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Phytochemical Screening and Pre-Clinical Evaluation of Analgesic, Anti-Inflammatory and Antipyretic Potentials of the Ethanolic Extract of Wedelia Trilobata

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ABSTRACT

Plants have been used as a source of natural products for various therapeutic purposes for a long time. Focus on plant research has increased all over the world due to easy accessibility and less adverse effect with much more contribution in various traditional systems of medicines. Wedelia trilobata (WT) has been used in folk medicine to alleviate several diseases such as cough, colds, flu, fevers and inflammations. The present study was investigated to evaluate the analgesic, anti-inflammatory and antipyretic effect of Wedelia trilobata. Swiss albino mice of either sex weighing 25-30 gm were used in this study. Analgesic activity was evaluated by using hot plate test, acetic acid induced writhing test and formalin induced paw licking test. Anti-inflammatory effect was assessed using cotton pellet and xylene test and carrageenan induced paw edema test. The antipyretic effect was investigated using yeast induced pyrexia test. The extract was used at 1000 and 2000 mg.kg body weight. The WT ethanolic extract showed significant effect against pain in acetic acid ($p<0.01$) and formalin test ($p<0.001$) and insignificantly in hot plate test. Inflammation was reduced by WT extract in cotton pellet test ($p<0.001$), xylene test ($p<0.01$) and significantly reduced the paw edema ($p<0.001$) in carrageenan induced paw edema test. Treatment with WT at both the doses reduced pyrexia very highly significantly ($p<0.001$) in yeast induced antipyretic test. Results from the studies suggested that the WT extract showed potent analgesic, anti-inflammatory and antipyretic effects in various animal models.

Keywords: Wedelia trilobata, Analgesic effect, Anti-inflammatory effect, Yeast induced pyrexia.

INTRODUCTION

Analgesia is a survival mechanism that serves as a warning sign of ongoing or impending tissue damage. Pain is an unpleasant sensory and produced by the excitation of particular receptors.Inflammation or phlogosis is a pathophysiological response of living tissue to injuries that leads to the local accumulation of plasmatic fluid and blood cells. Although it is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can be induced, maintain or aggravate many diseases. However, studies have been continuing on inflammatory diseases and the side effects of the currently available anti-inflammatory drugs pose a major problem during their clinical use.Therefore, development of newer and more powerful anti-inflammatory drugs with lesser side effects is necessary.

Natural products especially which are derived from plants have been used as a source for various therapeutic processes for long times from the human civilization. The most common strategy of drug development from plants is careful observation of use of natural resources in flock medicine in different cultures by ethnopharmacology. Plant secondary metabolites play an important role in health care for about 80% of the world's population. Approximately, half of the world's 25 best-selling pharmaceutical agents are derived from natural products (Baker et al. 1995). Thus,

emphasis is now given on the standardization of herbal medication active principles from them.

Wedelia trilobata of Asteraceae family is commonly known as Mohavringaraj or Vringaraj in Bengali, is native to the South America, also distributed to Central America, Mexico and the West Indies but is also widely found in Bangladesh, India, China, Hong-Kong, Malaysia and Indonesia. In Bangladesh it is widely grows in Chittagong, Dhaka, Mymensingh, Patuakhali, Tangail and Nijum Deep (Invasive Species Compendium 2015; Weeds of Australia 2011). W. trilobata is a vigorous perennial herb capable of forming a continuous herbaceous ground cover. Flowering takes place year round. It produces the most flowers in open, frost-free locations (Invasive Species Compendium, 2015). The whole plant of W. trilobata revealed the presence of steroids, triterpenoids, glycosides, tannins, alkaloids, saponins, phenols, carbohydrates, ent-kaurane diterpenes, sesquiterpene lactones and triterpenes (Wu and Zhang 2008). Traditionally the plant is used in muscle cramp, rheumatism, stubborn wounds, common cold, cough, hepatitis, indigestion, infections, tumor, CNS problem, headache, inflammations and fever (Invasive Species Compendium, 2015; Ghosh 2014; Wu and Zhang 2008).

In order to determine the potential use of herbal medicine, it is important to emphasize the study of medicinal plants that was found in folklore. With these backgrounds of the uses of W. trilobata, the experiments were conducted to evaluate analgesic and anti-inflammatory activity of the plant extract.

MATERIALS AND METHODS

Plant collection and Extraction

Wedelia trilobata was collected, identified and authenticated by the department of Botany, Jahangirnagar University, Savar, Dhaka. The collected materials were thoroughly washed in water, cut into smaller parts and shed dried at 35–40ºC for a week and pulverized in electric grinder to get coarse powder. Then powders were extracted with ethanol. Finally, a solid mass was obtained and preserved in a Petridis in the refrigerator for further analysis.

Experimental animals

For the experiment female Swiss albino mice, 6-7 weeks of age, weighing between 25-30g, were collected from the animal research lab in the Department of Pharmacy, Jahangirnagar University, Savar, Dhaka. Animals were maintained under standard environmental conditions (temperature: 27.0±1.0°, relative humidity: 55-65% and 12 h light/12 h dark cycle) and had free access to feed and water ad libitum. The animals were acclimatized to laboratory condition for one week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

Toxicity studies

by screening of biological activities of medicinal plants and isolating

Toxicity studies of the extracts were carried out in Swiss Albino mice of either sex weighing between 20 and 25 g. No mortality was found till 5000 mg/kg p.o.

Analgesic activity evaluation

Hot plate test

The hot plate test was used to measure response latency according to the method described by Eddy and Leimbach (1953) with slight modification. The temperature of the hot plate (model 7280; Ugo Basile, Italy) was maintained at 55 ± 2°C. Animals were placed in a Perspex cylinder on a heated surface, and the time between placement of the animal on the hot plate and the occurrence of discomfort, indicated by either licking of the paws or jumping off the surface, was recorded as response latency. The latency of discomfort was measured at 0, 30, 60, 120, and 180 min after test solution administration.

Acetic acid induced writhing test

The method according to Koster et al. (1959) was employed for this test. Forty five minutes later of administration time each mouse was injected with 0.7% acetic acid at a dose of 10 mL/kg body weight. The number of writhing responses was recorded for each animal during a subsequent 5 min period after 15 min of the I.P. administration of Acetic acid and the mean abdominal writhes for each group was obtained.

Formalin-induced Paw licking test

The method of Hunskaar and Hole (1987) was used for the study. After 1 hour of drug administration, 2.7% formalin was injected into the dorsal surface of the left hind paw. Time spent licking the injected paw was recorded. Animals were observed for the 5 min post formalin (acute phase) and for 5 min starting at 20th min post formalin (delayed phase)

Anti-inflammatory activity evaluation

Cotton pellet-induced granuloma formation test

The method of Swingle and Shideman (1972) was used. Sterilized cotton pellets of 10±1 mg of each weight were impregnated subcutaneously, one on each side of the abdomen of the animal, under light chloroform anesthesia and sterile technique. Test drugs were administered orally to male mice weighing 25-30g in once-daily dose regimen for 7 days; the control group received vehicle only. The mice were sacrificed at the 8th day & removed cotton pellets & dried at 60ºc for 24 hrs & weighed dry cotton weight (Swingle and Shideman 1972). The lowering of the weight of cotton pellet indicates the inhibition of inflammation.

Xylene-Induced Ear Edema test

The Xylene-induced ear edema test was performed as described in

Dai et al. (1995). One hour later of administration time, each animal received 20µl of xylene on the anterior and posterior surfaces of the right ear lobe. One hour after Xylene application circular sections were taken and weighed. The percentage of ear edema was calculated as inflammation based on the weight of left ear without xylene.

Carrageenan Induced paw edema test

The test was used to determine the anti-inflammatory activity of the extract by the method of Winter et al. (1962). The animals pretreated with 1% acacia (as vehicle), Indomethacin and WT extract one hour before were injected with 0.1 ml of 1% carrageenan (in 0.9% saline) solution into the sub planter region of right hind paw of each rat. Paw volume was measured by dislocation of the water column in a Plethysmometer (Ugo Basile, Model No. 7141, Italy) immediately after carrageenan application at 0, 1, 2, 3 and 4 hour(s) after the stimulus. Reduction in the paw volume compared to the vehicle-treated control animals was considered as anti-inflammatory response.

Antipyretic activity evaluation

The antipyretic study was done by using the brewer's yeast induced pyrexia model in rats (Loux et al. 1972). Fever was induced by injecting 20 ml/kg body weight of 20 % suspension of brewer's yeast subcutaneously. In our set up the rats developed fever after 10 hours of yeast injection. Only those animals which developed fever were taken for further study and rest were rejected. Both the standard and test drugs were given intra-peritoneally after development of initial pyrexia and the volume of injection were kept constant at 0.5 ml/rat. Paracetamol 100 mg/kg body weight was taken as standard drug for comparison. FI was given at the doses of 500 mg and 1000 mg/kg body weight intra-peritoneally to different sets of rats. The rectal temperatures were recorded 15 min, 30 min, 1 hour, 2 hours, 3 hours, 4 hours after the drug treatment.

Phytochemical Screening Tests

Phytochemical screening tests are given below (Table 1).

Constituents	Test	Procedure	Observation
Carbohydrates	Molisch's test (General test for Carbohydrates)	Two drops of Molisch's reagent (10% alcoholic solution of α-naphthol) were added to 2ml of aqueous extract. Allow 2ml of conc. sulfuric acid to flow down the side of the inclined test tube so that the acid forms a layer beneath the aqueous solution.	A red or reddish violet ring is formed at the junction of the two layers if a carbohydrate is present. On standing or shaking a dark purple solution is formed.
	Barfoed's test (General test for Monosaccharides)	1 ml of an aqueous extract of the plant material was added to 1 ml of Barfoed's reagent in a test tube and heat in a beaker for boiling water.	Red precipitate of cuprous oxide is formed within 2 minutes if a monosaccharide is present.
	Fehling's test	2. ml of an aqueous extract of the plant material was added to 1ml of a mixture of equal volumes of Fehling's solutions A and B then for a few minutes.	A red or brick-red precipitate is formed if a reducing sugar is present.
	Benedict's test	Test solution is treated with few drops of Benedict's reagent (alkaline solution containing cupric citrate complexes) and upon boiling on water bath.	Reddish brown precipitate forms if reducing sugars are present.
Glycoside	General Test	A small amount of an alcoholic extract of the fresh or dried plant material was dissolved in 1 ml of water and add few drops of aqueous sodium hydroxide solution.	A yellow color develops in the presence of glycoside.

Glucoside	Test for glucosides & glycosides with glucose as the glycose	A small amount of an alcoholic extract of the plant material was dissolved in water and alcohol, solution was divided into two portions and were treated in the following ways: One of them was boiled with a mixture of equal volume of Fehling's solution A and B was boiled. Note any brick-red precipitate. Other portion was boiled with a few drops of dilute sulphuric acid for about 5 minutes, neutralized the mixture with sodium hydroxide solution, add an equal volume of mixture of Fehling's solution A and B was boiled.	Production of a brick-red precipitate in the second experiment (carried out with the hydrolyzed extract) and no production of such a precipitate in the first experiment show the presence of glucosides in the extract.
Fats & Fixed Oils	Satin Test	The small quantity of extract is pressed between two filter papers.	The stain on 1 filter paper indicates the presence of fixed oils.
	Saponification test	A few drops of 0.5N of alcoholic potassium hydroxide was added to small quantities of various extracts along with a drop of Phenolphthalein separately and heated on a water bath for 1-2 hrs.	The formation of soap or partial neutralization of alkali indicates the presence of Fixed oils and Fats.
Proteins & Amino Acids	Ninhydrin test	A small amount of extract was boiled with 0.2% solution of Ninhydrin (Indane 1, 2, 3 trione hydrate)	Violet color appears indicates the presence of proteins and amino acid.
Alkaloids	Tests for alkaloids	A small volume of each extract is neutralized by adding 1 or 2 drops of dilute H2S04. This neutralized solution is treated with a very small amount of the following reagents and the respective color and precipitate formation is observed:	
		a) Mayer's reagent (Potassium-mercuric iodide solution)	For Mayer's reagent white or creamy white precipitate.
		b) Hager's reagent (1% solution of picric acid)	For Hager's reagent yellow crystalline precipitate.
		c) Dragendorff's reagent (Bismiuth potassium iodide solution)	For Dragendorff's reagent orange or orange-red precipitate is observed
		d) Wagner's reagent	For Wagner's reagent formation of brownish-black precipitate indicates the presence of alkaloids.

Table 1: Phytochemical screening tests.

Statistical Analysis

Statistical analysis for animal experiments was carried out by one way ANOVA following Dunnet's post hoc test using SPSS 23.0. Data were presented as Mean±SEM. The results obtained were compared with the control group. $p<0.05$, $p<0.01$ and $p<0.001$ were considered to be statistically significant, highly significant and very highly significant respectively.

RESULT

The hot-plate test was selected to investigate central analgesic activity, because it had several advantages, particularly the sensitivity to strong analgesics, limited tissue damage. In this test, ethanolic extract of WT at both doses slightly increased the pain threshold compared to control group whereas Tramadol increased the latency significantly (Figure 1).

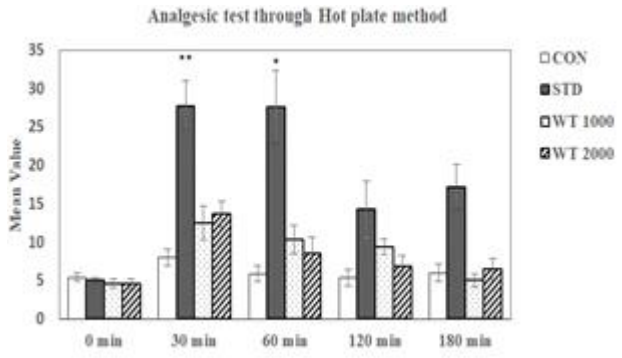

Figure 1: Effect of W. trilobata (1000 mg/kg and 2000 mg/kg bd.wt) in latency time in hot plate test.

Group	Total writhing (Mean ± SEM)	% Inhibition
CON	38.375 ± 3.615	
STD	8.125 ± 2.039***	78.83
WT 1000 mg	18.250 ± 1.250**	52.44
WT 2000 mg	18.875 ± 2.183**	50.81

Table 2: Effect of W. trilobata (1000 mg/kg and 2000 mg/kg bd.wt) in acetic acid induced writhing test.

Formalin test is biphasic, and measures pain of both neurogenic (first phase) and of inflammatory origin (second phase). Result of this study, indicated that WT extract at both the doses decreased licking time very highly significantly ($p<0.001$) at both the phases. The ability of WT extract to inhibit late phases of the formalin test more prominently indicates its involvement in peripherally mediated activity, probably by prostaglandin synthesis inhibition.(Table 3).

Group	First phase (0 to 5 min) (Mean ± SEM)	% Inhibition	Second phase (20 to 25 min) (Mean ± SEM)	% Inhibition
CON	20.500 ± 1.268		11.125 ± 1.109	
STD	8.875 ± 1.231***	56.71	1.375 ± 0.885***	87.64
WT 1000mg	7.375 ± 1.870***	64.02	1.625 ± 0.865***	85.39
WT 2000mg	7.375 ± 0.865***	64.02	0.250 ± 0.164***	97.75

Table 3: Effect of W. trilobata (1000 mg/kg and 2000 mg/kg bd.wt) in formalin induced paw licking test.

Cotton pellet granuloma model was used to evaluate the anti-inflammatory activity of WT in sub-acute inflammation. The dry weight of cotton pellet granuloma was reduced very highly significantly ($p<0.001$) at both doses of WT (1000mg and 2000 mg/kg) (Table 4).

Group	Inflammation (mg) (Mean ± SEM)	% Inhibitions
CON	31.500 ± 1.035	
STD	12.833 ± 0.600***	59.26
WT 1000mg	17.375 ± 0.944***	44.84
WT 2000mg	15.250 ± 1.424***	51.59

Table 4: Effect of W. trilobata (1000 mg/kg and 2000 mg/kg bd.wt) in cotton pellet pleurisy test.

In xylene induced ear edema test, the results showed that WT extract at 1000 and 2000 mg/kg dose reduced inflammation very highly significantly at 61% and 69% respectively. Diclofenac (100 mg/kg, p.o.) showed marked anti-inflammatory activity with a 76% reduction compared to the control, and indicted it might reduce the release of substance P or antagonize its action (Table 5).

Group	Ear inflammation (mg) (Mean ± SEM)	% Inhibition
CON	0.013 ± 0.001	
STD	0.003 ± 0.000**	76.92
WT 1000mg	0.005 ± 0.000**	61.54
WT 2000mg	0.004 ± 0.000**	69.23

Table 5: Effect of *W. trilobata* (1000 mg/kg and 2000 mg/kg bd.wt) in xylene induced ear edema test.

Administration of WT produced a very highly significant ($p<0.001$) and marked inhibition of the paw edematous response induced by carrageenan injection in paw edema test, at both the doses after 120, 180 and 240 minutes of administration (Figure 2).

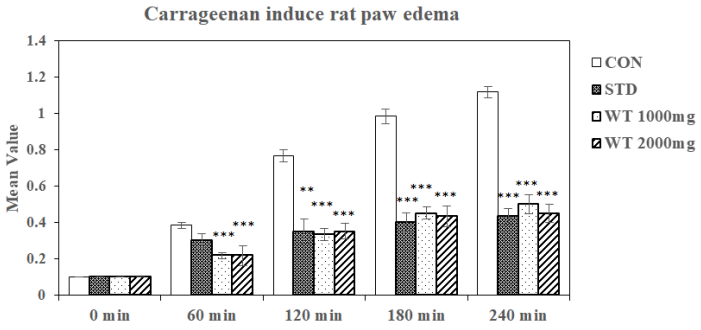

Figure 2: Effect of W. trilobata (1000 mg/kg and 2000 mg/kg bd.wt) in carrageenan induced paw edema test.

In the antipyretic test, WT at 1000mg/kg showed deceased temperature after 30 minutes very highly significantly ($p<0.001$) which was somehow poor at 60 minutes but constantly showed very highly significant effect from 120 to 240 minutes of administration. At 2000mg/kg, WT reduced temperature at 30 minutes significantly ($p<0.05$), highly significantly at 60 minutes and very highly significantly at 120 minutes to till 240minutes (Figure 3).

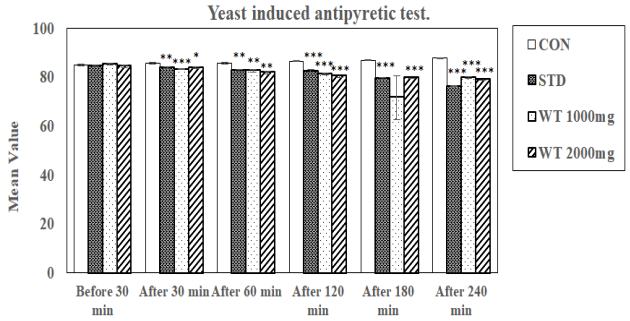

Figure 3: Effect of W. trilobata (1000 mg/kg and 2000 mg/kg bd.wt) in yeast induced antipyretic test.

Phytochemical screening

Phytochemical screening of WT revealed the presence of alkaloids, carbohydrates, glycosides, flavonoids and tannins, which may be responsible for their various pharmacological activities shown in in-vitro and in-vivo investigations (Table 6).

Phytochemicals	Name of the test	Observed Changes	Result
Alkaloids	Mayer's test	Creamy white precipitate	+
	Hager's test	Yellow crystalline precipitate	+
	Wagner's test	Brown or deep brown precipitate	+
	Dragendorff's test	orange or orange-red precipitate	+
Carbohydrates	Molisch's test	A red or reddish violet ring is formed at the junction of two-layer andon shaking a dark purple solution is formed	+
	Barfoed's test	Red precipitate of cuprous oxide is formed within 2 minutes if a monosaccharide is present	+
	Benedicts test	Reddish brown precipitate forms if reducing sugars are present	+
	Fehling test	A red or brick red precipitate is formed if a reducing sugar is present	+
Glycosides	General test	Yellow color	+

	Bromine water test	Yellow precipitate	+
	Test for glucoside	Brick red precipitate	+
Flavonoids	General test	Red color	+
	Shinoda test (Magnesium hydrochloride reduction test)	Green to blue color	+
	Zinc hydrochloride reduction test	Red color after few minutes	+
Saponins	Frothing test	Formation of stable foam	-
Steroids	Libermann-Burchard's test	Greenish color	-
Tannins	Lead acetate test	A yellow or red precipitate	-
	Frerric chloride test	Blue green color	+
	Alkaline reagent test	Yellow to red precipitate	+
Terpenoids	Salkowski test	Yellow color appears at the lower layer	-

(+) = Presence; (-) = Absence.

Table 6 : Result of phytochemical screening test of *WedeliaTrilobata*.

DISCUSSION

The analgesic activities were evaluated by two animal models, which could provide response to two different grades of noxious stimuli (in the thermal stimulus and chemically induced tissue damage) (Victor et al. 2004). Acetic acid causes an increase in peritoneal fluids of PGE2 and PGF2a, serotonin and histamine involved in part, which is a model commonly used for screening peripheral analgesics.

The hot-plate and tail-clip tests are useful in elucidating centrally mediated antinociceptive responses, which focuses mainly on changes above the spinal cord level (Vongtau et al. 2004) possibly acting on a descending inhibitory pain pathway (Richardson et al. 1998). Pain is centrally modulated via a number of complex processes including opiate, dopaminergic, descending noradrenergic and serotonergic systems.

In traditional system of medicine, certain plants are claimed to provide relief of pain and inflammation. Such a plant possess analgesic and anti-inflammatory effect and used by traditional practitioner for these purposes (Halliwell and Gutteridge 1999) was taken for the study. Inflammation is a complex process and ROS play an important role in the pathogenesis of inflammatory diseases (Conner and Grisham 1996). Thus antioxidants which can scavenge ROS are expected to improve these disorders.

In general, acetic acid causes pain by liberating endogenous substances such as serotonin, histamine, prostaglandins (PGs), bradykinins and substance P, which stimulate nerve endings. Local peritoneal receptors are postulated to be involved in the abdominal constriction response (Bentley et al. 1983). The method has also been associated with prostanoids, that is, increased levels of PGE2 and PGF2α in peritoneal fluids (Derardt et. al 1980), as well as lipoxygenase products (Roberts and Morrow 2001). The significant reduction in acetic acid-induced writhes suggests that the analgesic effect may be peripherally mediated via the inhibition of synthesis and release of PGs and other endogenous substances.

Formalin test is believed to be a more valid analgesic model which is better correlated with clinical pain. The first phase (0-5 min) being a result of direct stimulation of nociceptors measures centrally while the second phase (15 – 30 min) which is dependent on peripheral inflammation due to release of chemical mediators from damaged cells that stimulate nociception and thus induced pain. In general, the test measures the response to a long-lasting nociceptive stimulus (Tjolsen et al. 1992) and is recommended as a tool in basic pain research for studying the mechanisms of analgesic agents. Agents that act primarily on the CNS inhibit both phases equally while peripherally acting drugs inhibit the late phase.

The cotton pellet model is an indication for the proliferative phase of inflammation. It has been widely employed to assess the transudative, exudative and proliferative components of chronic inflammation . The suppression of proliferative phase

of sub-acute inflammation could result in decrease in the weight of granuloma formation.

Xylene induced neurogenous swelling, a common inflammatory model, was selected for evaluating vascular permeability which was partially associated with substance P (Luber-Narod et al. 1997). Xylene causes instant irritation of the mouse ear, which leads to fluid accumulation and edema characteristic of the acute inflammatory response (Atta and Alkofahi 1998).

Carrageenan is widely used to induce acute inflammation. Thus Carrageenan-induced rat paw oedema is a suitable test for determining anti-inflammatory action of drugs and natural products (Manueli et al. 1994). The inflammation induced by carrageenan involves cell migration and plasma exudation mediated by the production of inflammatory mediators such as histamine, serotonin, bradykinin, nitric oxide, interleukin (IL)-1β and IL-6, tumor necrosis factor (TNF)-α, and prostaglandins (Salvemini et al. 1996; Loram et al. 2007). Therefore, the compound is extensively used in studies assessing the anti-inflammatory effect of steroidal and non-steroidal drugs (Vinegar et al. 1987). It can be suggested WT action on reducing cell migration and paw edema is associated with the inhibition of the biosynthesis of the inflammatory mediators previously mentioned.

Therefore, WT like other non-steroidal anti-inflammatory/analgesic agents, acts by the inhibition of the synthesis of prostaglandins that are responsible for pain and pyrexia (Simmons et al. 2004). Therefore, WT was good antipyretic agent because this was able to decrease body temperature in albino rats even below its baseline temperature.

CONCLUSION

On the basis of the outcome of the present study, it is concluded that, the WT extract has inhibitory action on pain as well as inflammatory activity. Also, it confirmed that the WT is endowed with antipyretic effect. Therefore, it may be a potential therapeutic option in the effective management of problems associated with pain, inflammation and fever. This finding provides a scientific support for the traditional uses of these plants.

Declaration of Interest: Authors declare that they have no identified competing interests that could have influenced the work stated in this article.

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