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(Research Article)

Anti-inflammatory and Analgesic Activity of Acetone Extract of Polyherbal Antipyretic Drug

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ABSTRACT

The traditional polyherbal drug consisting of stems of Tinospora cordifolia Willd, fruits of Emblica officinalis Gaertn and rhizomes of Cyperus rotundus Linn was evaluated for anti-inflammatory and analgesic properties. The acetone extract at 400 and 600 mg/kg dose dependently exhibited significant anti-inflammatory properties during carrageen induced paw oedema test. The extract also exhibited substantial central analgesic effect during Hot plate and Tail immersion tests and marked peripheral analgesic activity during the Writhings test as compared to the control group and standard drugs. No toxic effect was observed up to the dose of 1000 mg/kg during acute toxicity studies. These anti-inflammatory and analgesic properties of the extract may be related to the presence of its active constituents especially flavonoids.

Key Words: Anti-inflammatory, Analgesic, Toxicity, Polyherbal, Pharmacognosy

INTRODUCTION

The traditional Indian system of Ayurvedic Medicine relies upon the effective use of several medicinal plants, mostly in combination, for preventive and curative treatment of ailments. One such traditional drug mentioned in the renowned text Charak Samhita consists of equal amounts of stems of Guduchi (*Tinospora cordifolia* Willd), fruits of Amlaki (*Emblica officinalis* Gaertn) and rhizomes of Mustak (*Cyperus rotundus* Linn), which has been prescribed as a cure for primary and associated symptoms of fever, inflammation and bodyache [1].

Emblica officinalis Gaertn belonging to the family Euphorbiaceae is a small or medium sized deciduous tree locally known as "Amlaki" or "Amla" in India whose fruits are known to be a rich source of Vitamin C. It contains tannin, ellagic acid and gallic acid, and is used for treatment of diabetes, anaemia, peptic ulcer, inflammation, skin diseases and cardiac problems. The fruit juice is also known for its lipid lowering and anti-atherosclerotic effect [2, 3, 4, 5].

Tinospora cordifolia known locally as "Guduchi" or "Gulancha" belongs to the Menispermaceae family and is mostly found in deciduous and dry Indian forests as a glabrous climbing shrub. Its stem is creamy white to grey in color, with deep clefts spotted with lenticels having aerial roots. The principal constituents found in stem are Tinosporin, Tinocordiside, Tinocordifolioside, Cordioside and alkaloids like Berberine and Palmatine. It has been described in Ayurveda as a rejuvenator and diuretic and prescribed for treatment of various ailments like leprosy, fever, asthma, jaundice, diabetes, skin infections, diarrhea and dysentery [2, 3, 4, 6].

"Mustak" or purple nutsedge or nutgrass (*Cyperus rotundus* Linn) is a perennial Indian weed spread in tropical, subtropical and temperate regions which belongs to the family Cyperaceae. Its rhizomes are bluntly conical crowned with the remains of stem and leaves forming a dark brown or black scaly covering with internal creamish-yellow colour. It contains polyphenols like Cyperone, Cyperenone and Cyperene and carbohydrates like D-glucose and D-fructose. The rhizomes are cooling, nervine tonic, and diuretic and traditionally used to treat diarrhoea, dysentery, leprosy, bronchitis and blood disorders [2, 3]. The rhizome is reported to possess analgesic, anti inflammatory and antipyretic properties [4, 7].

This polyherbal drug has already exhibited significant antipyretic action on rats when compared with standard drug in earlier studies [8]. Inflammation, pain and fever like symptoms are very much related to each other due to similar

type of pathogenesis in the body. In fact, the non-steroidal anti-inflammatory drugs are normally having anti-inflammatory, analgesic as well as antipyretic pharmacological actions due to inhibition of the biosynthesis of the prostaglandin and other prostanoids in the body [9]. Therefore, the present study was carried out to evaluate the anti-inflammatory and analgesic effect of the traditional polyherbal test drug using the standard scientific mechanisms of carrageen induced paw oedema, Hot plate, Tail immersion and writhing method on rodents.

MATERIAL AND METHODS

The chemical analysis and experimental studies were conducted in the laboratory and the animal experiment facility respectively of Dravyaguna department of the Institute of Post Graduate Ayurvedic Education and Research, Kolkata which is registered with CPCSEA, Government of India.

Plant Material

Fresh fruits of *Emblica officinalis,* stems of *Tinospora cordifolia,* and rhizomes of *Cyperus rotundus* were purchased from the reputed herb supplier of the pharmacy of S.V.S.P Hospital, Kolkata. They were authenticated by the Research Officer, Botanical Survey of India, Howrah (Ref No- BSI/CNH/AD/Tech/2010, Date- 21.07.2010). The herbarium of these plants is stored in the museum of Dravyaguna Department of I.P.G.A.E.R., Kolkata, India.

Chemicals

All chemicals used during the experiment were of analytical grade. Chemicals for phyto-chemical screening, along with standard reagents were purchased from M/s Merck Specialities Pvt. Ltd, Mumbai and Sisco Research Laboratories Pvt. Ltd, Mumbai, India.

Animals

The anti-inflammatory experiments were performed on adult Wistar rats of either sex weighing between 100 to 120 gm whereas Swiss albino mice weighing 20-30 gm were used for acute toxicity study. All the animals were procured from M/s Ghosh Scientific, Kolkata. All experimental animals were housed in polypropylene cages and maintained under environmentally controlled room provided with a 12: 12 hr light and dark cycle for each 24hr period at a temperature of approximately 25°C. They were fed with standard pellet diet and water *ad libitum.* All the experimental studies were carried out under appropriate conditions in animal house of IPGAER, registered under CPCSEA (Reg. No-1180/ac/08/CPCSEA), after obtaining the necessary permission from the Institutional Animal Ethical Committee. Prior to each experiment, the animals were fasted overnight but allowed free accesses to water [10].

Preparation of Acetone Extract of Polyherbal Drug

All the raw herbs were thoroughly washed to remove any extraneous matter such as dirt, foreign matter and adulterants. They were sun dried and crushed into particle size of 40 mesh. The powdered fruits of *Emblica officinalis,* stems of *Tinospora cordifolia,* and rhizomes of *Cyperus rotundus* were mixed in equal proportion to prepare the test drug using acetone as solvent in a Soxhlet Apparatus for 48 hours. The extract obtained was filtered, concentrated in a rotary evaporator and finally stored in refrigerator for further analysis.

Pharmacognostic Study of the Crude Drug Powder

The macroscopic and microscopic study of the powder of test drug was done according to standard procedures [11]. The final crude drug powder was mounted in glycerine, observed under optical microscope (40X) of Dewinter, Italy and photographed.

Physiochemical Analysis

Physiochemical parameters such as extractive value, moisture content, acid insoluble ash, water soluble ash and total ash content of the test drug powder were assessed according to the standard steps described in the Ayurvedic Pharmacopoeia, Government of India [11]. The research drug was treated in different solvents and observed under Visible and Ultra Violet lamps (at 254 nm and 365 nm) for standard fluorescence analysis.

Estimation of Total Phenolic Compound

The total phenolic content was determined by Folin Ciocalteu method using gallic acid as standard compound at different concentrations between 100 to 500µg/ml [11]. The total phenol values were expressed in terms of gallic acid equivalent (mg/g of dry mass).

Phyto-chemical Screening

Preliminary phyto-chemical screening for ascertaining the presence of different constituents like alkaloids, flavonoids, tannin, carbohydrates, glycosides, saponin, fats, oils, proteins and amino acids was done following standard procedures [11].

EXPERIMENTAL STUDY

Acute Toxicity Study

Acute oral toxicity study was carried out according to OECD guideline 423 [12]. The animals of both sexes were selected for the study by random sampling technique and were divided into 5 groups of 3 animals each. A single oral dose of the extract was administered at the level of 100mg, 300mg, 500mg, 700mg, 900mg and 1000mg/kg body weight and the animal groups were observed for appearance of toxic symptoms including behavioural changes, locomotion, muscle spasm, loss of righting reflex, tremor, convulsions and mortality for 24 hrs. They were further supervised for a period of 14 days for occurrence of toxic symptoms and mortality.

Anti-inflammatory Studies (Carrageenan-Induced Paw Oedema in Rats)

The paw oedema was induced by injecting 0.1 ml of 1% (w/v) Carrageenan suspension into the sub-planter region of right hind paw of rats according to methods described by Winter et al. [10, 13] .The control group (A) was orally administered saline (10ml/kg) while the standard group (B) was given Indomethacin (5mg/kg) and Groups C and D were given 400 mg/kg and 600 mg/kg of the test drug extract 1 hour before Carrageenan injection [14].

The measurement of paw oedema was carried out by displacement technique using Plethysmometer to find out the circumference of paw oedema immediately before and

after 1 hr, 2 hr, 3 hr and 4 hr following the Carrageenan injection.

The inhibitory activity was calculated according to the formula:

$$\% \text{ Inhibition} = \frac{(Ct\text{-}Co)_{control} - (Ct\text{-}Co)_{treated}}{(Ct\text{-}Co)_{control}} \times 100$$

Where Ct is the paw circumference at time t, Co is the paw circumference before Carrageenan injection and (Ct- Co) is oedema or paw size after Carrageenan injection.

Analgesic Studies

(A) Assessment of central analgesic effect

(i) Hot plate method

The central analgesic activity against thermal stimulus was evaluated in mice following hot plate method as well as tail immersion method [10, 15]. Morphine sulphate (2.5 mg/kg i.m.) was used as a standard drug. The control group (A) was orally administered 10ml/kg saline and the standard group (B) was given intramuscular injection of 2.5 mg/kg Morphine. Similarly, Groups C and D were orally administered 400 mg/kg and 600 mg/kg of test drug respectively 1 hour before applying the thermal stimulus, which was maintained at 55 ± 0.2 ^{o}C. The latency in hind paw licking was recorded in seconds as responses after 10, 30 and 60 minutes of drug administration in the hot plate method. Maximum reaction time of observation was about 60 seconds throughout to avoid tissue damage [14].

(ii) Tail immersion method

The tail immersion method was followed to find out the central analgesic effect of acetone extract in different dosages [10, 15, 16]. Morphine sulphate (2.5 mg/kg i.m.) was used as the standard drug and injected into group B. The control group, group C and group D were orally administered 10 ml/kg of saline, and 400 mg/kg and 600 mg/kg of test drug respectively 1 hour before applying the thermal stimulus by placing the tail 5cm. in the glass beaker having water temperature maintained at 55 ± 0.2 ^{o}C. The withdrawing of the tail from the glass beaker was recorded in seconds as responses after 10, 30 and 60 minutes of drug administration in this method. Maximum reaction time of observation would be about 60 seconds throughout to avoid tissue damage.

(B) Assessment of peripheral analgesic effect using Writhing analysis

The peripheral analgesic activity of test drug was evaluated in acetic acid induced writhing experiments using mice. The abdominal constriction writhings resulting from intra-peritoneal injection of acetic acid (10 ml/kg of 0.6% v/v glacial acetic acid solution in water) were observed according to standard procedure [16, 17]. Saline (10 ml/kg) was orally administered to group A (control group) whereas Standard Aspirin (100 mg/kg) was prescribed for group B and 400 mg/kg and 600 mg/kg test drug extract was orally given for Groups C and D respectively. Acetic acid solution was administered after 30 minutes and number of writhings counted in each animal for 15 minutes. Percentage inhibition response was calculated as the reduction in the number of abdominal constrictions between control group and test drug treated groups as a percentage of the number of writhes observed in case of the control group.

Statistical Analysis

The differences in the parametric data of rectal temperature(s) were examined by two-way analysis of variance (ANOVA) followed by Dennett's t test, to compare a set of experimental data against control mean. The level of significance was fixed between $p < 0.05 – p < 0.01$.

RESULTS

Pharmacognostic Study

The macroscopic study of the test drug powder revealed that its texture is slightly rough and colour is light brown. It possesses a sweet odour and sour and slightly astringent taste.

The microscopic characteristics of the powder showed the presence of crystals and starch grains in large amount, cork cells, raphides and parenchymatous cells.

Physiochemical Analysis

The yield of dried acetone extract was 2.57 % w/w while the moisture content was observed to be 8.0 % w/w. The total ash content (5.005% w/w) along with the acid insoluble ash content (1.67% w/w) and water soluble ash content (3.34% w/w) values were also determined [8].

Phytochemical screening

Preliminary Phytochemical screening of the acetone extract revealed the presence of flavonoids, tannins and carbohydrates. The total phenol content in the test drug was found to be 26.0 mg/gm of dry mass as calculated from the standard curve of Gallic acid [8].

Experimental Study

Acute Toxicity Study

The animals tested up to 1000 mg/kg dose in acute toxicity experiments showed no significant toxic symptoms like sedation, convulsion, diarrhoea, irritation, etc. There were no signs of any behavioural changes and no mortality was observed up to 24 hrs and later for 14 days.

Anti-inflammatory Studies (Carrageenan - induced Paw Oedema in Rats)

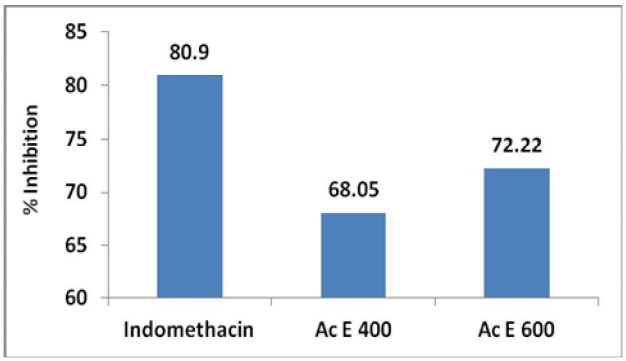
The circumference of paw oedema in rats induced by the carrageen agent to evaluate the acute anti-inflammatory effect at 400 mg/kg and 600 mg/kg dose showed significant decrease of 68.05% and 72.22% respectively ($p < 0.05$) in inflammation up to 4 hours as compared to the control group (fig.1). The percentage inhibition of the acute inflammation in the paw of rats was found to be less in 600 mg/kg of test drug dosage (72.22% inhibition) compared to the standard drug Indomethacine (80.90% inhibition) after 4 hours of treatment (table-1).

Table 1: Effect of acetone extract on paw oedema caused by carrageen–induced inflammation in rats

Group	0 hr	1 hr	2 hr	3 hr	4 hr	% inhibition after 4 hr
Control (10ml/kg)	0.45 ± 0.08	1.32 ± 0.04	2.36 ± 0.08	2.87 ± 0.09	2.88 ± 0.07	
Indomethacin (5mg/kg)	0.43 ± 0.04	0.76 ± 0.06	0.70 ± 0.05	0.63 ± 0.07	0.55 ± 0.08	80.90
400 mg/ kg test drug	0.48 ± 0.07	1.02 ± 0.05	0.98 ± 0.06	0.96 ± 0.06	0.92 ± 0.03	68.05
600 mg/ kg test drug	0.47± 0.05	0.97 ± 0.07	0.92 ± 0.05	0.86 ± 0.07	0.80 ± 0.07	72.22

Results are presented as Mean+ SEM. ($p < 0.05$) compared to control n = 6

Figure-1: Percentage inhibition in circumference of paw oedema in carrageen–induced inflammation in rats



Analgesic studies

(i) Hot plate Method

The reaction time was increased significantly up to 60 minutes after giving thermal stimulus following the hot plate method in case of the 400 mg/kg drug group (increasing from 3.38 sec to 5.15 sec) and 600 mg/kg test drug group (increasing from 3.22 sec to 5.92 sec) ($p < 0.05$) when compared with the control group (where it actually decreased from 3.01 sec to 2.86 sec). The test drug at the dose of 600 mg/kg showed similar type of pattern up to 90 minutes (reaction time increasing from 3.22 sec to 6.23 sec) but slightly lower central analgesic effect when compared with the standard drug morphine sulphate (where the reaction time increased from 3.25 sec to 6.62 sec) (table 2).

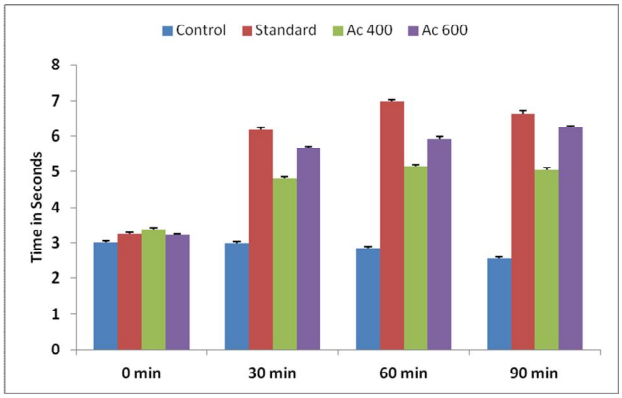
Table 2: Average reaction time in seconds using Hot plate method

Group	Average reaction time after				% increase in reaction time after 60 min
	0 min	30 min	60 min	90 min	
Control	3.01 ± 0.042	3.00 ± 0.040	2.86 ± 0.034	2.56 ± 0.048	- 4.98%
Standard (Morphine)	3.25 ± 0.035	6.17 ± 0.067	6.98 ± 0.058	6.62 ± 0.089	114.77%
400 mg / kg test drug	3.38 ± 0.040	4.82 ± 0.033	5.15 ± 0.043	5.05 ± 0.073	52.37%
600 mg/ kg test drug	3.22 ± 0.031	5.67 ± 0.049	5.92 ± 0.078	6.23 ± 0.046	83.85%

Results are presented as Mean+ SEM. ($p < 0.05$) compared to control n = 6

The results of the hot plate method showed that the percentage increase in reaction time after oral administration of drug and giving of thermal stimulus to the animals up to 60 minutes was 114.77 % in case of standard group, 52.37 % in case of 400 mg/kg test drug group and 83.85 % in case of 600 mg/kg group. In comparison to this, the control group actually showed a 4.98 % decrease in reaction time after 60 minutes. The test drug thus exhibited significant central analgesic effect at the dose of 400 mg/kg and 600 mg/kg when compared with the control group. However, the results of test drug assessed in terms of the average reaction time at the dose of 600 mg/kg (83.85 % increase) showed less central analgesic effect as compared with the standard drug Morphine (114.77 % increase) (fig. 2).

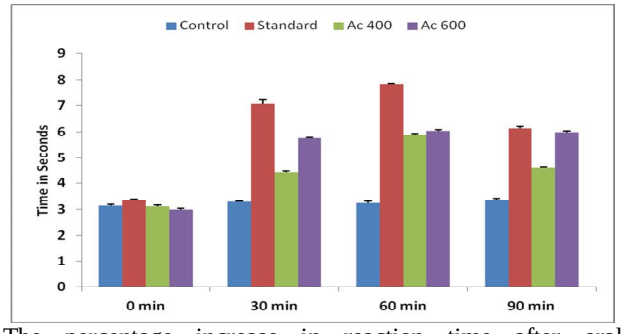
Figure 2: Average reaction time using Hot plate method



(ii) Tail Immersion Method

The results of the tail immersion method showed significant increase ($p < 0.05$) in reaction time up to 60 minutes after giving thermal stimulus in case of both the 400 mg/kg drug group (increasing from 3.12 sec to 5.88 sec) and the 600 mg/kg test drug group (increasing from 2.98 sec to 6.03 sec) when compared with the control group (where it increased from 3.15 sec to 3.35 sec). The 600 mg/kg test drug group showed similar type of pattern up to 90 minutes (reaction time increasing from 2.98 sec to 5.97 sec), however its overall central analgesic effect was a little lower when compared with the standard drug Aspirin (where the reaction time increased from 3.34 sec to 6.14 sec during this period) (fig. 3).

Figure 3: Average reaction time in Tail immersion method



The percentage increase in reaction time after oral administration of the drug and giving of thermal stimulus to the animals in case of tail immersion method up to 60 minutes was 134.13 % in case of the standard group, 88.46 % in case of the 400 mg/kg test drug group and 102.35 % in

case of the 600 mg/kg group. The control group showed only 3.18 % increase in reaction time over the same time period, indicating significant impact of the test drug and standard drug during the experiment. The results indicated that the test drug exhibited significant central analgesic effect at both the doses of 400 mg/kg and 600 mg/kg as compared to the control group. However, the overall impact of the test drug assessed in terms of the average reaction time at the dose of 600 mg/kg (102.35 % increase) showed less central analgesic effect as compared with the standard drug Morphine (134.13 % increase) (table 3).

Table 3: Average reaction time in seconds in Tail immersion method

Group	0 min	30 min	60 min	90 min	% Increase in reaction time after 60 min
Control	3.15 ± 0.042	3.29 ± 0.034	3.25 ± 0.058	3.35 ± 0.067	3.18%
Standard Aspirin	3.34 ± 0.054	7.08 ± 0.143	7.82 ± 0.049	6.14 ± 0.060	134.13%
400 mg / kg test drug	3.12 ± 0.034	4.42 ± 0.069	5.88 ± 0.052	4.60 ± 0.037	88.46%
600 mg/ kg test drug	2.98 ± 0.046	5.74 ± 0.044	6.03 ± 0.052	5.97 ± 0.049	102.35%

Results are presented as Mean+ SEM. ($p < 0.05$) compared to control n=6

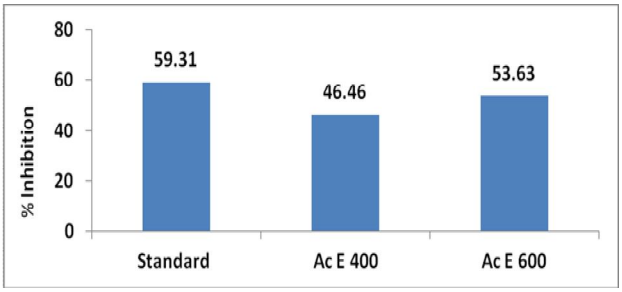
Writhing Test

The peripheral analgesic effect in Writhing test was evaluated on the basis of the average number of abdominal constrictions indicated by the extension of hind paw of animals. The observed inhibition in writhings as a result of administration of the test drug up to 15 minutes was significantly higher ($p < 0.05$) at the dose of 400 mg/kg (46.46 %) as well as at 600 mg/kg (53.63%) when its activity was compared with the control group (fig. 4). Comparing the performance of the test drug with the standard drug, the observed peripheral analgesic effect was slightly less at the test drug dose of 600 mg/kg as indicated by 53.63% inhibition in writhings as compared to the standard drug Aspirin which resulted in 59.31% inhibition (table 4).

Table 4: Analgesic activity during Acetic acid induced Writhings test

Group	Average no. of writhings in 15 mins	% Inhibition
Control	56.33 ± 1.25	0.00
Standard Aspirin	22.92 ± 0.67	59.31
400 mg / kg test drug	30.16 ± 0.82	46.46
600 mg/ kg test drug	26.12 ± 1.08	53.63

Figure 4: Analgesic activity during Acetic acid induced Writhings test



DISCUSSION

The oral administration of different dosages (400 mg/kg and 600 mg/kg) of the test drug during the carrageenan induced paw oedema test in rats resulted in marked and significant anti-inflammatory response in terms of reduction in paw circumference over various time intervals. The acute and sustained effect on the induced inflammation up to four hours depends upon the decreased production of pro-inflammatory cytokines and PGE_2 in the tissue of the effected part of the body. The inflammation induced by phlogostic agent is related to the production of histamine, bradykinin and cyclooxygenase products while delayed phase is related to neutrophil infiltration, as well as to the continuing of the production of arachinoic acid metabolites [18, 19].

The central analgesic effect of the acetone extract of test drug at both doses of 400 mg/kg and 600 mg/kg assessed using the Hot plate and the Tail immersion methods in mice showed significant analgesic activity as compared to the control group. The results obtained using both the abovementioned methods clearly showed that the test drug at the dose of test drug 600mg/kg is having a pronounced and noticeable central analgesic effect which is comparable to that of the standard drug morphine in these experiments.

The percentage inhibition in the average numbers of writhings or abdominal contractions which were observed through extension of the hind paw in mice is a clear indication of the peripheral analgesic effect. During the acetic acid induced writhing method, the test drug exhibited 46.46 % and 53.63 % inhibition at 400 mg/kg and 600 mg/kg dosage as compared to 59.31% inhibition in the standard (Aspirin) group. Thus, the test drug showed a marked peripheral analgesic effect for curing the visceral pain at 600 mg/kg dosage which was comparable to the effect obtained with the synthetic standard drug Aspirin in this study.

The acetone extract of the test drug has already exhibited significant antipyretic effect [8]. The anti-inflammatory and analgesic activities of the test drug evaluated in comparison with the control and standard drug groups during the present study could be co-related to its antipyretic effect. Pyrexia, pain and inflammation are the result of secondary impact of infection, tissue damage, inflammation, graft rejection, malignancy or other diseased states. Mediators like interleukin 1β, α, β and TNF-α increase the synthesis of PGE_2near pre-optic hypothalamus area thereby triggering the hypothalamus to elevate the body temperature [18, 20]. All these pharmacological activities of the test drug may be due to the presence of high concentration of phenolic compounds such flavonoids and tannin which are mostly found in plant samples and exhibit may pharmacological actions such as anti-inflammatory, analgesic, antipyretic, antioxidant and cardio-protective properties. Flavonoid is known to target

prostaglandins which are involved in pyrexia. Many such activities of the flavonoidic compounds result from the inhibition of arachidonic acid, prostaglandin and cytokine like substances in the body [9, 17, 21, 22].

CONCLUSION

The test drug which is a traditional Ayurvedic antipyretic formulation as described in the ancient text Charak Samhita was subject to rigorous scientific evaluation using standard procedures. The results of the study indicate its significant and substantial anti-inflammatory, peripheral analgesic effect and marked central analgesic effect vis-à-vis standard modern antipyretics. The test drug also exhibited no toxic effect up to the dose of 1000 mg/kg body weight during acute toxicity studies, validating its non-toxic effect even in higher doses whereas use of most NSAIDs is associated with complications like gastric ulceration, hepatic and kidney disorders.

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