ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Effect of *Trapa bispinosa* on HDAC Level in Animal Tissues for its Anti-arthritic Activity.

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ABSTRACT

The objective of this work was to evaluate the Hydroethanolic extract of Trapa bispinosa for its antiarthritic activity. Arthritis is inflammation of one or more joints which results in pain, swelling, and limited movement cartilage damage and erosions of the underlying bone. The severe side effects of steroidal and non-steroidal anti-inflammatory drugs evoked us to search for new anti-inflammatory drugs from the indigenous source. The hydroethanol extract of fruits of *Trapa bispinosa* Roxb was evaluated for anti- arthritic activity against the carrageenan, histamine and Freund's complete adjuvant induced rat paw edema and HDAC1 Assay. The hydroethanolic extract of fruits of *Trapa bispinosa* Roxb (200 and 400 mg/kg body weight) exhibited significant activity ($p< 0.01$) against all phlogistic agent used in dose dependant manner. All these effects were compared with reference drug diclofenac sodium (20 mg/kg) and methotrexate (0.75 mg/kg).

Keywords: *Trapa bispinosa,* anti-arthritic, Anti-inflammatory, Carrageenan Histamine, freunds complete adjuvant, Paw edema, HDAC1

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ISSN: 0975-8585

INTRODUCTION

Incidences of inflammatory diseases, especially arthritis are increasing widely. Rheumatoid arthritis (RA) is characterized by persistent joint synovial tissue inflammation. Over the period of time, bone erosion and irreversible joint damage can occur leading to permanent disability. Early inflammatory arthritis can be a self-limiting disease, develop into RA or differentiate into another form of chronic arthritis. As is the case for other forms of arthritis RA is thought to result from the combination of genetic susceptibility and exposure to an appropriate environmental trigger. It is the second most common form of arthritis and the most common autoimmune disease [1].

Many of current drugs for the treatment of arthritis and inflammation have been reported to have undesirable side effects. Side effects and adverse effects caused by the treatments are mainly stomach ulcers, gastrointestinal bleeding, kidney failure, heart attacks and strokes. In spite of newer advances made in drug therapy of inflammation, the search for an ideal drug is still on(the treatment of RA have been developed in the past few decades, there is still and urgent need for more effective drugs with lower side effects [1].

Natural or alternative, medicine is often thought of as a phenomenon of new age; in reality much of it is older than human history. Every society has herbal cure and folk remedies, many of which have been incorporated into orthodox medicine. In fact, it is esteemed that many of the modern drugs originated with natural plant sources [2].

Traditionally, *Trapa bispinosa* has been used in India for several important medicinal purposes. It has been used as nutritive, astringent, aphrodisiac, cooling, appetizer, tonic, anti-diarrheal etc. In Unani system of medicine it is being used in various diseases like sexual weakness, sore throat, bilious affections, bronchitis, tuberculosis, renal calculi and fatigue etc[1] (Shaikh*et. al.,* 2013). Experimental and clinical studies explored its hepatoprotective [3], anti-microbial [4], anti-bacterial, anti-tumor [5], antioxidant [6], free radical scavenging [7], analgesic [8], anti-bacterial [9], antidiabetic [10], neuroprotective [2], immunomodulatory [2] and neuropharmacological activity [11].

MATERIALS AND METHODS

Plant Material

Fruits of *Trapa bispinosa* Roxb.were procured from crude drug supplier M/s. GokuldasGoverdhandas (237, Budhawarpeth Pune). The plant material was identified and authenticated by Dr. R. B. Bhagat, Head of Botany, Poona District Education Association's Arts, Commerce and Science College, Pirangut Tal. Mulshi, Dist. Pune.

Preparation of seed extract for anti-iflammatory Activity

Dried fruits of *Trapa bispinosa* Roxb.were extracted with 50% mixture of hydro ethanol and concentrated. The concentrated material was washed several times with petroleum ether to remove the resinous matter. Then the mass was filtered and concentrated,dried to get the powder from the extract [2].

Animals

Adult Wistar Albino rats of either sex (150-200g) were used for the present investigation. Animals were housed under standard environmental conditions at temperature (25±20 C) and light and dark (12:12 h). Rats were fed with standard pellet diet and water ad libitum.

Acute toxicity study

The selected adult albino rats were used to determine the dose. The animals were divided in to 8 groups of 6 in each. The animals were fasted overnight prior to the acute experimental procedure. The karbers method [12,13] was used to determine the dose. Gum acacia (2% w/v) was used as vehicle to suspend the extracts and administered intraperitoneally. The control group received 2ml/kg of the vehicle intraperitoneally. The other group received the extract as test drug in one of the following doses – 100, 200, 400, 800, 1000, 2000 and 3000 mg/kg body weight of animal in a similar manner. Immediately after dosing, the animals were observed continuously for first four hours for behavioral changes and for mortality at the end of 24^{th}hrs, 48^{th}hrs and 72 hrs respectively. The toxicity study showed that the hydroethanol extract of drug at a minimum dose of 200 mg/kg onwards shows the reaction in experimental animals. However, no mortality was reported even after 72 hours. This indicates that the hydroethanol extract of *Trapa bispinosa* is safe up to a single dose of 3 g/kg body weight [14].

Antiinflammatory Activity

Carrageenan induced hind paw edema

Male Wistar rats of either sex weighing 150-200 grams were divided into five groups of six animals each. The dosage of the drugs administered to the different groups was as follows. Group I -Control (Normal Saline 0.1ml/kg), Group II – Diclofenac (20 mg/kg, p.o.), Group - III ,IV and V – *Trapa bispinosa*(100,200 mg/kg and 400 mg/kg, p.o.). All the drugs were administered orally. Diclofenac served as the reference standard anti-inflammatory drug. After one hour of the administration of the drugs, 0.1 ml of 1% W/V carrageenan solution in normal saline was injected into the subplantartissue of the left hind paw of the rat and the right hind paw was served as the control. The paw volume of the rats were measured in the digital plethysmograph (Orchid scientific PLM02, Nashik, India), at the end of 0 hr, 1 hr, 3 hr,5 hr. The percentage inhibition in paw edema of the treated groups was compared with that of the control and the inhibitory effect of the drugs was studied. The relative potency of the drugs underinvestigation was calculated based upon the percentage inhibition of the inflammation.

Percentage inhibition

$$\frac{\text{Control (\% increase in paw volume in 3rd hour) -Test (\% increase in paw volume in 3rd hour)}}{\text{Control (\% increase in paw volume in 3rd hour)}} \times 100$$

Histamine induced hind paw edema

Male Wistar rats of either sex weighing 150-200 grams were divided into five groups of six animals each. The dosage of the drugs administered to the different groups was as follows. Group I -Control (Normal Saline 0.1ml/kg), Group II – Diclofenac (20 mg/kg, p.o.), Group - III , IV and V – *Trapa bispinosa* (100,200 mg/kg and 400 mg/kg, p.o.). All the drugs were administered orally. Diclofenac sodium served as the reference standard anti-inflammatory drug. After one hour of the administration of the drugs, 0.1 ml of 1% W/V histamine (1%) solution in normal saline was injected into the subplantar tissue of the left hind paw of the rat and the right hind paw was served as the control. The paw volume of the rats were measured in the digital plethysmograph (Orchid scientific PLM02, Nashik, India), at the end of 0 hr, 1 hr, 3 hr,5 hr. The percentage inhibition in paw edema of the treated groups was compared with that of the control and the inhibitory effect of the drugs was studied. The relative potency of the drugs under investigation was calculated based upon the percentage inhibition of the inflammation.

Antiarthritic Activity

Complete Freund's adjuvant Induced Arthritis in Rats

Male Wister rats weighing between 200-250 gm was selected and divided into 5 groups which are kept on standard diet. On day 1, animals were treated orally and same treatment was continued for 12 days as follows: control group received vehicle, 3 test groups received three different doses of test drug 200, 400 mg/kg p.o and standard group received marketed drug for comparison. All animal received injection of 0.1 ml of complete Freund's adjuvant injected in sub-plantar region of the left hind paw ½ or 1 hr after drug treatment. Paw volumes of both sides was recorded before and after drug treatment plethysmographically and also body weight was recorded. Again paw volume was recorded on day 5, 7, 14 plethysmographically using plethysmometer (Orchid Scientific PLM02, Nashik, India). From day 13 to 21, dosing was stopped. On day 21, the body weight and the severity of the secondary lesions were recorded visually.

HDAC ASSAY; Effect of *Trapa bispinosa* on HDAC Level in Animal Tissues

Male wistar rats weighing between 200-250 gm was selected and divided into 2 groups for each tissue (i.e paw, uterus and lung tissue). Animals were treated orally as follows: 1 test group received hydroethanolic extract of fruit of *Trapa bispinosa* 400 mg/kg body wt .p.o and control group received vehicle for comparison. HDAC1 level was measured by procedure[15] given in rat-specific HDAC1, ELISA kit.

Preparation of reagent

All kit components and sample were brought to normal room temperature (18-25°C) before use. Standard solution (50 ng/ml) was reconstituted with standard diluents (0.5ml) in 7 tubes to get concentration of 50, 25, 12.5, 6.25, 3.12, 1.56 ng/ml and last tube was blank with standard diluents as 0 ng/ml and kept for 10 minutes. Assay diluents A & B were diluted with 6 ml of distilled water to make up the volume to 12 ml of both. Detection

reagent A& B were diluted with prepared assay diluents A and B respectively in 1:100m proportion. Wash solution (20 ml) was diluted with 580 ml of distilled water.

HDAC Assay procedure

100 µl of prepared standard dilutions (7 wells) and standard diluents (1 well as blank), samples were added in wells and incubated for 2 hrs at 37 °C by covering it with plate sealer. Liquid was removed completely from each well by snapping plate onto absorbent paper. 100 µl of detection reagent A & B were added in each plate and incubated for 1 hr and 30 min respectively by covering it with plate sealer. Solution was aspirated and washed with 350 µlwash solution for 3-5 min after addition of each detection reagent A & B. 90 µl of substrate solution was then added to each well and incubated for 15-25 mins by covering with plate sealer. Liquid was turned blue after addition of substrate solution. Finally 50 µl of stock solution was added to each well. Liquid turned yellow after addition of solution. Absorbance was taken at 450 nm immediately on ELISA reader [15].

Statistical analysis

Data obtained were statistically analyzed by using suitable parametric or non-parametric test by ANOVA Software and Results considered significant at $p< 0.05$.

RESULTS AND DISCUSSION

The hydroethanol extract of did not exhibit any mortality up to the dose level of 2000 mg/kg. So at this concentration the extracts were found to be safe for long term administration. The hydroethanolextract of *Trapa bispinosa*at the dose level of 200 and 400 mg/kg decreased the edema significantly ($p <0.001$) at 3rdand 4thhrs after administration of the extract when compared to the control group. The effect was compared to the activity ($p <0.001$) produced by standarddrug Diclofenac sodium at 3rdand 4thhrafter administration (Table 1)

Table 1: Effect of Hydroethanol Extract of *Trapa bispinosa* on Paw Volume Changes (ml) in Carrageen Induced Paw Edema in Rat For 1 Day Treatment.

Groups	Treatment and Dose at mg/kg body wt. p.o.	Mean Paw edema (ml) ±SEM			
		Before injection	1 hr	3hr	5hr
Group I; Normal Control	Tween 80 (1%)	1.25±0.03	2.34±0.15	2.55±0.09	3.06±0.19
Group II; Std Drug	Diclofenac Na 20	1.34±0.06	2.26±0.05	2.19±0.05*	1.75±0.03*
Test Group III; Plant Extract	*Trapa bispinosa* 100	1.36±0.05	2.34±0.04	2.31±0.04*	2.24±0.02*
Test Group IV; Plant Extract	*Trapa bispinosa* 200	1.40±0.05	2.07±0.05*	2.29±0.05*	2.30±0.07*
Test Group V; Plant Extract	*Trapa bispinosa* 400	1.35±0.09	1.9±0.12*	2.35±0.12*	2.09±0.09*
n=6	Whereas, SEM=Standard Error Mean All values are expressed as mean ±SEM (n=6) using the ANOVA followed by Dunnet's test. Result considered as significant at * $p <0.05$ compared with control).				

ISSN: 0975-8585

In the present study, the antiinflammatory activity of the hydroethanol extract of *Trapa bispinosa*has been established. The hydroethanol extract is found to significantly inhibit the carrageenan induced rat paw edema, a test which has significant predictive value for anti-inflammatory agents acting by inhibiting the mediators of acute inflammation. Carrageenan induced inflammation is useful in detecting orally active anti-inflammatory agents.

The development of carrageenan induced edema is believed to be biphasic. The initial phase is attributed to the release of histamine and serotonin. The edema produced at the peak 3rdhour is thought to be due to the release of kinin-like substances, especially bradykinin. The second phase of edema is due to the release of prostaglandins, protease and lysosomes and it is sensitive to most anti-inflammatory drugs [16]. Results of the present study are suggesting that the drugs under investigation predominantly inhibit the release of prostaglandin like substances. The seed extract of Trapa *bispinosa*possesses varying degree of antiinflammatory activity when tested at three different doses. The seed extract of *Trapa bispinosa*at the dose of 200, 400 mg/kg shows highsignificant anti-inflammatory activity at 3rd and 4thhours, where it caused 52.48% and 56.38 % inhibition, as compared to that of 20 mg/kg of diclofenac sodium (74.15%). The tannins, saponines and flavonoids may have the role in anti-inflammatory effect.

Hydroethanol extract of *Trapa bispinosa* at dose 100 mg/kg showed decrease in paw edema at all intervals but significantly (P<0.01) only at 1 hr compare to normal control, however, 200 & 400 mg/kg body wtp.o significantly (P<0.01) decreased paw edema at 1st, 3rd and at 5th hr (0.05) intervals compare to normal control (Table: 2).

Table 2: Effect of Hydroethanol Extract of *Trapa bispinosa* on Paw Volume Changes (in ml) in Histamine Induced Paw Edema in Rats(1 Day Treatment)

Groups	Treatment and Dose at mg/kg body wt. p.o.	Mean Paw edema (ml) ± SEM			
		Before injection	1 hr	3hr	5hr
Group I; Normal Control	Tween 80 (1%)	1.16±0.185	1.85±0.240	1.76±0.21	1.51±0.214
Group II; Std Drug	Diclofenac Na 20	0.55±0.114	0.73±0.105*	0.83±0.1406*	0.73±0.125*
Test Group III; Plant Extract	*Trapa bispinosa* 100	0.86±0.149	1.78±0.157*	1.73±0.211	1.49±0.207
Test Group IV; Plant Extract	*Trapa bispinosa* 200	0.85±0.128	1.65±0.201*	1.59±0.183*	1.40±0.200*
Test Group V; Plant Extract	*Trapa bispinosa* 400	0.71±0.179	1.55±0.095*	1.46±0.115*	1.32±0.070*
n=6	Whereas,SEM=Standard Error Mean All values are expressed as mean ±SEM (n=6) using the ANOVA followed by Dunnet's test. Result considered as significant at * p <0.05, **p<0.01compared with control Group				

ISSN: 0975-8585

The percentage inhibition in paw edema by hydroethanol extract of *Trapa bispinosa* 100 mg/kg body wtp.o. was found to be 47.02% and by 200 & 400 mg/kg of body wtp.o. was found to be 51.35 %, 45.94% respectively (Table:3)

Table 3: Effect of Hydroethanol Extract of *Trapa bispinosa* on Percentage Inhibition (%) At Various Time Intervals InHistamine Induced Paw Edema in Rats (1 Day Treatment).

Groups	Treatment and dose at mg/kg of body wt. p.o.	Percentage inhibition (%) at various time interval		
		1 hr	3 hr	5 hr
Group I; Normal Control	Tween 80 (1%)	---	-----	-----
Group II; Std Drug	Diclofenac Na 20	48.22	55.13	56.02
Test Group III; Plant Extract	*Trapa bispinosa* 100	47.02	5.11	1.32
Test Group IV; Plant Extract	*Trapa bispinosa* 200	51.35	22.72	3.97
Test Group V; Plant Extract	*Trapa bispinosa* 400	45.94	34.65	5.96

In the present study of histamine induced paw edema, administration of histamine subcutaneously in rat paw induced inflammation of paw in control group. In present investigation administration of *Trapa bispinosa* (100, 200 and 400 mg/kg p.o) significantly decreased paw edema. Results of present study suggest that the anti-inflammatory activity of *trapa bispinosa* may be due to inhibition of neutrophil recruitment induced by histamine. Histamine is one of the important inflammation mediators since it has a potent vasodilation and vascular permeability action. Histamine H1 receptors are involved in mediating the inflammation induced by various inflammatory agents [17]. Histamine induced paw edema is a well-established model to study inflammation and neutrophil infiltration in paw tissue. Several reports have confirmed that histamine alone and in association with chemo-attractants such as platelet activating factor, interleukin 8 and leukotriene B4 are involved in the regulation of neutrophil recruitment [18].

Administration of Complete Freund's adjuvant (0.1ml) showed development of paw edema which reached peak on 21st day of injection. Methotrexate treated group showed significant inhibition of paw edema on day 4th ($P<0.05$), 8th ($P<0.01$), 14th ($P<0.01$) and day 21st ($P<0.01$). Hydroethanol extract of *Trapa bispinosa* (200 mg/kg body wtp.o) showed significantly inhibition of paw edema on day 14th and day 21st with $P<0.01$. Also rats treated with *Trapa bispinosa* (400 mg/kg body wtp.o) showed significantly inhibition of paw edema on day 4th ($P<0.05$), 8th ($P<0.05$), 14th ($P<0.01$) and day 21st ($P<0.01$) (Table: 4).

Hematological findings showed that increased in level of ESR (Erythrocyte sedimentation rate) and decrease in level of HB (Haemoglobin) in control group. Rats treated with Methotrexate showed significantly change in ESR and HB with $P<0.01$. Hydroethanol extract *Trapa bispinosa* (200 mg/kg body wtp.o) showed significantly increase in HB with $P<0.05$. Hydroethanol extract *Trapa bispinosa* (400mg/kg body wtp.o) showed significant changes in ESR and HB ($P<0.01$) (Table: 5).

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Table 4: Effect of Hydroethanol Extract of *Trapa bispinosa* on Paw Volume Changes (in ml) in Complete Freund's Adjuvant Induced Arthritic Rats For 21 Days Treatment.

Groups	Treatment and dose at mg/kg of body wt. p.o.	Mean changes in paw edema (± SEM)				% inhibition of paw swelling on 21st day
		4th day	8th day	14th day	21st day	
Group I; Normal Control	CFA(0.1ml)	2.15±0.039	2.10±0.04	1.99±0.036	1.95±0.033	0
Group II; Std Drug	Methotrexate 0.75	1.87±0.034	1.53±0.03	1.26±0.016**	1.03±0.022**	47.17
Test Group III; Plant Extract	*Trapa bispinosa* 200	2.07±0.178*	1.812±0.07*	1.75±0.11*	1.608±0.05**	17.91
Test Group IV; Plant Extract	*Trapa bispinosa* 400	1.87±0.049**	1.510±041*	1.31±0.019**	1.21±0.08**	37.94
n=6	Whereas, CFA=Complete freund's adjuvant, SEM=Standard Error Mean. All values are expressed as mean ±SEM (n=6) using the ANOVA followed by Dunnet's test. Result considered as significant at * p <0.05, **p<0.01compared with control.					

Table 5: Effect of Hydroethanol Extract of *Trapa bispinosa* on Hematological Parameters in FCA Induced Arthritis in Rats (21 Days Treatment).

Groups	Treatment and dose at mg/kg of body wt. p.o.	Hematological Parameters			
		Hb (g/dL)	RBC (x106/mm3)	WBC (x103/mm3)	ESR
Group I; Normal control	FCA (0.1 ml)	9.00±0.18	3.76±0.02	17.43±0.16	10.67±0.42
Group II; Std drug	Methotrexate0.75	8.67±0.25	3.78±0.01	12.78±0.27*	10.50±0.43
Test Group III; Plant Extract	TB 400	10.33±0.21*	4.49±0.02*	9.36±0.19*	7.53±0.26*
n=3	Whereas, FCA= freunds Complete adjuvant, SEM=Standard Error Mean, HB=Hemoglobin, RBC=red blood cells, WBC=white blood cells, ESR=erythrocytes sedimentation rate. All values are expressed as mean ±SEM (n=3) using the ANOVA followed by Dunnet's test. Result considered as significant at * p <0.05, **p<0.01compared with control.				

In complete Freund's adjuvant induced arthritis, administration of hydro ethanol extract of *Trapa bispinosa* 200, 400 mg/kg p.o significantly decreased edema in rat paws and the maximum effect was observed on 14th, 18th and 21st day.

Freund's adjuvant induced arthritis model was used to assess the anti-arthritic activity. In the present study, the rats were selected to induce arthritis because rats develop a chronic swelling in multiple joints with the influence of inflammatory cells, erosion of joint cartilage and bone destruction. It has close similarities to human rheumatoid disease. The determination of rat paw swelling is apparently simple, sensitive and one of the quick procedures for evaluating the degree of inflammation and the therapeutic effects of drugs [19]. Significantly decreased levels of RBC and Hemoglobin were observed in arthritic rats (Normal vehicle control group). There was a significant improvement in the levels of Hemoglobin and RBC in *Trapa bispinosa (*400 mg/kg) treated rats. The increased levels of WBC, ESR were significantly suppressed by the hydro ethanol extract of *Trapa bispinosa* (400 mg/kg) administered arthritic group.

ISSN: 0975-8585

The chronic inflammation involves the release of number of mediators like cytokines, GM-CSF (Granulocyte macrophage-colony stimulating factor), interferon's and PDGF (platelet-derived growth factor). These mediators are responsible for the pain, destruction of bone and cartilage that can leads to severe disability. In arthritic condition, there is a mild to moderate rise in WBC count due to the release of IL-IB inflammatory response, IL-IB increases the production of both granulocyte and macrophages colony stimulating factors [19].

Hydroethanol extract *Trapa bispinosa* (400 mg/kg body wt p.o.) significantly (P<0.01) decreased HDAC1 levels in paw tissues of rats as compared to the control group (Figure 1 & table 6).

Table 6: Effect of Hydroethanol extract of *Trapa Bispinosa* on $HDAC_1$ Level in Rats by ELISA Reader Test.

Groups	Treatment and dose at mg/kg body wt .p.o.	Tissues		
		Paw	Uterus	Lungs
Group I; Normal control	Tween 80 (1%)	4.1±0.240	1.147±0.0	0.587±0.03
Group II; Plant extract	*Trapa bispinosa* 400	2.54±0.03	2.45±0.17	1.83±0.7
n=6	All values are expressed as mean ±SEM (n=3) using the ANOVA followed by Dunnet's test. Result considered as significant at * p <0.05, **p<0.01compared with control.			

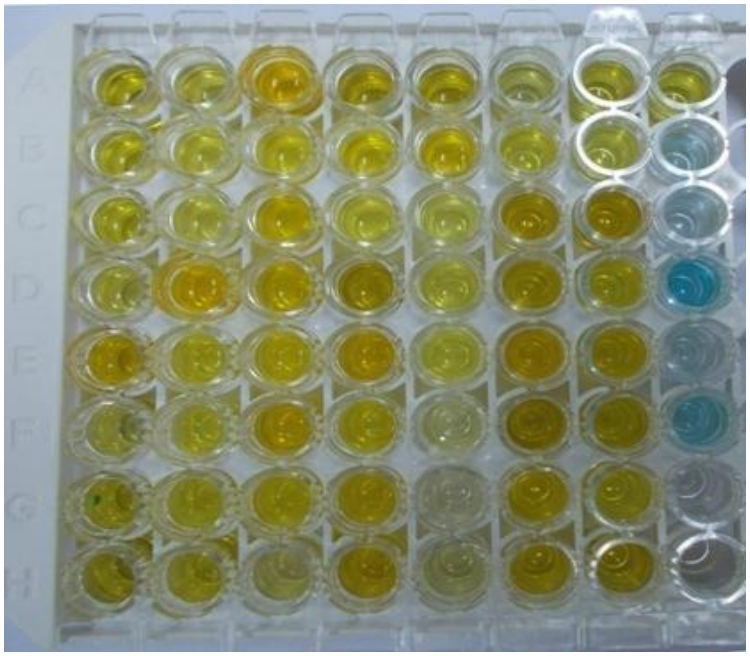

Figure 1: Effect of hydroethanol extract of *Trapa bispinosa* on rat tissue viz; paw , uterus, lungs tissue indicates the development of Yellow colour on ELISA plate.

ISSN: 0975-8585

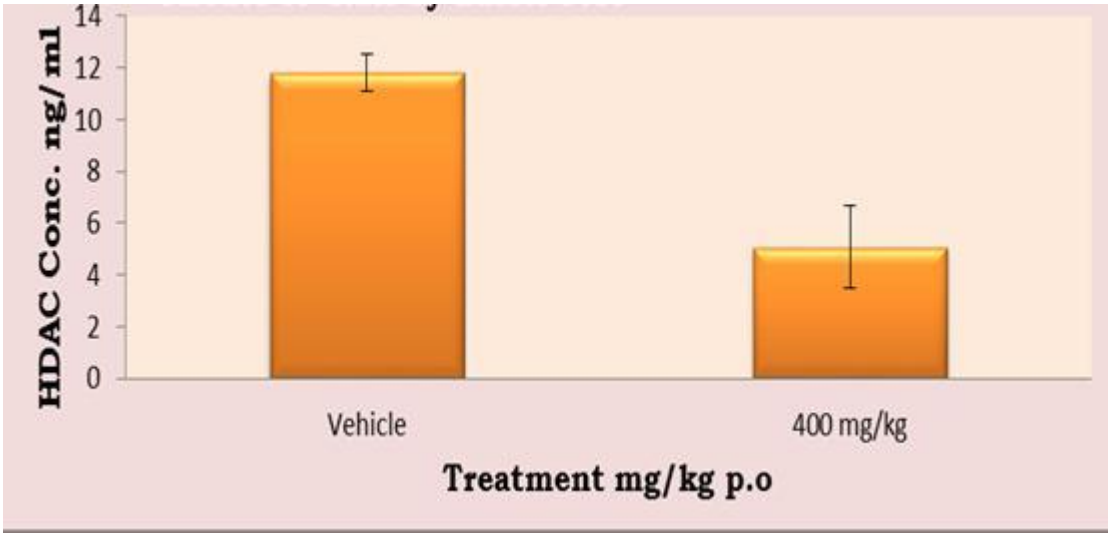

Figure 2: Effect of Trapa bispinosa on HDAC levels in paw tissues of rats by ELISA method.

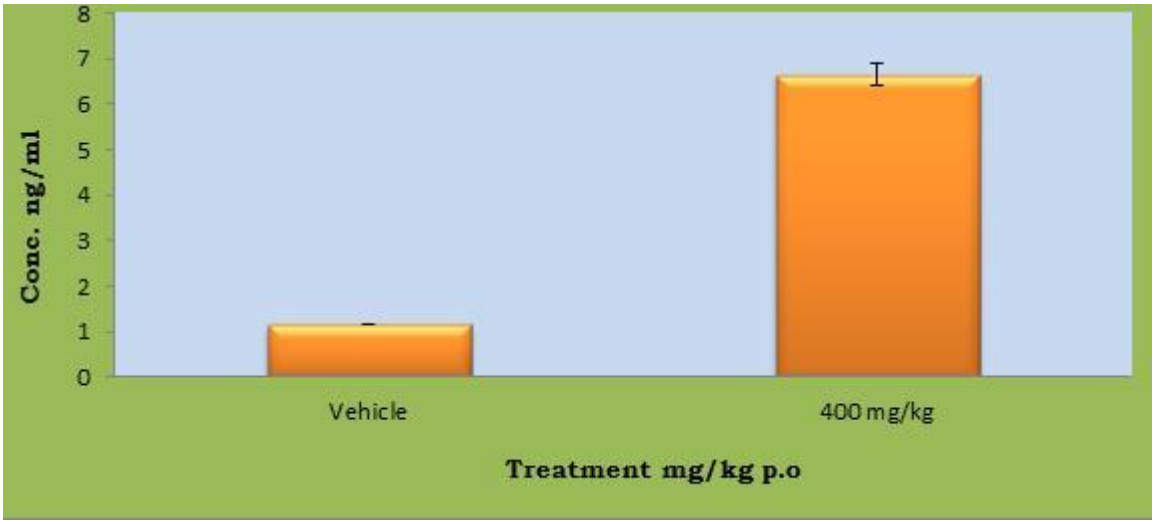

Figure 3: Effect of Trapa bispinosa on HDAC levels in female rats by ELISA method.

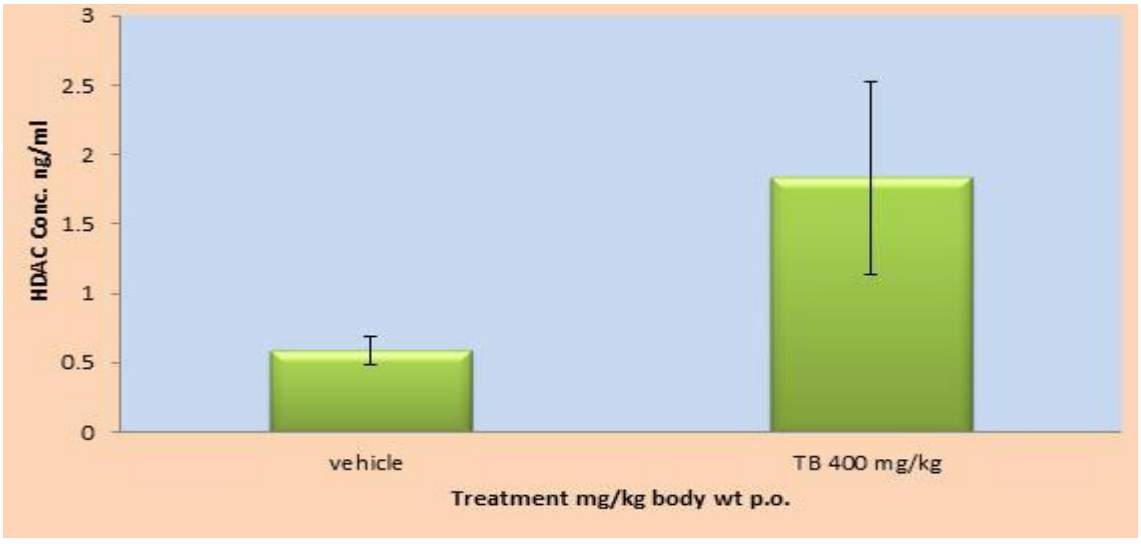

Figure 4 : Effect of Trapa bispinosa on HDAC levels in lung tissues of rats by ELISA method.

Hydroethanol extract of *Trapa bispinosa* (400 mg/kg body wt p.o.) significantly increased HDAC levels in uterus of female rats (P<0.01)(Figure 2).

Hydroethanol extract *Trapa bispinosa* (400 mg/kg body wt p.o.) significantly (P<0.01) decreased HDAC1 levels in lung tissues of rats as compared to the control group (Figure 3).

Significant decrease in HDAC1 levels in paw tissues of rats after administration of Hydroethanol extract of *Trapa bispinosa* indicates that the drug may act through HDAC1 inhibition mechanism. In female rat uterus tissues, HDAC1 level was increased after administration of Hydroethanol extract *Trapa bispinosa* and it does not interfere with the process of inflammation but it indicates its possible potential to cure infertility. Hydroethanol extract of *Trapa bispinosa* significantly increases the level of HDAC1. Further detailed chronic study required to clarify HDAC inhibition mechanism.

Inhibition of histone deacetylases (HDAC) has been shown to modulate gene expression and cytokine production after stimulation with several stimuli. HDAC inhibitor reduces the production and/or activity of pro-inflammatory cytokines in vitro or to exert a potent effect in animal models of inflammatory diseases.

CONCLUSION

The present study on hydroethanol extract of *Trapa bispinosa*has demonstrated that this plant has significant anti-inflammatory and antiarthritic properties, and it justifies the traditional use of this plant in the treatment of various types of pains and inflammation.

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