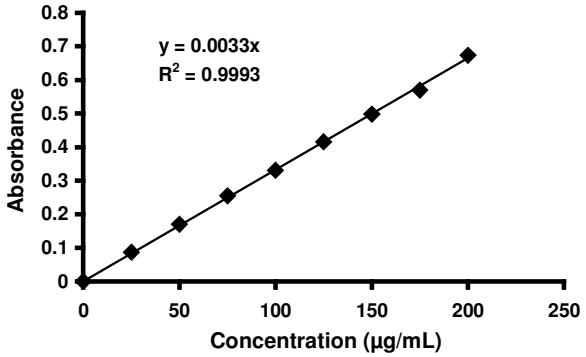

Fig. 1. Calibration curve for tannic acid

chamber immediately for behavioural responses to nociception including biting, licking and scratching of the injected paw, were noted and time was recorded till 1 h. The early and short lasting first phase (0-10 min) was considered as the early phase and the period of 15 to 60 min as the late phase of the nociceptive response.

Hot plate induced pain: In the hot plate method[16], animals were divided into 4 groups (n = 5). Group 1 control (received water, 0.1 mL, i.p.), group II received pentazocine (10 mg/kg, i.p.) served as positive control while group III and IV received MEW (50 and 100 mg/kg, i.p.).

All animals were selected 1 h before the test on the basis of their reactivity for the paw-lick or jump response, eliminating mice that remained on thermostatically controlled the hot plate maintained at 55-56 ºC for up to 15 s. The pain threshold is considered to be reached when the animals lift and lick their paws or attempt to jump out. The latency for paw licking was recorded at 0, 30 and 60 min.

Acetic acid-induced writhing in mice: In acetic acid-induced writhing[16], mice were divided into 4 groups (n = 5). Mice were placed in individual observation boxes for 0.5 h to acclimatize to the new environment before commencement of the experiment. Group 1 control (received water, 0.1 mL, i.p.), group II received pentazocine (10 mg/kg, i.p.) while group III and IV received MEW (50 and 100 mg/kg, i.p.). After 0.5 h, the animals were injected with 0.1 mL of 0.6 % v/v acetic acid, the number of abdominal constrictions was cumulatively counted over a period of 0.5 h.

The writhing effect was indicated by stretching of abdomen with simultaneous stretching of at least one hind limb. The percentage protection was calculated as:

$$\frac{\text{No. of writhing in control animals} - \text{No. of writhing in treated animals)}}{\text{(No. of writhing in control animals)}} \times 100$$

Stastical analysis: All the results were expressed as mean ± standard error. p values < 0.05 were considered as significant. The analgesic activities for above models were analyzed stastically by using one way ANNOVA followed by Dunett's test.

RESULTS AND DISCUSSION

Total phenolic content: The calibration curve for tannic acid was found linear from 2.5 to 200 µg/mL. The correlation coefficient (Table-1) indicates good linearity between concentration and absorbance. The total phenolic content in methanolic extracts were estimated as wood, bark, fruit and leaf (22.97, 14.00, 2.63 and 1.75 %), expressed as % tannic acid (Table-2).

TABLE-1
STATISTICAL PARAMETERS FOR TANNIC ACID

Parameter	Value
Absorption maxima	765 nm
Beer's law limit (µg/mL)	25-200
Regression equation	y = 0.0033x
Intercept (a)	0.0
Slope (b)	0.0033
Correlation coefficient	0.9993

TABLE-2
ESTIMATION OF PHENOLIC CONTENT IN VARIOUS PLANT PARTS

Part used	Extractive value (%)	*Absorbance at 765 nm	Phenolic content in extract (% w/w)
Leaf	17.90	0.058	1.7576
Bark	12.54	0.462	14.000
Fruit	11.88	0.087	2.6364
Wood	04.10	0.758	22.9697

*Averages of three readings.

Formalin test: Formalin administration produced a typical pattern of flinching behaviour. The first phase started immediately after administration of formalin and then diminished gradually in *ca.* 10 min. The second phase started at 15 min and lasted until 1 h. Intraperitoneal administration of methanolic extract of wood, fruit, bark and leaf (50-100 mg/kg) dose-dependently reduced the flinching behaviour in both phases. The degree of reduction was more in late phase than early phase as compared to control. The results signify anti nociceptive effect of plant extract in the doses examined.

The degree of anti nociceptive response produced by the methanolic wood extract (100 mg/kg, i.p.), was more significant in the early phase and the late phase (3.80 ± 0.26# and 3.40 ± 0.45#, $p < 0.001$) compared with pentazocine (24.60 ± 2.45* and 3.60 ± 2.5*, $p < 0.05$) at the dose of 10 mg/kg, i.p. The plant extract at the dose of 50 mg/kg significantly reduced both phases (8.20 ± 1.23 # and 4.20 ± 0.96 #) as compared to control group (30.00 ± 2.12 and10.00 ± 1.26) (Table-3).

Acetic acid induced writhing: The methanolic extract of wood (50 mg/kg, i.p.) showed significant activity with percent protection of 21.43 %. At the dose of 100 mg/kg, i.p., produced comparable protection (42.86 %) with pentazocine, 10 mg/kg, i.p., (50.00 %, $p < 0.01$) (Table-4).

TABLE-3
SCREENING OF VARIOUS PLANT FRACTIONS FOR FORMALIN-INDUCED PAIN IN MICE

Phase	Vehicle	Treatment (mg/kg)								
		Pentazocine	Fruit		Leaf		Wood		Bark	
		10	50	100	50	100	50	100	50	100
I	30.00 ± 2.12	24.60 ± 2.45	18.20 ± 2.47*	14.80 ± 2.13*	19.20 ± 3.16*	10.80 ± 1.89*	8.20 ± 1.23*	3.80 ± 0.26*	16.20 ± 2.56*	11.80 ± 1.89*
II	10.00 ± 1.26	3.60 ± 2.5#	7.80 ± 1.79	4.60 ± 0.45	7.80 ± 1.96	6.40 ± 1.45	4.20 ± 0.96	3.40 ± 0.45#	9.80 ± 1.96	8.00 ± 1.45

n = 5, *p < 0.001, #p < 0.05, One-way ANOVA followed by Dunnett's test.

TABLE-4
EFFECT OF MEW ON ACETIC ACID-INDUCED WRITHING IN MICE

Treatment	Number of writhings (Mean ± SEM)	Percentage protection
Vehicle (water-0.1 mL)	28 ± 0.12	–
MEW (50 mg/kg, i.p.)	22 ± 0.14*	21.42
MEW (100 mg/kg, i.p.)	16 ± 0.19*	42.86
Pentazocine (10 mg/kg, i.p.)	14 ± 0.11*	50.00

n = 5, *p < 0.01, One way ANOVA followed by Dunnett's test.

Hot plate test: The methanolic extract of wood produced a dose dependent antinociception at doses of 50 and 100 mg/kg, i.p. The latency was significantly ($p < 0.05$) potentiated at 0.5 and 1.0 h in MEW (100 mg/kg, i.p.) treated group (17.80 ± 2.48* and 14.20 ± 2.65, *$p < 0.05$) compared to vehicle treated group (6.80 ± 1.95 and 5.00 ± 0.54). Pentazocine (10 mg/kg, i.p.) a standard analgesic drug, alone significantly potentiated antinociception (18.20 ± 3.12# and15.40 ± 3.26#, $p < 0.05$) (Table-5).

TABLE-5
EFFECT OF MEW ON PAIN INDUCED BY HOT PLATE ANALGESIOMETER IN MICE

Treatment	Latency for paw licking (second) (Mean ± SEM)		
	0 min	30 min	60 min
Vehicle (water-0.1 mL)	5.20 ± 1.21	6.80 ± 1.95	5.00 ± 0.54
MEW (50 mg/kg, i.p.)	6.40 ± 1.54	10.60 ± 2.65	9.20 ± 1.12
MEW (100 mg/kg, i.p.)	4.40 ± 0.97	17.80 ± 2.48*	14.20 ± 2.65*
Pentazocine (10 mg/kg, i.p.)	5.80 ± 0.54	18.20 ± 3.12*	15.40 ± 3.26*

n = 5, *p < 0.05, One-way ANOVA followed by Dunnett's test.

The results showed that the methanolic wood extract exerts significant inhibitory effect on nociceptive response in both phases of formalin test as compare to bark, fruit and leaf extract. The second phase of formalin test results from an inflammatory reaction in the peripheral tissue, attenuating the associated inflammation, may be due to activation of opiate receptors and nitric oxide release[17]. The wood extract (100 mg/kg, i.p.) has resulted in significant analgesic action examined in chemical

(formalin and acetic acid induced writhing) and thermal (hot plate test) models in a dose dependent manner comparable to pentazocine. The writhing effect induced by chemical substances is due to sensitization of nociceptors by prostaglandins. The abdominal constriction response induced by acetic acid is thought to involve local peritoneal receptors to establish peripherally acting analgesic drug[18]. The antinociceptive effect in 2nd phase suggested in the treatment of acute inflammatory pain, suppressing the formation of pain inducing substances in the peripheral tissues, prostaglandins and bradykinin[19].

In conclusion, the data obtained in this study demonstrated good central and peripheral analgesic properties of wood extract, may be due to presence of highest amount of phenolic compounds (29.97 % w/w).

ACKNOWLEDGEMENT

The authors are thankful to the Prof. Dr. V.R. Gudsoorkar, Principal, NDMVPS College of Pharmacy, Nashik for providing necessary facilities.

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(*Received*: 26 May 2009; *Accepted*: 16 January 2010) AJC-8301