Available online at www.elixirpublishers.com (Elixir International Journal)

Pharmacy

Elixir Pharmacy 44 (2012) 7265-7270

ISSN: 2229-712X

Analgesic and anti-inflammatory activities of Indian medicinal plant *Ziziphus xylopyrus* stem barks in experimental animal models

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ARTICLE INFO

Article history:

Received: 3 December 2011;

Received in revised form:

17 February 2012;

Accepted: 9 March 2012;

Keywords

Ziziphus xylopyrus,
Hot plate,
Tail immersion,
Acetic acid- induced writhing,
Carrageenan.

ABSTRACT

In this study chloroform and methanol extracts of *Ziziphus xylopyrus* stem barks were tested for analgesic (Hot plate, Tail immersion and Acetic acid- induced writhing method) and anti-inflammatory activity (paw edema induced by carrageenan) in mice and rats, respectively. The methanolic extract in doses of 50, 100 and 200 mg/kg showed 10.91, 34.95 and 57.61 percentage of protection from writhing respectively and the percentage inhibition of paw edema at the end of four hours was 1.26, 24.05 and 35.42 respectively. In the Hot plate and Tail immersion models, methanolic extract in the above doses increased the pain threshold significantly after 30 min, 1, 2 and 3h of administration. Methanolic extract showed dose-dependent action in all the experimental models in different doses whereas chloroform extract was not able to show such remarkable significant activities.

Introduction

As presently available synthetic analgesic and anti-inflammatory drugs pose several health problems during their clinical use, search to develop new and more effective drugs with fewer side effects is necessary. The use of natural products is growing in the world especially in developing countries like India where over 75% of the population relies mainly on plants and plant extracts for healthcare.

Ziziphus Xylopyrus (family Rhamnaceae) is such a plant which is commonly found in various parts of north-western India, Uttar Pradesh, Bihar and Central and South India[1]. As per the ethnomedicinal information, various parts of this plant possess several medicinal properties. The fruit powder with pinch ginger powder thrice in a day is useful for stomachache and indigestion[3]. It also possess antidepressant, antimicrobial and anthelmintic activities as per the available information[4,5,6]. The reported chemical constituents present in this plant are quercetin and quercitrin in leaves; catechol-type of tannins (8-12%), oleanolic acid, l-epicatechin, l-leucocyanidin, 3, 3, 4-tri-O-methyl-ellagic acid in fruits; tannin (7.2%), d-7, 3', 4'-trihydroxyflavan-3, 4-diol and oleanolic acid in barks[1]. The stem wood of the plant is reported to contain triterpenoid compounds[7], alkaloids (xylopyrine-A & B)[8,9] and flavonoids[4]. On this basis, the objective of the present investigation was to study the analgesic and anti-inflammatory effects of stem barks of chloroform and methanol extracts of *Z. xylopyrus*.

Materials and methods

Plant material

The stem barks of the selected plant were collected from the forest of Similipal Biosphere Reserve, Mayurbhanj, Orissa, India in August 2006. The plant material was identified and authenticated taxonomically at the Central National Herbarium, Botanical Survey of India, Botanical Garden, Howrah-711103, West Bengal, India (Ref no-CNH/I-I(59)/2006/Tech II, dated-27.10.2006). A voucher specimen of the collected sample was deposited in the institutional herbarium for future reference.

Preparation of extracts

The said plant parts were cleaned, dried under shade and powdered by a mechanical grinder. Hundred grams of the pulverized stem bark was extracted with petroleum ether, chloroform and methanol successively in a soxhlet apparatus. Petroleum ether was used in initial step of extraction for defatting the plant materials. The successive extracts were separately filtered and concentrated at reduced temperature on a rotary evaporator. The yield of petroleum ether, choloform and methanol extract was found to be around 3.21, 5.10 and 15.13% (W/W) respectively. For the pharmacological tests the extracts were soluble in 1% DMSO solution.

Phytochemical Studies

The successive extracts were subjected to phytochemical screening tests for the detection of various constituents using conventional protocol[10,11].

Animals

Swiss albino mice (25-30g) and albino rats of Wistar strain (150-200g) of either sex were procured from the central animal house of Royal College of Pharmacy and Health Sciences, Berhampur, Orissa, India. They were housed in standard Poly propylene cages and kept under controlled room temperature in a 12h light-dark cycle. The animals were given a standard laboratory diet and water *ad libitum*. Food was withdrawn 12h before and during the experimental hours. All experimental protocols were approved by the Institutional animal ethics committee.

Acute toxicity studies

Acute toxicity studies were carried out according to OECD (Organization for economic cooperation and development

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guidelines no. 425, 2006). The LD_{50} of the test drug was calculated using a computer assisted statistical programme (OECD 2006).

Analgesic activity study:

Hot-plate method (Thermal stimulus)

The mice selected were weighted (25-30g) and groups into eight of six in each and the normal basal reaction time were taken by repeating for 5 times. Group-3 to Group-5 received chloroform extract and Group-6 to Group-8 received methanol extracts respectively at a dose of 50mg/kg, 100 mg/kg and 200mg/kg body weight (p.o.). Group- 2 received Morphine sulphate 5mg/kg body weight (s.c.) and served as standard. Group- 1 administrated 1% DMSO in the dose of 10ml/kg body weight (p.o.) served as control. All animals were lowered onto the surface of a hot plate (50 ± 1.0^0C) enclosed with cylindrical glass and the time for the animal to jump or lick the fore limb was noted as the reaction time (RT). Cut off time in the absence of a response was 15 sec to prevent the animals from being burnt[12]. The observations were made before and after administration of respective drugs at 30 min, 60 min, 120 min, and at the end of 180 min[13,14].

Tail immersion Test (Thermal stimulus)

The Swiss albino mice were selected by immersing the tail in hot water at temperature 55^0C $\pm5^0$C and the basal reaction time was noted. The mice which showed a positive response within a span of 5 seconds for withdrawal of the tail clearly out of water were selected for further studies. The mice selected were weighted (25-30g) and groups into eight of six in each and the normal basal reaction time were taken by repeating for 5 times. Group 3 to Group 5 received chloroform extract and Group 6 to Group 8 received methanol extract respectively at a dose of 50mg/kg, 100 mg/kg and 200mg/kg body weight. Group 2 received Morphine sulphate 5mg/kg body weight (s.c.) and served as a positive control. Group 1 served as solvent control (1% DMSO) and received the dose of 10ml/kg body weight. The observations were made before and after administration of respective drugs at 30 min, 60 min, 120 min, and at the end of 180 min[13,14].

Writhing Test (Chemical stimulus)

Aspirin like non-narcotic analgesic activity of the test extracts was investigated by the ability to protect a painful writhing syndrome in mice. The syndrome is characterized by abdominal torsion, drawing up of hind limbs to the abdominal wall, marked contraction of the abdominal area and periodical arching of the back to rub the abdominal wall on the glazed surface on which the mouse is kept. Writhing was consistently produced in mouse by an intraperitoneal injection of 0.6% aqueous acetic acid. Overnight fasted, healthy adult male albino Swiss mice weighing between 18 to 25gm in groups of six each were taken for present investigation. DMSO 1% solution of the test extracts were administered orally in a dose of 50mg/kg, 100mg/kg and 200mg/kg body weight respectively to the test groups animal.

The control group of animals were given only DMSO 1% solution in the dose of 10ml/kg body weight. One group of animal was administered with Diclofenac sodium as standard, orally in a dose of 5mg/kg (b.w). After a gap of 30 minutes of the administration of the test extracts, all the groups of mice were given the writhing agent, 0.6% aqueous acetic acid, in a dose of 1ml/100gm (b.w) intraperitoneally. Five minutes after administration of acetic acid the number of writhing produced in these animals were counted for next 10 minutes and the number of writhing produce in the treated groups were compared with those in the control group and the percentage protection was calculated as show below[13,14].

Percentage protection
= [(No.of writhes in control - No. of writhes in test)/ No. of writhes in control] x 100

Anti-inflammatory activity study

Carrageenan induced Rat paw edema

The anti-inflammatory activity of the test compounds were evaluated in Wistar rats employing the method[15,16]. Animals were fasted overnight and were divided into control, standard and different test groups each consisting of six animals. The different test extracts were administrated to the animals in the test groups at the dose of 50,100 and 200mg/kg by oral route. Animals in the standard group received Indomethacin at the dose of 4mg/kg, by oral route. Control group animals were received 1% DMSO at the dose of 10ml/kg body weight. Thirty minutes after administration of the respective drugs, all the animals were challenged with 0.1 ml of 1% carrageenan in the sub planter region of left hind paw. Paw volume was measured by using digital plethysmometer before administration of carrageenan and after 30min, 1, 2, 3 and 4 hrs intervals. The efficacy of different drug was tested on its ability to inhibit paw edema as compared to control group.

Volume of edema = Final Paw Volume - Initial Paw Volume

The Percentage inhibition of paw edema was calculated by the formula as below.

% Inhibition of Paw edema = $[(V_C - V_T)/ V_C] \times 100$

Where, V_C = Paw edema of control group and V_T = Paw edema of treated group

Statistical analysis

The results were analysed for statistical significance using One-way ANOVA followed by post Bonferroni test. A *P* value <0.05 was considered significant.

The statistical analysis was performed for all experimental animal models by using one-way ANOVA followed by post test Bonferroni (Graph pad prism 5.0.3.0 version). The mean value ± SEM was calculated for each parameter[17]. All the test samples and standard drug's parameter were compared with control group at respective time. Finally, the test drugs were compared with standard drug to know the significance difference between two groups at respective time.

Results and Discussion

Preliminary phytochemical analysis of different extracts of stem bark of the plant indicated the presence of alkaloids, tannins, steroids and fixed oils in petroleum ether extract. Chloroform extract contains amino acids, flavonoids and steroids whereas methanol extract shows the presence of proteins, carbohydrates, Gums & mucilage, flavonoids, saponins and triterpenoids respectively.

No adverse effect or mortality was detected in albino rats up to 3gm/kg, p.o. of all the extracts of *Z. xylopyrus* during the 24h observation period basing on which the respective doses are selected for further study.

While evaluating analgesic activity of different extracts by hot plate method, it was observed that Morphine sulphate showed significant analgesic effect at 30, 60,120 and 180 minutes. Peak effect was observed at 120 minute. Normal 1% DMSO solution (group-1) did not have any significant change in

basal reaction time. The different dose of methanolic extract of *Z. xylopyrus* showed highly significant effect (P<0.0001) at 30, 60,120 and 180 minutes as compared with control group. The chloroform extract 200mg/kg showed a significant activity (P<0.001) at 30minute and highly significant activity (P<0.0001) at 60,120 and 180 minutes. As compared to standard drug, the methanolic extract at a dose of 200mg/kg was found to have no significant differences (P<0.05) in basal reaction time at different time periods. The methanolic extract at a dose of 200mg/kg showed peak effect 13.2± 0.185 at 120 minute (Table-1).

During the search of analgesic effect of selected extracts of the plant by tail immersion method, it was observed that Morphine sulphate showed highly significant analgesic effect at 30, 60, 120 and 180 minutes. Peak effect was observed at 120 minute. Normal 1% DMSO (group-1) did not have any significant change in basal reaction time. Methanolic extract at a dose of 100mg/kg and 200mg/kg showed highly significant activity (P<0.0001) at different time interval as compared to control group. The methanol extract at 50mg/kg was found to be highly significant (P<0.0001) at 60,120 and 180 minutes as compared to control group. The chloroform extract at 100mg/kg and 200mg/kg showed highly significant activity (P<0.0001) at 60,120 and 180 minutes. The chloroform extract at 50mg/kg was showed significant activity (P<0.001) at 120 and 180 minutes as compared to control. There were no significant differences (P<0.05) in basal reaction time with standard was observed in methanolic extract 200mg/kg at 60,120 and 180 minutes.The methanolic extract of *Z. xylopyrus* at a dose of 200mg/kg showed peak effect of 13.6±0.173 at 180 minute (Table-2).

While searching the analgesic efficacy of different extracts by acetic acid induced writhing method, it was found that the standard drug Diclofenac sodium showed highly significant analgesic activity in acetic acid induced writhing method in Swiss albino mice. Normal 1% DMSO (group-1) did not have any significant decrease in average numbers of writhes. *Z. xylopyrus* methanolic extract at a dose of 100mg/kg and 200mg/kg showed highly significant activity (P<0.0001) as compared to control group. The methanol extract at 50mg/kg was found to be significant analgesic (P<0.001) activity. The chloroform extract at 200mg/kg showed highly significant activity (P<0.0001). There was no significant differences (P<0.05) in the average numbers of writhes with standard as was observed in methanolic extract at 200mg/kg dose level.

Diclofenac sodium at the dose of 5mg/kg body weight showed significant inhibition (62.73%) of writhes in acetic acid induced writhing method in Swiss albino mice while methanolic extract at a dose of 100 and 200mg/kg body weight showed the percentage inhibition of 34.95% and 57.61% (Table-3).

During the search for anti-inflammatory efficacy of selected extracts of the plant using Carrageenan induced Rat paw edema method, it was quite evident that, a gradual increase in paw volume was observed after carrageenan administration and which reached maximum at 3hour and then declined. The standard drug Indomethacin at a dose level of 4mg/kg body weight showed highly significant activity (P<0.0001) as compared to control group at 1, 2, 3 and 4 hours.

Methanolic extract at a dose of 200mg/kg showed highly significant anti-inflammatory activity (P<0.0001) as compared to control group at 2, 3 and 4 hours respectively. The methanol extract at 100mg/kg was found to have significant activity (P<0.001) at 3 hour. There were no significant differences (P<0.05) in paw edema volume when standard was observed along with methanolic extract 200mg/kg dose level (Table 4 & 5).

The standard drug Indomethacin at a dose of 4mg/kg body weight inhibited the development of edema significantly from 0.5 hour onwards. It showed maximum percentage reduction (50.63%) in paw edema at 4 hour. Methanolic extract at the dose of 100 and 200mg/kg body weight showed percentage of inhibition of paw edema at 4 hour 24.05% and 35.42% respectively (Table-6).

Conclusion

On the basis of the outcome of the present study, it is concluded that the selected plant *Z. xylopyrus* is endowed with potential analgesic and anti-inflammatory activities and the results of the study further scientifically justifies the use in the folklore remedies as analgesic and anti-inflammatory agent since ancient times. The stem bark of the plant possessing both analgesic and anti-inflammatory properties, suggested the presence of non-steriodal anti-inflammatory property, which may be mediated through the prostaglandin inhibition in the living system. To study the mechanism of the action in depth requires further studies and conformations.

The phytochemical study of biologically active methanolic extract of the plant showed the presence of proteins, carbohydrates, flavonoids, saponins and triterpenoids which have been reported to be promising anti-inflammatory and analgesic agents in animal models as per the literature research[18,19,20]

However, the exact active constituent(s) responsible for the analgesic and anti-inflammatory actions may further be isolated and characterized as future work.

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Table 1. Evaluation of analgesic activity of chloroform and methanol extracts of *Z. xylopyrus* bark by hot plate method

Group	Treatment	Dose (mg/kg bw)	Basal reaction time	Reaction time(in sec) after administration of drugs at different time (minutes)			
				30	60	120	180
1	Control (1% DMSO)	10	4.5±0.115	5.0±0.208	4.6±0.145	4.8±0.152	5.1±0.152
2	Morphine sulphate	5	4.7±0.115	9.7±0.057†	12.8±0.115†	13.9±0.100†	13.4±0.115†
3	Choloform Extract	50	4.6±0.152	5.1±0.100	5.5±0.152‡	5.4±0.173	5.3±0.115
4		100	4.8±0.152	5.4±0.173	5.8±0.115†	6.0±0.360↑	6.1±0.057†
5		200	5.1±0.208	6.1±0.057‡	6.3±0.057†	6.5±0.115†	7.7±0.120†
6	Methanol Extract	50	4.5±0.100	5.8±0.115↑	6.9±0.100†	7.6±0.057†	7.9±0.100†
7		100	5.0±0.100	7.9±0.152†	9.4±0.115†	10.8±0.305†	11.0±0.152†
8		200	4.9±0.100	9.0±0.208#†	12.2±0.173#†	13.2±0.185#†	12.9±0.057#†

All values are expressed in Mean ± SEM, n=6; †p< 0.0001, ‡p< 0.001 and ↑p< 0.05compare with control and '#'- Indicates there is no significant difference between standard and test drug at P< 0.05 significant level.

Table 2. Evaluation of analgesic activity of chloroform and methanol extracts of *Z. xylopyrus* bark by Tail immersion method

Groups	Treatment	Dose (mg/kg body wt)	Basal reaction time	Reaction time(in sec) after administration of drugs at different time (minutes)			
				30	60	120	180
1	Control (1% DMSO)	10	5.3±0.115	5.1±0.057	5.3±0.115	4.9±0.208	5.4±0.173
2	Morphine sulphate	5	4.9±0.100	9.4±0.057†	12.5±0.152†	14.4±0.351†	14.2±0.152†
3	Chloroform Extract	50	4.5±0.152	4.9±0.115	5.6±0.057	6.2±0.100‡	6.4±0.057‡
4		100	4.7±0.115	5.5±0.152	6.4±0.057†	7.1±0.152†	7.0±0.115†
5		200	4.3±0.152	5.4±0.173	6.8±0.057†	7.7±0.115†	8.2±0.115†
6	Methanol Extract	50	5.1±0.100	5.8±0.115†	6.7±0.152†	7.8±0.115†	7.9±0.100†
7		100	4.8±0.100	6.4±0.057†	8.2±0.120†	9.9±0.152†	10.6±0.057†
8		200	4.6±0.100	8.4±0.100†	11.9±0.145$^{\#\dagger}$	13.5±0.133$^{\#\dagger}$	13.6±0.173$^{\#\dagger}$

All values are expressed in Mean ± SEM, n=6; †p< 0.0001, ‡p< 0.001 and ↑p< 0.05compare with control and '#'- Indicates there is no significant difference between standard and test drug at P< 0.05 significant level.

Table 3. Evaluation of analgesic activity of chloroform and methanol extracts of *Z. xylopyrus* bark by Acetic acid induced Writhing method

Groups	Treatment	Dose	Avg. no. of Writhing	% Inhibition
1	Control (1% DMSO)	10ml/Kg	76.66±0.594	-
2	Diclofenac sodium	5mg/kg	28.57±1.225†	62.73%
3	Chloform Extract	50 mg/kg	75.32±0.614	1.74%
4		100mg/kg	69.45±1.644†	9.40%
5		200mg/kg	61.25±0.797†	20.10%
6	Methanol Extract	50 mg/kg	68.29±1.087‡	10.91%
7		100mg/kg	49.86±1.942†	34.95%
8		200mg/kg	32.49±0.701$^{\#\dagger}$	57.61%

All values are expressed in Mean ± SEM, n=6; †p< 0.0001, ‡p< 0.001 and ↑p< 0.05compare with control and '#'- Indicates there is no significant difference between standard and test drug at P< 0.05 significant level.

Table 4. Determination of paw volume of rats at different time for *Z. xylopyrus* extracts

Groups	Initial paw volume	Paw volume at different time interval (in ml)				
		0.5hr	1hr	2hr	3hr	4hr
Control(1%DMSO)	1.22±0.182	1.50±0.122	1.63±0.109	1.89±0.370	2.08±0.106	2.01±0.185
Indomethacin (4mg/kg)	1.25±0.303	1.40±0.101	1.48±0.158	1.61±0.110	1.70±0.121	1.64±0.135
ZXCH (50 mg/kg)	1.27±0.101	1.55±0.100	1.67±0.121	1.94±0.143	2.10±0.221	2.07±0.070
ZXCH (100 mg/kg)	1.23±0.110	1.50±0.092	1.63±0.102	1.91±0.143	2.09±0.271	2.02±0.043
ZXCH (200 mg/kg)	1.25±0.124	1.50±0.094	1.64±0.111	1.88±0.106	2.04±0.269	1.98±0.064
ZXME (50 mg/kg)	1.24±0.105	1.47±0.096	1.63±0.144	1.92±0.106	2.09±0.206	2.02±0.072
ZXME (100 mg/kg)	1.25±0.117	1.50±0.133	1.61±0.079	1.79±0.060	1.93±0.015	1.85±0.015
ZXME (200 mg/kg)	1.24±0.106	1.46±0.115	1.53±0.045	1.69±0.058	1.83±0.146	1.75±0.076

ZXCH = *Z. xylopyrus* chloroform extract, ZXME = *Z. xylopyrus* methanol extract
All values are expressed in Mean ± SEM, n=6

Table 5. Differences in paw edema volume of rats at different time for *Z. xylopyrus* extracts

Groups	Paw edema volume at different time interval (in ml)				
	0.5hr	1hr	2hr	3hr	4hr
Control(1%DMSO)	0.28±0.025	0.41±0.020	0.67±0.011	0.86±0.020	0.79±0.030
Indomethacin(4mg/kg)	0.15±0.005‡	0.23±0.020†	0.36±0.026†	0.45±0.025†	0.39±0.040†
ZXCH (50 mg/kg)	0.28±0.017	0.40±0.026	0.67±0.025	0.83±0.015	0.80±0.011
ZXCH (100 mg/kg)	0.27±0.020	0.40±0.035	0.68±0.020	0.86±0.020	0.79±0.020
ZXCH (200 mg/kg)	0.25±0.020	0.39±0.020	0.63±0.030	0.79±0.030	0.73±0.020
ZXME (50 mg/kg)	0.27±0.020	0.39±0.040	0.68±0.020	0.85±0.020	0.78±0.025
ZXME(100 mg/kg)	0.25±0.011	0.36±0.041#	0.54±0.023†	0.68±0.040‡	0.60±0.036†
ZXME(200 mg/kg)	0.22±0.017#	0.29±0.020#	0.45±0.011#†	0.59±0.005†	0.51±0.005#†

ZXCH = *Z. xylopyrus* chloroform extract, ZXME = *Z. xylopyrus* methanol extract, All values are expressed in Mean ± SEM, n=6; †p< 0.0001, ‡p< 0.001 and ↑p< 0.05 compare with control and '#'- Indicates there is no significant difference between standard and test drug at P< 0.05 significant level.

Table 6. Data of % Inhibition of rat paw edema by *Z. xylopyrus* extracts

Groups	% Inhibition of rat paw edema at different time				
	0.5hr	1hr	2hr	3hr	4hr
Indomethacin (4mg/kg)	46.42	43.90	46.26	47.67	50.63
ZXCH (50 mg/kg)	-	2.43	-	3.48	-
ZXCH (100 mg/kg)	3.57	2.43	-	-	-
ZXCH (200 mg/kg)	10.71	4.87	5.97	8.13	7.59
ZXME (50 mg/kg)	3.57	4.87	-	1.16	1.26
ZXME (100 mg/kg)	10.71	12.19	19.40	20.93	24.05
ZXME (200 mg/kg)	21.42	29.26	32.83	31.39	35.42

ZXCH = *Z. xylopyrus* chloroform extract, ZXME = *Z. xylopyrus* methanol extract, '-'Indicates no inhibition.