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Pharmacological Screening of *Wrightia tinctoria* Bark Hydro-Alcoholic Extract

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Abstract : The objective of the present study was to investigate the pharmacological profile of hydro-alcoholic extract of *Wrightia tinctoria* (Roxb) R. Br. Linn., (Family- Apocynaceae) in mice and rats using various models. The effects of the extract were observed in three different dose levels 300, 500 & 1000 mg/kg as extract does not show any sign of toxicity up to 3000 mg/kg dose. Investigations were carried out against thermal, chemical and mechanical noxious stimuli to study antinociceptive activity and on pentobarbitone induce hypnosis. Carrageenan-induced paw edema and cotton pellet induced granuloma model were employed to test anti-inflammatory activity. The parameters taken for diuretic activity was urine volume and renal excretion of Na^+, Cl– and K^+ ions.

Study revealed moderate analgesic effect against thermal (P<0.001 to 0.01) and chemical ($p < 0.05$) noxious stimuli and anti-inflammatory activity (P<0.001 to 0.01) at the 1000 mg/kg dose. Extract is devoid of any sedative activity. *W. tinctoria* extract considerably increases urine volume, acting as strong kaliuretic.

Key words: *Wrightia tinctoria*, Antinociceptive, Antiinflammatory, Hypnosis, Diuretic, Kaliuretic.

Introduction

Wrightia tinctoria (Roxb.) R.Br. is a small deciduous tree of the family Apocynaceae distributed in Central India, Burma and Timor (Chary, 1980). This plant is extensively used in the Indian system of medicine. Fresh leaves are pungent and are chewed for relief from toothache (Kirtikar and Basu, 1975; Anonymous, 1976). Bark and seeds are antidysenteric, carminative, astringent, aphrodisiac and diuretic, used in flatulence, stomach pain and bilious affections. The plant is very useful as stomachic, in the treatment of abdominal pain, skin diseases, antidiarrhoeal and antihaemorrhagic (Nadkarni, 1976; Shah and Gopal, 1988; Singh *et al*., 1980). Oil emulsion of *W. tinctoria* pods is used to treat psoriasis and also have fungicidal activity against *Pityrosporum ovale* recovered from dandruff (Mitra *et al*., 1998; Krishnamoorthy and Ranganathan, 2000; Reddy *et al*., 2000). *W tinctoria* is commonly used as adulterant of an important antidysentric drug *Holarrhena antidysentrica* another apocynaceae plant (Chopra *et al*., 1958). The therapeutic properties of *W. tinctoria* are

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similar to that of *H. antidysentrica*, which contains several steroidal alkaloids (Glasby, 1975; Glassman, 1971). Ethyl acetate, acetone and methanol extracts of *W. tinctoria* bark showed antinociceptive activity in mice (Chopra *et al*., 1956). *W. tinctoria* bark ethanolic extract showed immunomodulatory and good antiulcer activity against experimentally induced acute gastric ulcers on rat (Bigoniya *et al*., 2006).

W. tinctoria have been widely used to treat a number of ailments in traditional system of medicine in north and central India. Sufficient scientific data is not available to support these above said claims. Based on literature information the present study was undertaken with the aim to confirm its uses in folklore medicine as analgesic, anti-inflammatory and diuretic.

Material and Methods

Collection and identification of plant material

W. tinctoria bark was collected from Hoshangabad district of Madhya Pradesh, India in the month of Sept-Nov. 2003. The plants were identified with the help of available literature and authenticated by Dr. A. P., Shrivastava, Principal, P. K. S. Govt. Ayurveda College and Institute, Bhopal, India. A voucher specimen was deposited in the herbarium department (*W. tinctoria*; No. 1084).

Preparation of hydro-alcoholic extract

Ethanolic (70%) extract of dried, milled coarse bark powder was prepared by cold maceration. The extract was filtered through muslin cloth and evaporated at 40°C up to one third of initial volume, remaining solvent was completely evaporated using a rotary vacuum evaporator (Superfit, India). The extract was then weighed and percentage yield calculated. The color and consistency of the extract was noted and subjected to different tests to detect the presence of various phytoconstituents.

Materials

Pentobarbitone sodium and carrageenan were obtained from Sigma Chemical, USA. The drugs used in the study were gift samples from the following sources - Morphin (Pharma Chemico Lab., Solan), aspirin (Alpa Lab., Indore), Diazepam (Ranbaxy, Dewas) and frusemide (Lupin Lab., Mandideep). All the other chemicals were of analytical grade.

Test Animals

Laboratory bred Wistar albino rats (150 – 200 gm) and Albino mice (20-25 gm) of either sex were maintained under standard laboratory conditions at 22±2°C, relative humidity 50±15% and photoperiod (12 h dark and light), were used for the experiment. Commercial pellet diet (Hindustan Lever, India) and water were provided *ad libitum*. Institutional Animal Ethical Committee approval was obtained before carrying out the experiments.

Dried crude extract was freshly suspended in 2% (w/v) carboxy methyl cellulose (CMC) prepared in distilled water and used as vehicle control. Based on the OECD guidelines a **Limit test** was performed to categorize the toxicity class (LD_{50}) of the compound (Diener *et al*., 1995; Roll *et al*., 1986). **Limit test** was performed at 2000 mg/kg, p.o and repeated at 5000 mg/kg in which it does not show mortality in rats. LD_{50} is greater than 5000 mg/kg. A dose range of 300 mg/kg, 500 mg/kg and 1000 mg/kg were selected for evaluation of pharmacological activity. For all the studies overnight fasted animals of either sex were divided randomly six per group.

Analgesic activity Study

Effect of extracts against thermal stimulus (Eddy's hot plate method)

Morphin (5 mg/kg, s.c) was used as standard drug. The initial screening was done by selecting rats, which showed reaction time of 3-5 sec. Analgesia test was carried out by placing a rat on Eddy's hot plate (Techno, India) at 55±0.5°C for a maximum period of 30 sec.

and noting the basal reaction time i.e. licking front paws or making an effort to jump out of the chamber (Wu *et al.*, 2003). Increase in reaction time after drug administration against basal reaction time was noted in sec. Cut-off time of 30 sec. was selected to avoid tissue damage. The percentage of pain inhibition after 60 minutes of drug treatment were calculated according to the following formula (Rumana and Vorora, 2000).

Pain inhibition percentage (PIP) = $(T_1 - T_0) / T_0 \times 100$. T_1 = Post drug latency (reaction time after drug treatment) and T_0 = Pre drug latency (basal reaction time).

Effect of extracts against thermal stimulus (Tail flick method)

Analgesia test was carried out by placing the tail tip (last 1-2 cm) of rat on radiant heat of the Tail Flick Analgesiometer (Techno, India) heated at 55±0.5°C for a maximum period of 15 sec and noting the basal reaction time (flicking of tail). The initial screening was done by selecting rats, which showed reaction time of 3-5 sec (Wu *et al.*, 2003). Increase in tail flick response after drug treatment was noted and percentage increase in reaction time calculated. The observations were made 45 min after drugs administration.

Effect of extracts against mechanical stimulus

45 min after administration of extracts and standard drug (morphin, 5 mg/kg, s.c) an arterial clip (with jaws covered by a rubber tubing) was applied to the tail base of all the rats. Compression by the tail clip induces physical pressure and act as a mechanical stimulus. Attempt to dislodge the clip (biting or kicking etc.) in <15 sec were considered as positive response (Witkin *et al.*, 1961). Failure of such response in treated animals was taken as criteria for analgesia. Basal reaction time and reaction time after drug administration was noted and percentage increase in reaction time calculated.

Effect of extracts against chemical stimulus

The analgesic activity of extracts on acetic acid-induced writhing was screened in mice following method of Witkin *et al.* (1961). All the drugs were given orally in the form of suspension. After 30 minutes of administration of extracts or standard drug (aspirin, 100 mg/kg, p.o) all groups of mice were given the writhing agent, 3% aqueous acetic acid in a dose of 2 ml/kg body weight intraperitoneally. Writhing is defined as a stretch, torson or constriction of abdomen and extension or drawing up of a hind leg etc. The writhing episodes produced for 10 minutes were counted and percentage protection was calculated as shown below.

Percentage protection = (100 - no. of wriths in test / no. of wriths in control) × 100

Effect on pentobarbitone-induced hypnosis in mice

Pentobarbital (45 mg/kg, i.p.) was administered to control and extract treated animals. Onset of sleep (loss of righting reflux) was noted and duration of sleep measured, which is the period between loss of righting reflux and its revival (Harris and Spencer, 1962). Diazepam (2 mg/kg, i.p) and extract at different doses were given subsequently 30 min and 45 min prior to pentobarbitone injection.

Anti-inflammatory activity study

Against carrageenan-induced rat hind paw edema

One hour after the administration of vehicle, different doses of extract and standard drug aspirin (100 mg/kg, p.o), inflammation was produced by injecting 0.1 ml of 1% w/v carrageenan solution in normal saline beneath the sub-plantar surface of right hind paw of all the animals. For assessment of anti-inflammaroty activity, the volume of the paw before and three h after carrageenan treatment was measured by mercury displacement

technique using plethysmometer. Percentage inhibition in paw edema or inflammation after 3 h was calculated (Winter *et al*., 1962).

Against cotton pellet induced granuloma

The rats were anaesthetized with pentobarbitone sodium (30 mg/kg) after shaving off the fur on the back. Sterilized pre weighed cotton pellets were implanted in both axillae and groin regions through a single midline incision on the dorsal surface according to the method of D'Arcy *et al*. (1960). Drugs were administered 3 h after implantation and continued for seven days. On the 8th day the pellets were dissected out, dried at 60°C the dry weights were determined. The difference between the initial and final weight of cotton pellet was considered to be the weight of granulomatous tissue produced.

Effect of extracts on urine output along with electrolyte concentration

Animals were deprived of food and water for 16 hours. All the rats received priming dose of normal saline 25 ml/kg orally. Immediately after administration of vehicle, different doses of extract and standard drug frusemide (5 mg/kg, p.o) all the rats were placed in metabolic cages (group wise) specially designed to separate urine and faeces at room temperature of 25±0.5°C (Leander, 1983). The urine was collected in measuring cylinder up to 5 h after drug administration. During this period no food and water was made available to animals. Collection time for first drop of urine and total volume of urine collected from both control and treated groups were measured. Concentration of Na^+ and K^+ in urine was measured by Flame photometer (Elico, India). Chloride ion concentration was estimated by titration with silver nitrate solution (N/50) using 3 drops of potassium chromate solution as indicator (Jeffery *et al*., 1989).

Statistical Analysis

Experimental data were analyzed using one way ANOVA followed by Turkey-Kramer multiple comparison test. P value less than 0.05 were considered statistically significant. Graph Pad Prism Version 3.02 was used for statistical calculations.

Results

Qualitative phytochemical investigation of crude plant extract (dark brown in colour, yield 19.145% w/w) revealed the presence of steroidal saponin, alkaloid, reducing sugar, tannins, flavonoids and absence of glycoside.

In Eddy's Hot Plate reaction *W. tinctoria* extract in a dose of 1000 mg/kg body weight showed a highly significant (P<0.001) prolongation of the reaction time in hot plate test upto two hours compared to its pre-treatment values. The results presented in table. I demonstrates that the pain inhibition percentage of extract is 101.12 (1000 mg/kg) compared to 496.42 of morphine at 1st h after drug administration.

Table. II depict the effect on tail flick algesia. *W. tinctoria* extract at 300 and 500 mg/kg dose showed significant (P<0.01) analgesic activity where as at 1000 mg/kg dose analgesic activity was highly significant (P<0.001) and percentage increase in reaction time is 159.32 compared to 490.55 of morphine. There was no significant alteration in the tail flick reaction time in any of the extract treated rats.

Antinociceptive effects of *W. tinctoria* extract on the abdominal writhes of mice induced by acetic acid are summarized in table. III. The ethanolic extract of *W. tinctoria* significantly (P<0.05) increased the pain threshold in rats at 1000 mg/kg dose and demonstrated inhibition of the abdominal writhes by 43.86% as compared to 66.19% of morphine in relation to the control value. The extract did not have any effect over pentobarbitone-induced hypnosis at any tested dose level.

Table 1 : Effect of *W. tinctoria* extract on eddy's hot plate reaction time on rats

Treatment (mg/ kg) p.o	Mean basal reaction time in sec. (M ± SEM)	Average reaction time in sec. (M ±				Pain inhibition % (PIP) at 60 min.
		30 min.	60 min.	90 min.	120 min.	
Morphin (5), s.c	5.03 ± 0.04	20.75 ± 1.48	30.00*** ± 1.06	28.12 ± 1.37	24.60 ± 2.57	496.42
W. t extract (300)	4.92 ± 0.23	5.66 ± 0.41	7.16ns ± 1.05	6.26 ± 1.32	5.08 ± 0.69	45.52
W. t extract (500)	5.66 ± 0.56	7.25 ± 1.04	9.08* ± 1.34	8.24 ± 1.62	7.12 ± 1.18	60.42
W. t extract (1000)	5.33 ± 0.37	7.34 ± 1.52	10.72*** ± 1.63	9.02 ± 1.67	8.42 ± 1.42	101.12

*n = 6 per group. ***P<0.001, *P<0.05 and ns = not significant as compared to respective mean basal reaction time.*

Table 2 : Effect of *W. tinctoria* extract on tail flick latency in rats

Treatment (mg/ kg) p.o	Basal reaction time in sec. (M ± SEM)	Reaction time in sec. after 45 min. (M ± SEM)	% Increase in reaction time
Morphin (5), s.c	2.54 ± 0.02	15.00 ± 0.97***	490.55
W. t extract (300)	2.98 ± 0.14	3.86 ± 0.23**	29.53
W. t extract (500)	2.70 ± 0.05	5.75 ± 0.75**	112.96
W. t extract (1000)	3.22 ± 0.09	8.35 ± 1.02***	159.32

*n = 6 per group. ***P<0.001, **P<0.01 and ns = not significant as compared to respective mean basal reaction time.*

Effect of extract treatment on carrageenan-induced edema is shown in figure. I. The edema suppressant effect of was significant (P<0.05) at 500 mg/kg and highly significant (P<0.001) at 1000 mg/kg dose compared to control. The percentage edema volume inhibition of 1000 mg/kg dose of extract is 69.47 compared to 75.78 of aspirin.

Figure. II showed the effect of drug treatment on the mean weight of cotton pellet. *W. tinctoria* extract at 1000 mg/kg dose inhibited the granuloma tissue formation significantly (P<0.01) and shows 46.36% inhibition of granuloma tissue weight where as diclofenac produces 60.58% inhibition compared to vehicle control.

The mean basal urine output was 6.65 ± 0.96 ml over 5 h. Frusemide 5 mg/kg induced a brisk and significant diuresis within 15 min of administration. *W. tinctoria* extract produced dose dependent diuretic activity, with increasing dose onset of diuresis and total volume of urine formed was increased. The electrolyte changes induced by the standard drug, vehicle and different doses of extract are shown in table. IV. Frusemide caused the expected increase in the renal excretion of Na^+, Cl– and K^+ ions. The extract induced a highly significant increase in urine Na^+ (0.01), Cl– and K^+ (0.001) ion loss. Extract at all the tested dose levels produce highly significant (P <0.001) loss of urine K^+ ion as a result with increasing dose urine Na^+/K^+ ratio was decreased to 0.62 (1000 mg/kg) compared to 1.91 of control. The effect of extract on sodium excretion is comparatively less then that of potassium. Extract at 1000 mg/kg dose

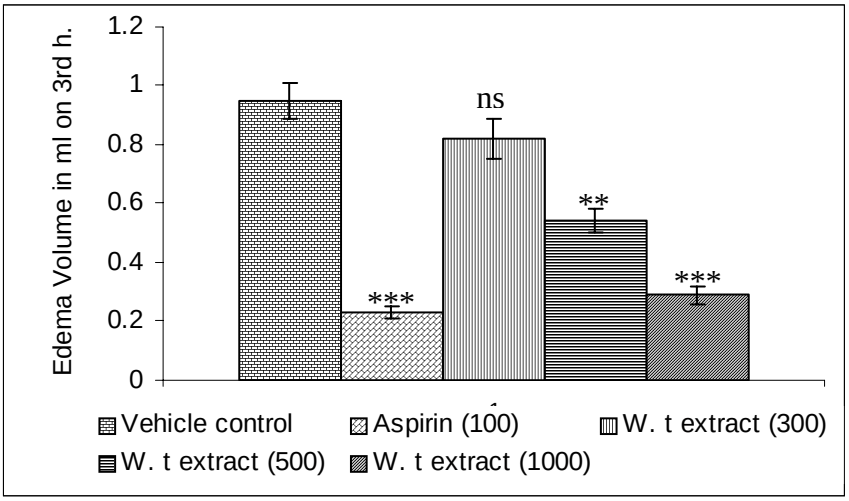

Fig. 1 : Effect of *W. tinctoria* extract on carrageenan-induced paw edema in rats. n = 6 per group. **P<0.01, ***P<0.001 and ns = not significant as compared to control values. Aspirin and W.t extract (1000mg/kg) treatment showed 75.78% and 69.47% inhibition of edema volume respectively compared to vehicle control group.

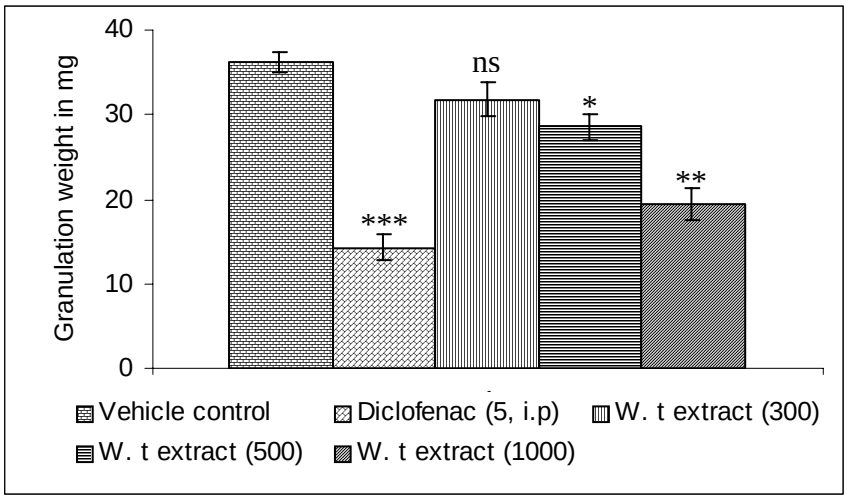

Fig. 2 : Effect of W. tinctoria extract on cotton pellet induced granuloma in rats. n = 6 per group. *P<0.05, **P<0.01, ***P<0.001 and ns = not significant as compared to control values. Diclofenac and W.t extract (1000mg/kg) treatment showed 60.58% and 46.36% inhibition in respectively compared to vehicle control group.

Table 3 : Effect of *W. tinctoria* extract on acetic acid induced writhes in rats

Treatment (mg/ kg) p.o	Writhing in 10 min. (M ± SEM)	% Protection
Vehicle control (0.5ml/100gm)	22.57 ± 2.89	--
Aspirin (100)	7.63 ± 1.79***	66.19
W. t extract (300)	21.32 ± 3.54^{ns}	5.54
W. t extract (500)	19.16 ± 2.36^{ns}	15.11
W. t extract (1000)	12.67 ± 1.76*	43.86

*n = 6 per group. *P<0.05, ***P<0.001 ns = not significant as compared to control value.*

Table 4 : Effect of W. tinctoria bark extract on urine output and electrolyte excretion parameters of rats

Treatment (mg/kg) p.o	Urine collection starts (min.)	Total volume of urine on 5th h.	Electrolyte excretion (M ± SEM)				
			Na^+ (mEq/L)	K^+ (mEq/L)	Cl (mEq/L)	Na^+/K^+ ratio	% increase in Na^+ excretion
Vehicle control	12.5 ± 1.03	6.65 ± 0.96	189.25 ± 12.72	99.08 ± 12.33	195.31 ± 6.08	1.91	--
Frusemide (5)	2.8 ± 0.12***	15.72 ± 1.25***	310.53 ± 14.64***	564.85± 12.76***	360.05 ± 10.57***	0.55	64.08
W.t extract (300)	7.4 ± 1.13**	10.84 ± 1.12^{ns}	191.62 ± 12.86^{ns}	227.36 ± 13.49***	209.42 ± 12.80^{ns}	0.84	1.25
W.t extract (500)	6.2 ± 0.97***	11.95 ± 1.10*	225.06 ± 13.43^{ns}	312.21 ± 19.09***	261.16 ± 14.02**	0.72	18.92
W.t extract (1000)	5.6 ± 0.56***	13.28 ± 1.73**	269.54 ± 8.07**	434.55 ± 8.20***	310.57 ± 14.74***	0.62	42.43

*n = 6 in each group. *P<0.05, **P<0.01, ***P<0.001 and ns = not significant when compared to control group.*

produces 42.43% increase in Na^+ excretion relatively against 64.08% of frusemide when compared to control. *W. tinctoria* extract has significant kaliuretic activity.

Discussion

The pharmacological activities of the *W. tinctoria* bark ethanolic (70%) extract was evaluated in the study. The results show that the ethanolic bark extract of *W. tinctoria* has antinociceptive effects in normal rats when given orally. The antinociceptie effect of the extract was dose-dependent. Study revealed moderate analgesic effect against thermal and chemical noxious stimuli, but such action was not observed against the mechanical stimulus at the doses used. The hot plate model is commonly used to assess analgesic activity of narcotic analgesics, other drugs such as sedative and hypnotics or phychomimetic drugs which act centrally (Vaz *et al*., 1996). The hot plate test is considered to be selective for opioids like compounds in several animal species (Janssen *et al*., 1963). The extract

shows analgesic activity in hot plate test may be due to presence of steroids in the extract. The antinociceptive activity of *W. tinctoria* was found only when assessed using the hot plate and tail flick but not on the tail clip technique of nociception. This indicates that the extract is effective against acute phasic pain and the antinociceptive effect is mediated centrally (Wong *et al*., 1994).

The abdominal writhing elicited by acetic acid has been reported to be a very sensitive and less selective model that enables the detection of antinociceptive activity of compounds in laboratory animals. Collier *et al*. (1968) proposed that acetic acid acts indirectly by releasing endogenous mediators, which stimulate neurons that are sensitive to other drugs such as narcotics and centrally acting agents. The abdominal constriction response is thought to involve local peritoneal response. Several studies demonstrated that steroids produce antinociception when assessed in several chemical models of nociception in animals (Santos *et al*., 1995).

The fact that many neurosedative drugs tend decrease sleep latency and increase sleeping time led us to assay the effect of extract on sleeping time induced by pentobarbital sodium. *W. tinctoria* extract was employed in a pentobarbital-induced sleeping time study in order to test if it exhibits sedative or hypnotic effects. *W. tinctoria* bark ethanolic extract does not have any effect on the sleeping latency or sleeping time induced by pentobarbital administration. These results confirm that the antinociceptive effect of extract against thermal and chemical stimuli is not due to sedative property, rather indicating extract induced analgesia is genuine.

The edema development method is biphasic, the initial phase is due to release of histamine, serotonin and kinins in the first few hours after injection of carrageenan (Mujumdar *et al*., 2000). The more pronounced second phase occurs in 2-3 hours due to release of prostaglandin like substances. Carrageenan-induced paw edema model is prototype of exudative phase inflammation. Granuloma develops during several days in cotton pellet induced granuloma model. This method indicates the proliferative phase of inflammation, involving macrophases, neutrophils and fibroblasts proliferation. The significant anti-inflammatory activity of *W. tinctoria* extract may be due to flavonoids or alkaloids by exerting predominant inhibition of inflammatory mediators from phlogogenic stimuli. Extract moderately decreases granulation tissue weight indicating suppression of proliferation phase of inflammation. The association of both analgesic and anti-inflammatory effects is well documented for various non-steriodal anti-inflammatory agents.

Diuresis has two different connotations, increase in urine volume per se and net loss of solute and water involving suppression of renal tubular reabsorption of electrolytes, water and low molecular weight organic substances into blood stream and consequently promoting the urine formation (De'Stevens, 1963). *W. tinctoria* extract considerably increases urine volume, acting as strong kaliuretic. The 5 h cumulative urine output induced by the extract (1000 mg/kg) and the standard drug were highly statistically significant compared to control. Frusemide induced a brisk and significant diuresis with in 15 min of administration by increasing both Na^+ and Cl^-. The diuretic effect of extract was significant after 2 h of administration may be due to delay in the onset of diuresis as a result of poor absorption of the active principles in the crude preparation of the extract. The data presented in the study indicate that *W. tinctoria* ethanolic extract contained compounds that mediated diuretic effects by increasing the rate of urine output as well as electrolyte excretion.

Therefore, as envisaged by us the use of *W. tinctoria* species particularly the bark in folklore medicine may be due to its symptomatic relief of pain as validated by us.

However the mechanisms of action of the extract are uncertain but the results suggests that the bark extract have moderate analgesic and anti-inflammatory as well as potent diuretic activity at 1000 mg/kg dose. These finding suggest that isolation of the active constituent could be potentially useful for the development of antinociceptives useful in the management of pain.

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