



STUDIES OF ANTI INFLAMMATORY, ANTIPYRETIC AND ANALGESIC EFFECTS OF AQUEOUS EXTRACT OF TRADITIONAL HERBAL DRUG ON RODENTS

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ABSTRACT

Aqueous extract of combination of stems of *Tinospora cordifolia*, fruits of *Embelica officinalis* and rhizomes of *Cyperus rotundus* has been used as traditional herbal drug in Indian medicine system for treatment of fever, body ache, joint pain and inflammation. The collected botanicals were subject to physicochemical, pharmacological & phytochemical screening before animal experiments. After acute toxicity studies, anti-inflammatory effect was assessed using carrageen induced paw oedema test and antipyretic effect using yeast induced pyrexia method. Tail immersion, hot plate and writhings test were used for determining the analgesic properties. Phytochemical analysis revealed the presence of polyphenolic flavonoids, tannin and saponins. Significant anti-inflammatory, antipyretic and analgesic properties were noticed in dose dependant manner after aqueous extract administration especially at 600 mg/kg dose. These test drug activities were sustained and comparable to the standard drugs while exhibiting no acute toxicity. Aqueous extract of test drug possesses significantly high anti-inflammatory, antipyretic and analgesic properties without any acute toxicity possibly due to presence of flavonoids.

Keywords: *Tinospora cordifolia*, *Embelica officinalis*, *Cyperus rotundus*, anti-inflammatory, antipyretic, analgesic

INTRODUCTION

The Ayurvedic system of treatment approaches the whole problem of disease in a holistic fashion. It advocates correcting the imbalance through a combination of diet, exercise, regimen and herbal medicines to accelerate the process of healing and bringing the body-mind-sense complex to a normal natural state. According to WHO, traditional medicine has established and proved itself to possess promotive, preventive, curative and rehabilitative roles. The Traditional Herbal Medicinal Products Directive is changing the face of the botanicals extracts and supplements sector in the world for prevention and curing of diseases on the basis of scientific evaluation of evidence based Ayurvedic drugs. The test drug has been selected from the renowned Ayurvedic text Charak Samhita which is having the combination of three ingredients in equal amounts - stems of Guduchi (*Tinospora cordifolia* Willd), fruits of Amlaki (*Embelica officinalis* Gaertn) and rhizomes of Mustak (*Cyperus rotundus* Linn) ^{1,2}. It has been traditionally used for the treatment of fever, pain and inflammation in clinical practice but its aqueous extract has not yet been subject to scientific pharmacological evaluation.

Embelica officinalis Gaertn (family Euphorbiaceae), locally known as "Amlaki" or "Amla" is a medium sized deciduous tree found throughout India. Dried fruit of the plant is brown to blackish brown in colour with characteristic odour and sour and astringent taste. Its fruits are a rich source of Vitamin C, besides it also contains tannin, ellagic acid and gallic acid, phyllobins. They are also used in diabetes, anaemia, peptic ulcer, inflammation, skin diseases and cardiac problems. The fruit juice has been reported to possess lipid lowering and anti-atherosclerotic effect ^{3,4}.

Tinospora cordifolia belonging to the family of Menispermaceae locally known as "Guduchi" is a glabrous climbing plant mostly found in India, typically growing in

deciduous and dry forests. The succulent stem is creamy white to grey in color, with deep clefs spotted with lenticels often giving out aerial roots. The principal constituents found in its stem are Tinosporin, Tinocordiside, Tinocordifolioside, Cordioside and alkaloids like Berberine & Palmatine. This plant is used in Ayurvedic practice for treatment of various ailments like leprosy, fever, asthma, jaundice, diabetes, skin infections, diarrhea and dysentery ^{4,5}.

Cyperus rotundus Linn (Cyperaceae), locally known as "Mustak" or purple nutsedge or nutgrass, is a perennial weed plant indigenous to India. Its rhizomes are bluntly conical and vary in size and thickness, crowned with the remains of stem and leaves forming a scaly covering, dark brown or black externally, creamish-yellow internally and have pleasant odour. The rhizomes are cooling, nervine tonic, and diuretic and traditionally used to treat diarrhoea, leprosy, bronchitis and blood disorders. The rhizome contains polyphenols like Cyperone, Cyperenone and Cyperene and carbohydrates like D-glucose and D-fructose. The rhizome is reported to possess analgesic, anti-inflammatory and antipyretic properties ^{4,7,8}. The standard drugs used for analgesic, antipyretic and anti-inflammatory action such as NSAIDs have been associated with some side-effects and toxicity ⁹. Therefore, this traditional drug which has been mentioned for the treatment of fever, muscular pain, joint pain & inflammatory diseases was taken up for its biological evaluation. Its aqueous extract was subject to anti-inflammatory, antipyretic and analgesic experimental studies carried out on rodents to compare its efficacy with non-steroidal anti-inflammatory synthetic drugs.

MATERIALS AND METHODS

All the experimental studies and chemical examinations were performed in the laboratory and CPCSEA registered animal

house facility located in the Dravyaguna department of the Institute of Post Graduate Ayurvedic Education & Research, Kolkata, India.

Collection & identification of plant samples

The samples of all the test botanicals in raw form were collected from a reputed medicinal herb supplier of the pharmacy of S. V. S. P. Hospital attached to IPGAER, Kolkata. These samples were duly authenticated by the Research Officer, Botanical Survey of India, Shibpur, Howrah (Ref No- BSI/CNH/AD/Tech/2010 dated 21.07.2010) as per procedure laid down in the Ayurvedic Pharmacopoeia of India¹⁰.

Chemicals

Analytical grade chemicals for performing various types of chemical and phytochemical analysis during this research were purchased from a reputed company, namely M/s Merck Pvt. Ltd.

Animals

The animal house & experimentation facility of IPGAER which is registered with CPCSEA vide Reg. No 1180/ac/08/CPCSEA was used for all the experimental studies. The requisite permissions were duly obtained from the Institutional Animal Ethical Committee before commencing the experiments. The anti-inflammatory and antipyretic experiments were performed using adult Wistar rats of either sex (1 month old and weighing 80-120gm) while Swiss albino mice weighing 20-30 gm were used for the acute toxicity study and the analgesic tests. All the animals were procured from a reputed animal supplier of Kolkata, Mr. Satyen Ghosh. The 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. Prior to experiments, the animals were fasted overnight but allowed free access to water¹¹.

Extraction of Research drug

The ingredients of crude drugs were washed, sun dried and crushed to particle size of 40 mesh. The powdered fruits of *Emblica officinalis*, stems of *Tinospora cordifolia*, and rhizomes of *Cyperus rotundus* were mixed in equal fraction to prepare research drug. The research drug was then subsequently extracted by Petroleum ether (60°- 80°), Chloroform, Acetone, Methanol and Water using Soxhlet Apparatus. The extracts obtained were filtered, concentrated by rotary evaporator and finally stored in refrigerator for further analysis. The aqueous extract of the research drug has been used throughout the study^{10,12}.

Pharmacognostical study of the crude drug powder

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

The fluorescence analysis of the powder was done after treating it with several solvents and then observing it under Visible, UV 254 nm and UV 365 nm lamps.

Elemental analysis

Elemental analysis was performed to detect the presence of nitrogen, sulfur and halogens using routine chemical analysis techniques. A piece of metallic sodium was taken in a test tube, melted by slow heating and about 0.5 g of research drug powder was added which was strongly heated for about 2 min. Twenty ml of distilled water was taken in a mortar and pestle, the red-hot test tube was broken and ground in mortar distilled water. The aqueous solution was filtered through Whatman-40 filter paper and the filtrate was subjected to test for these elements.

Physiochemical Analysis

Physiochemical parameters such as extractive value, moisture content, acid insoluble ash, water soluble ash and total ash content of the powdered test drug were evaluated according to standard steps described in the Ayurvedic Pharmacopoeia, Government of India.

Phytochemical screening

Preliminary phytochemical screening for assessing the presence of different active constituents like alkaloids, flavonoids, tannins, carbohydrates, glycosides, saponins, fats and oils, protein and amino acids was performed following the standard procedures.

Estimation of total polyphenol content

Total polyphenol content was estimated using the Folin-Ciocalteu method calibrated on Gallic acid 20. Sample extracts of 500 µl were added to 500 µl of water, 5 ml of 0.2 N Folin-Ciocalteu reagent and 4 ml of 75 g/l saturated sodium carbonate solution and mixed in a cyclomixer. The absorbance was measured in the spectrophotometer at 765 nm after incubation for 2 h at room temperature. Quantification of total polyphenol content was done on the basis of a standard curve generated with 100, 200, 300, 400 and 500 mg/l of Gallic acid^{10,12,13}.

Thin Layer Chromatography (TLC) analysis

The extract of the research drug (2.5 mg/ml) and standard phenolic compound Gallic acid (0.6 mg/ml) were analysed using a glass plate coated with a thin layer of 60 F₂₅₄ silica gel (194015 G, Batch No. HX024736, SISCO Research laboratories Pvt. Ltd., Mumbai, India) using different solvent mixtures. The development was stopped when the solvent front had advanced about 7.5 cm. The different spots developed were visualized on coloration (like Iodine vapour exposure) and their R_f values were calculated. R_f values of components are indicative of specific character of molecule in the given environment of mobile and stationary phase. The best results were obtained with the solvent system: Toluene: Ethyl acetate: Formic acid: MeOH (3: 3: 0.8: 0.2 v/v/v). Gallic acid was purchased from M/s Nice Chemicals Pvt. Ltd.

Experimental study

Acute toxicity study

Acute oral toxicity study was carried out according to OECD guideline 423. Animals of both sexes were selected by random sampling technique for the study and divided into 5 groups of 3 animals each. A single oral dose of the extract starting at 200 mg/kg and progressively moving from 400, 600, 800 mg/kg up to 1000 mg/kg was administered. 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 and further supervised for a period of 14 days for occurrence of toxic symptoms and mortality¹⁴.

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. (1962) [23]. The control group (A) was orally administered saline (10 ml/kg) while the standard group (B) was given Indomethacin (5 mg/kg) and Groups C & D were given 400 mg/kg & 600 mg/kg of the test drug extract one hour before Carrageenan injection^{11, 15-18}.

The measurement of paw oedema was carried out by displacement technique using Plethysmometer to find out the circumference of paw oedema immediately before and at 1hr, 2hr, 3hr and 4 hours following the Carrageenan injection.

The inhibitory activity was calculated according to the formula-

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

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

Evaluation of Antipyretic activity

The antipyretic efficacy of aqueous extract was assessed using brewer's yeast induced pyrexia method. Pyrexia was induced by injecting 10.0 ml/kg of 20% w/v suspension of Brewer's yeast in normal saline subcutaneously 18 hours before the commencement of experiment. Only animals whose rectal temperature increased by at least 1.0°C after 18 hours of induced subcutaneous yeast injection were included in the study. The normal body temperature of each animal was measured by inserting a flexible tail thermostat probe coated with lubricant into rectum and recorded using digital IMCOP Telethermometer. The experimental animals were selected randomly and divided in four groups containing six animals each. The control group (A) was orally administered saline (10 ml/kg) while the standard group (B) was given 100 mg/kg Aspirin and Groups C & D were prescribed 400 mg/kg & 600 mg/kg of the aqueous extract of test drug respectively. The rectal temperature was recorded at time intervals of 1hr, 2hrs, 3hrs, 4hrs and 5 hours after drug administration^{11, 36}.

Evaluation of Analgesic activity

Assessment of central analgesic effect

Hot plate method

The central analgesic activity against thermal stimulus was evaluated in mice following hot plate method as well as tail immersion method. Morphine sulphate (2.5 mg/kg i.m.) was used as the standard drug in the hot plate method. The control group (A) was orally administered 10 ml/kg saline and the standard group (B) was given intramuscular injection of 2.5 mg/kg Morphine. Similarly, Groups C & D were orally administered 400 mg/kg & 600 mg/kg of the aqueous extract of test drug respectively 1 hour before applying the thermal stimulus, which was maintained at $55 \pm 0.2^\circ\text{C}$. 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^{19, 20}.

Tail immersion method

The tail immersion method was followed to find out the central analgesic effect of aqueous extract in different dosages^{11, 21, 22}. Morphine sulphate (2.5 mg/kg i.m.) was used as the standard drug and injected into group B. The control group, group C & group D were orally administered 10 ml/kg of saline, and 400 mg/kg & 600 mg/kg of the aqueous extract of test drug respectively 1 hour before applying the thermal stimulus by placing the tail 5 cm. in the glass beaker having water maintained at $55 \pm 0.2^\circ\text{C}$ temperature. The latency in tail withdrawal from the glass beaker was recorded in seconds as response after 10, 30 and 60 minutes of drug administration in this method. Maximum reaction time of observation was taken as about 60 seconds throughout to avoid tissue damage^{21, 22}.

Assessment of peripheral analgesic effect (acetic acid induced 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 procedure²². 10 ml/kg Saline was orally administered to group A (control group) whereas standard Aspirin (100 mg/kg) was prescribed for group B and 400 mg/kg & 600 mg/kg of the aqueous extract of test drug was orally given for Groups C & 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 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 $p < 0.05 - p < 0.01$ ²³.

RESULTS

Pharmacognostical study

The observations under microscope showed the presence of crystals and starch grains in large amount, cork cells, raphides and parenchymatous cells in the powder.

Fluorescence analysis

The colours observed during the fluorescence analysis of the drug sample in day light, UV 254 and UV 365 are outlined in table 1.

Physiochemical analysis

The moisture content was observed to be 7.5 % w/w while the pH was found to be 3.4. The total ash content was estimated as 5.01 % w/w, the acid insoluble ash being 1.58 % w/w and the water soluble ash content being 3.43 % w/w.

The extractive value (% w/w) in different solvent systems was found to be 0.65% in petroleum ether, 0.69% in Chloroform, 2.57% in Acetone, 4.1% in Methanol and 4.56% in aqueous system.

Table 1 Fluorescence analysis of drug sample

Reagent	Observation of colours		
	Day light	UV 254	UV 365
1M Sodium Hydroxide	light brown	Dark brown	Blackish brown
Acetic acid	Light brown	Light brown	Blackish brown
1M Hydrochloric acid	Light brown	Greenish brown	Blackish brown
Dil. Nitric acid	Light greenish brown	Dark greenish brown	Blackish red
5% Iodine	Reddish black	Dark brown	Blackish red
5% Ferric Chloride	Light green	Dark green	Dark brown
Methanol	Light brown	Green	Peaks
50% Nitric acid	Creamish yellow	Green	Black
1M Sulphuric acid	Light brown	Green	Black
Dil. Ammonia	Yellow	Dark green	Black
Sodium Hydroxide in Methanol	Yellow	Light green	Dark green

Table 2: Anti-inflammatory effect of Aqueous extract on carrageen -induced paw oedema

Treatment group	0 hour	1 hour	2 hour	3 hour	4 hour	% inhibition after 4 hr
Control (10ml/kg)	0.43 ± 0.05	1.24 ± 0.07	2.33 ± 0.06	2.79 ± 0.09	2.81 ± 0.05	
Indomethacin (5mg/kg)	0.49 ± 0.08	0.88 ± 0.06	0.80 ± 0.05	0.75 ± 0.07	0.60 ± 0.04	78.65
Aqueous extract (400mg/kg)	0.45 ± 0.04	1.07 ± 0.05	1.02 ± 0.04	0.99 ± 0.07	0.90 ± 0.08	67.97
Aqueous extract (600mg/kg)	0.47 ± 0.08	0.99 ± 0.04	0.93 ± 0.07	0.88 ± 0.05	0.81 ± 0.06	71.17

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

Table 3: Antipyretic effect of aqueous extract in comparison to Aspirin

	Initial Rectal Temp (°C)	Rectal Temperature in °C after 18hrs of Yeast Injection (Mean ± SEM)						Reduction in temperature after 4 hr
		0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	
Control	36.40 ± 0.04	37.22 ± 0.07	37.62 ± 0.03	37.53 ± 0.05	37.4 ± 0.03	37.31 ± 0.02	37.26 ± 0.04	- 0.24%
Standard	36.82 ± 0.06	37.99 ± 0.04	37.36 ± 0.02	37.18 ± 0.05	37.12 ± 0.04	36.97 ± 0.05	37.02 ± 0.03	2.69%
Aqueous Extract (400mg/kg)	36.51 ± 0.02	37.7 ± 0.05	37.12 ± 0.06	36.88 ± 0.03	36.82 ± 0.02	36.79 ± 0.07	36.81 ± 0.03	2.41%
Aqueous extract (600mg/kg)	36.65 ± 0.03	37.87 ± 0.02	36.89 ± 0.04	36.75 ± 0.03	36.72 ± 0.04	36.7 ± 0.05	36.68 ± 0.02	3.99%

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

Table 4: Average reaction time using Hot plate method

Groups	0 min	30 min	60 min	90 min	% increase in reaction time after 60 min
Control	3.67 ± 0.049	3.53 ± 0.043	3.48 ± 0.054	3.62 ± 0.060	-5.18%
Standard Morphine	3.45 ± 0.043	7.07 ± 0.076	7.58 ± 0.060	7.12 ± 0.031	107.89%
Aqueous Extract 400 mg/kg	3.35 ± 0.022	5.45 ± 0.060	5.66 ± 0.048	5.94 ± 0.033	68.96%
Aqueous Extract 600 mg/kg	3.36 ± 0.033	6.06 ± 0.026	6.36 ± 0.054	6.84 ± 0.031	89.29%

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

Table 5: Average reaction time during Tail immersion method

Group	0 min	30 min	60 min	90 min	% increase in reaction time after 60 min
Control	3.07 ± 0.056	3.25 ± 0.056	3.23 ± 0.042	3.35 ± 0.043	5.21%
Standard Aspirin	3.27 ± 0.049	6.08 ± 0.130	6.75 ± 0.056	6.27 ± 0.080	106.42%
Aqueous Extract 400 mg/kg	3.07 ± 0.059	5.63 ± 0.040	5.67 ± 0.031	5.19 ± 0.037	84.69%
Aqueous Extract 600 mg/kg	3.05 ± 0.043	5.82 ± 0.021	5.92 ± 0.033	5.40 ± 0.043	94.09%

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

Table 6: Analgesic effect during Writhing method

	Average number of Writhings /15 min	% Inhibition
Control	58.78 ± 1.35	0.0
Standard (Aspirin)	23.66 ± 0.88	59.78
Aqueous Extract 400 mg/kg	32.63 ± 1.03	44.53
Aqueous Extract 600 mg/kg	28.89 ± 0.98	50.89

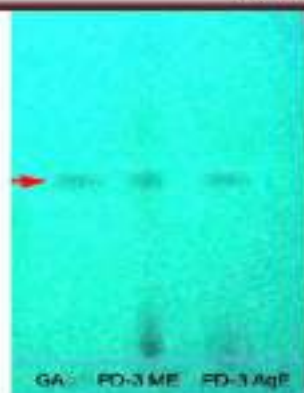


Figure 1: TLC plate obtained using the solvent system: Toluene: Ethyl acetate: Formic acid: MeOH (3: 3: 0.8: 0.2)

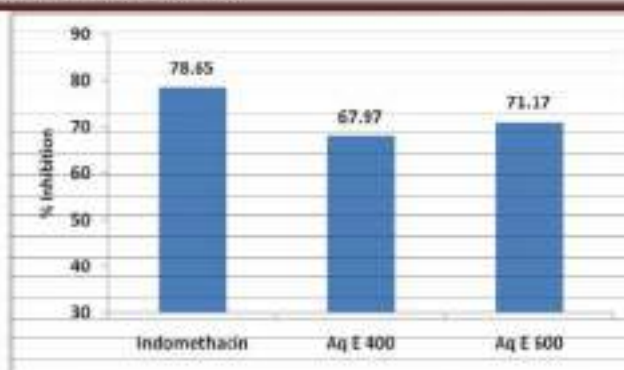


Figure 2: Inhibition of acute inflammation in rat paws during carrageen-induced paw oedema test

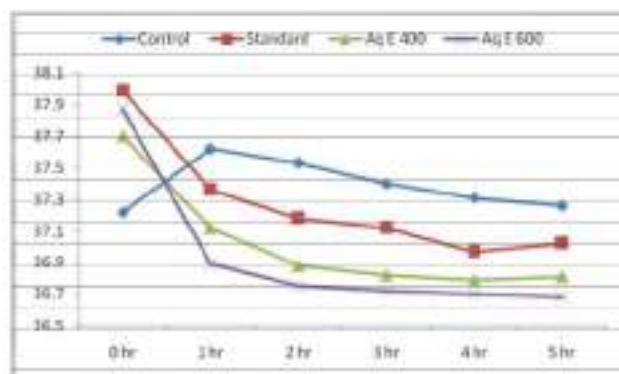


Figure 3: Rectal temperature of rats during yeast induced pyrexia test

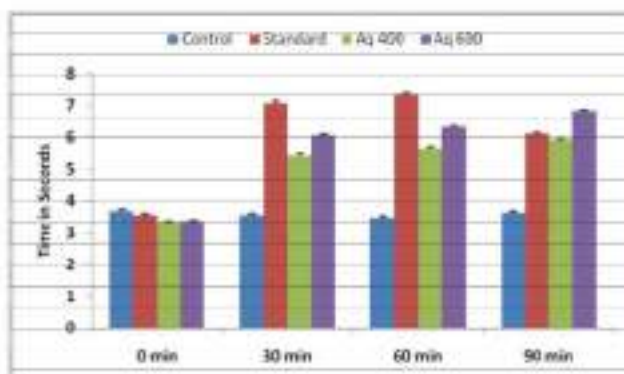


Figure 4: Reaction time using Hot plate method

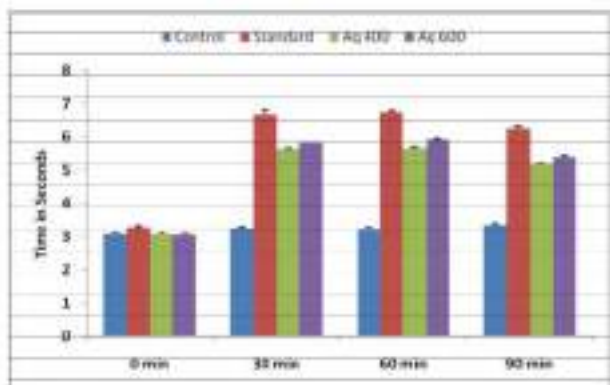


Figure 5: Central analgesic effect during Tail immersion method

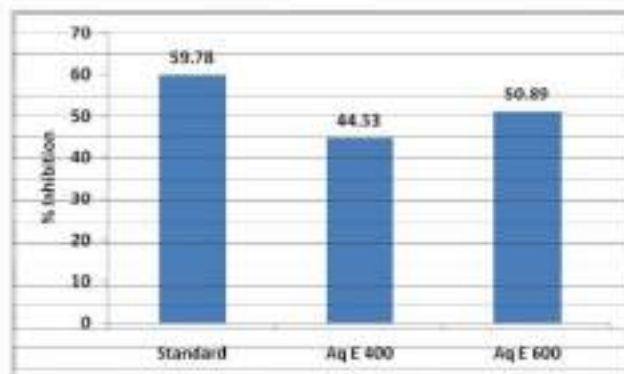


Figure 6: Peripheral analgesic effect in Writhing method

Elemental analysis

Only sulphur was found present in the research drug, while nitrogen and halogens were found to be absent.

Thin Layer Chromatography (TLC)

After several runs in different solvent environments, flavonoids in the hydrolyzate of the research drug moved distinctly in flavonoid-specific solvent front Toluene: Ethyl acetate: Formic acid: MeOH (3: 3: 0.8: 0.2) with R_f values of 0.344, 0.7444 and 0.967. Preliminary separation observed in TLC plate (Figure 1) is suggestive of more than one flavonoidic components in the research drug.

Estimation of total polyphenol content

The total phenol content was estimated to be 24.80 mg/gm of dry mass as calculated from the standard curve of Gallic acid.

Experimental Methods

Acute Toxicity study

The animals tested in acute toxicity study showed no significant toxic symptoms up to the dose of 600 mg/kg such as sedation, convulsion, diarrhoea, irritation, etc. At dosage level of 1000 mg/kg, some animals showed mild symptoms of irritation and minor behavioural changes but returned to normal condition during a few hours. Even at this high dose, no further toxic symptoms or mortality was observed for next 24 hrs and subsequently up to 14 days.

Anti-inflammatory studies (Carrageenan-induced paw oedema)

The circumference of paw oedema in rats induced by the carrageen agent at 400 mg/kg and 600 mg/kg dose showed significant decrease of 67.97% and 71.17% respectively ($p < 0.05$) in inflammation up to 4 hours as compared to the control group (table 2). The inhibition of acute inflammation in the rat paws was found to be a little lower in 600 mg/kg of test drug dosage (71.17% inhibition) as compared to the standard drug Indomethacine (78.65% inhibition) after 4 hours of treatment (Figure 2).

Antipyretic effect of aqueous extract on yeast induced pyrexia in rats

The yeast induced pyrexia experiment on rats showed an average mean increase of about 1.11 °C in rectal temperature after 18 hrs of yeast injection. The test drug extract showed significant antipyretic effect at both the doses (400 mg/kg and 600 mg/kg). The antipyretic effect of the extract at the dose of 600 mg/kg is highly significant ($p < 0.05$) and comparable to that of the standard drug, Aspirin. The test drug administration resulted in a sharp decrease in the elevated rectal temperature after 1hr of administration which was further followed by a gradual trend of decrease in a dose dependent manner. Unlike Aspirin and 400 mg/kg aqueous extract which showed antipyretic activity up to 4 hrs, the effect of the 600 mg/kg aqueous extract remained sustained throughout the test period of 5 hrs. Thus, by the end of the 5th hr of the experiment, the overall body temperature of rats in the 600 mg/kg dose group almost came down to the initial state while the animals in the other groups still showed an elevated body temperature. The effect of the aqueous extract of test drug on yeast induced pyrexia has been shown in table 3.

The overall reduction in the rectal temperature of animals after 4 hours of oral drug administration was 2.69 % in case of standard drug, 2.41 % in 400 mg/kg test drug and 3.09 % in 600 mg/kg test drug while the control group showed an increase of 0.24 % during this period. From the perspective of percentage reduction of rectal temperature after 4 hours, 600 mg/kg dose of the test drug showed more pronounced results as compared to the standard drug Aspirin. The test drug also exhibited more sustained and persistent antipyretic effect lasting up to 5 hours during this test (Figure 3).

Evaluation of Analgesic activity

Assessment of central analgesic effect

Hot plate method

The reaction time was increased significantly up to 60 minutes after giving the thermal stimulus in case of the 400 mg/kg drug group (increasing from 3.35 sec to 5.66 sec) & 600 mg/kg test drug group (increasing from 3.36 sec to 6.36 sec) when compared with the control group (where it actually decreased from 3.67 sec to 3.48 sec) (table 4). The test drug at the dose of 600 mg/kg showed similar type of pattern up to 90 minutes (reaction time increasing from 3.36 sec to 6.84 sec) but slightly lower central analgesic effect when compared with the standard drug morphine sulphate (where the reaction time increased from 3.55 sec to 7.12 sec) (Figure 4).

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 107.89 % in case of standard group, 68.96 % in case of 400 mg/kg test drug group and 89.29 % in case of

600 mg/kg group. In comparison to this, the control group actually showed a 5.18 % decrease in reaction time after 60 minutes. The test drug thus 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 results of test drug assessed in terms of the average reaction time at the dose of 600 mg/kg (89.29% increase) showed a lower central analgesic effect as compared with the standard drug Morphine (107.89% increase).

Tail immersion method

Significant increase in reaction time was observed up to 60 minutes after giving thermal stimulus during the tail immersion method in case of both the 400 mg/kg drug group, where the increase was from 3.07 sec to 5.67 sec, and the 600 mg/kg test drug group where the reaction time went up from 3.05sec to 5.92 sec (table 5). In comparison, the reaction time increased from 3.15 sec to only 3.35 sec in the control group. During observations up to 90 minutes, a similar pattern was observed – the reaction time of 600 mg/kg test drug group increased from 3.05 sec to 5.40 sec (Figure 5). However, its overall central analgesic effect was a little lower when compared with the standard drug Aspirin where the reaction time increased from 3.27 sec to 6.27 sec during this period.

The increase in reaction time up to 60 minutes after oral administration of drug and giving thermal stimulus in case of tail immersion method was 106.42 % in case of the standard group, 84.69 % in case of the 400 mg/kg test drug group and 94.09 % in case of the 600 mg/kg group. The control group showed only 5.21% 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 showed lower central analgesic effect as compared to the standard drug Morphine.

Assessment of peripheral analgesic effect (acetic acid induced writhing analysis)

The peripheral analgesic effect in the Writhing test was evaluated on the basis of the average number of abdominal contractions indicated by the extension of hind paw of animals. The observed inhibition in writhings as a result of administration of the test drug was significantly higher ($p < 0.05$) at the dose of 400 mg/kg (44.53%) as well as at 600 mg/kg (50.89 %) when compared with the control group (table 6). Comparing the performance of the test drug with the standard drug, the observed peripheral analgesic effect was slightly lower at 600 mg/kg test drug dose as indicated by 50.89 % inhibition in writhings as compared to the standard drug Aspirin which resulted in 59.78% inhibition (Figure 6).

DISCUSSION

Inflammation is clinically defined as a patho-physiological process characterized by redness, edema, fever, pain and loss of function. Inflammation can result in locally increased production of free radicals by inflammatory enzymes, as well as the release of inflammatory mediators that promote cell proliferation and angiogenesis and inhibit apoptosis. Although the currently used steroidal anti-inflammatory drugs (SAIDs) and non-steroidal anti-inflammatory drugs (NSAIDs) treat acute inflammatory disorders, these

conventional drugs have not been successful to cure chronic inflammatory disorders such as rheumatoid arthritis (RA) and atopic dermatitis (AD). Moreover, usage of many of such drugs has been associated with adverse impact upon the hepatic and renal functions^{24,25}.

The preliminary chemical analysis of aqueous extract of this botanical following standard method indicates high concentration of phenolic compounds such as flavonoids, tannins and carbohydrates. Flavonoids are a large family of compounds synthesized by plants that have a common chemical structure. The flavonoidic phenolic compounds have been known to exhibit anti-inflammatory, antioxidant and metal-chelating properties^{9,24}.

The Hot plate and tail immersion methods indicated that the central analgesic effect of the aqueous extract of test drug was significant and dose dependent as revealed by the increased reaction time after giving thermal stimulus to the mice. The latency in reaction time continued up to 90 minutes after the drug administration in both 400 mg/kg and 600 mg/kg extracts revealing sustained and pronounced central analgesic effect when compared with the control group. However, its overall central analgesic effect was a little lower when compared with the standard drug Morphine over different time periods, the higher test drug dosage resulting in a more pronounced effect.

The assessment of peripheral analgesic effect of the test drug exhibited significant percentage inhibition in the writhings which were induced by acetic acid in the mice at both the 400 mg/kg and 600 mg/kg doses of the aqueous extract when compared with the control group. The percentage inhibition of writhings at the higher dose of 600 mg/kg indicated the pronounced peripheral analgesic effect in the context of visceral pain which was comparable to the standard pure drug Morphine within 15 minutes of test. The significant analgesic effect at the higher dose was attributed to the presence of high concentration of flavonoidic compounds which inhibited the synthesis, release or receptor responses in prostaglandin mediated effects^{9,26,27}.

During antipyretic tests, the percentage reduction in rectal temperatures after yeast injection up to 4 hours was quite noticeable and comparable to the standard group in case of 600 mg/kg test drug. The observed antipyretic effect was found to be sustained and lasted up to at least 5 hours after oral administration of the drug sample in case of both the 400 mg/kg and 600 mg/kg aqueous samples which showed similar pattern of antipyretic efficacy. Pyrexia is a result of secondary impact of infection, tissue damage, inflammation, graft rejection, malignancy or other diseased states. Mediators like interleukin 1 β , α , β and TNF- α increase the synthesis of Prostaglandin E2 near pre-optic hypothalamus area thereby triggering the hypothalamus to elevate the body temperature. Flavonoids are known to target prostaglandins which are involved in pyrexia^{28,29}.

The evaluation of acute inflammation which was induced by the carageenan in the paw of rats demonstrated the significant percentage decrease in circumference and oedema resulting from administration of the aqueous extract of test drug in doses of 400 mg/kg and 600 mg/kg. The percentage inhibition of acute inflammation in the paw of rats was found to be lower in case of 600 mg/kg of test drug dosage when compared to the standard drug Indomethacine after 4 hours of treatment. The acute and sustained effect on the induced inflammation up to four hours depends upon the decreased production of pro-inflammatory cytokines and PGE₂ in the tissue of the effected part of the body. The inflammation

induced by phlogistic 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 arachidonic acid metabolites. Prostaglandins and nitric oxide biosynthesis is involved in inflammation, and isoforms of inducible nitric oxide synthase (iNOS) and of cyclooxygenase (COX-2) are responsible for the production of a great amount of these mediators. It has been demonstrated that flavonoids are able to inhibit both enzymes, as well as other mediators of the inflammatory process such as reactive C protein or adhesion molecules³⁰.

The variety of flavonoids have been found to have antimicrobial, antiviral, anti-ulcerogenic, cytotoxic, anti-neoplastic, mutagenic, antioxidant, antihypertensive, antihypertensive, hypolipidemic, antiplatelet and antipyretic anti-inflammatory activities. Flavonoids also have biochemical effects, which inhibit a number of enzymes such as aldose reductase, xanthine oxidase, phosphodiesterase, Ca²⁺-ATPase, lipoxygenase, cyclooxygenase, etc.^{9,11}.

The detailed scientific evaluation of the pharmacological properties namely the analgesic, antipyretic and anti-inflammatory properties of the botanical medicine clearly exhibit its therapeutic efficacy which was found to be comparable to that of the standard drugs. The test drug was found to be non toxic, significantly effective and having sustained effect during this study. Many of these activities could be attributed to the presence of flavonoids in the aqueous extract. However, further detailed studies are needed to identify the responsible bio-chemical marker compound in the test drug.

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