ANALGESIC AND ANTI-INFLAMMATORY ACTIVITY OF METHANOLIC EXTRACT OF *VITIS VINIFERA* LEAVES

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Summary

The methanolic extract of *Vitis vinifera* leaves at 100, 200 and 400 mg/kg body weight were evaluated for anti-inflammatory and analgesic activities in rats and mice. Anti-inflammatory activity was studied by using carrageenan and histamine induced oedema right hind paw volume while the analgesic effect was evaluated using formalin-induced pain and tail flick nociception response. Methanolic extract showed significant ($p<0.01$) and dose dependent analgesic and anti-inflammatory activity in comparison to control group. The potential analgesic and anti-inflammatory by methanolic extract was comparatively less than that of diclofenac (10 mg/kg *p.o.*). Methanolic extract did not show any toxicity and mortality up to maximum dose of 2 gm/kg of body weight. Preliminary phytochemical screening revealed the presence of flavonoids, saponins and carbohydrate, tannins and phenolic compounds in methanolic extract. Thus it could be concluded that methanolic extract *Vitis vinifera* possess significant analgesic and anti-inflammatory activity.

Key words: *Vitis vinifera,* analgesic, anti-inflammatory.

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Introduction

V. vinifera a large deciduous climber, climbing by means of intermittent, leaves opposed, large, often bifid tendrils, cultivated in many part of India.[(1)] The ripe fruit is cooling, laxative and purgative, fattening, diuretic, aphrodisiac, appetizer, and the throat; cures thirst, asthma, “vata” and “vatarakta”, jaundice, strangury, blood disease. The ashes of stem are good for pains in joints, swelling of the testicle, and piles. [(2)] The flowers are expectorant, emmenagogue and haematinic, and are useful in bronchitis.[(3)] Its leaves are consumed in some traditional foods (Dolmathes) and used for diarrhoea, vomiting and varicose treatment.[(4)] The chemical analysis has shown the presence of procyanidins, anthocyanins, Flavanoids, hydroxylcinnamic acid derivatives, triterpenes, sterols, tannins, polysaccharides, monosaccharide’s and non-alkaloid nitrogen containing compounds. [(5)] The stilbene groups, as resveratrol and viniferins, have also been isolated from leaves. [(6)] 3-oxo-a-ionol, vomifoliol and dehydrovomifoliol were identified for the first time in fruit from *Vitis vinifera.* [(7)]

Pain is an ill-defined, unpleasant sensation, usually evoked by an external or internal noxious stimulus. Pain is warning signal and primarily protective in nature, but causes discomfort. Excessive pain may be unbearable and cause other effects – sinking sensation, apprehension, sweating, nausea, palpitation, and rise or fall in BP, tachypnoea.[8] Pain is classified as one of two types, based on speed of onset, quality of the sensation, and duration: acute (fast) and chronic (slow). Acute pain occurs very rapidly, usually within 0.1 second after a stimulus is applied, and is not felt in deeper tissues of the body. Impulses for acute pain conduct along large diameter, myelinated A fibers. Chronic pain by contrast, begins after a second or more and then gradually increases in intensity over a period of several second or minutes. Impulses for chronic pain conduct along smaller diameter, unmyelinated C fibers. [9]

Inflammation is considered as a primary physiologic defense mechanism that helps body to protect itself against infection, burn, toxic chemicals, allergens or other noxious stimuli. An uncontrolled and persistent inflammation may act as an etiologic factor for many of these chronic illnesses. [10] Kinins play an important role in the inflammatory process. Kallikreins and kinins can produce redness, local heat, swelling, and pain, and the production of kinins is increased in inflammatory lesions produced by a variety of methods. [11]

Methods

Collection and authentication of plant:

The leaves of the plant were collected from the Balaji nursery, Jagatpura, Jaipur District, Rajasthan state, India in month of March 2009. The identity of the collected plant was confirmed by P.J. Parmar, Joint Director in Botanical Survey of India (BSI), Jodhpur (Rajasthan, India). The Herbarium of the plant was deposited in the BSI against voucher specimen no. JNU/JPR/PC/ JS-1.

Preparation of plant extract:

The leaves of plant were washed, shade dried and powdered. The powdered material was defatted with petroleum ether and then extracted with methanol by cold maceration process. The extract was concentrated for further studies at reduced pressure and temperature in a rotary evaporator. Methanolic extract was tested for presence of secondary metabolites by different phytochemical tests.

Experimental animals:

Swiss albino mice (25-30 gm) and Healthy Wistar albino rats of either sex (150-200 gm) were taken for the study. They were housed in polypropylene cages in an air-conditioned area at 25±2 ºC with 12/12 h light/dark cycle. All animals had free access to standard pellet diet (Mahavir industries, Delhi) and clean water *ad libitum*. The norms for Good Laboratory Practice (GLP) were followed for care of laboratory animals. The present studies were duly approved by IAEC (Institutional Animal Ethical Committee clearance) 002/2009/IAEC/jnu.

Drugs and Chemicals used:

Carrageenan, Commercial Grade, Type I (Sigma Aldrich, Co.), formalin (Merck Specialties Pvt. Ltd., Mumbai), Diclofenac (Gift sample from Glenmark Laboratories Ltd. Mumbai), Sodium chloride (Quqligens Fine Chemicals A division of GlaxoSmithKline) were used in this study. Other chemicals used for extraction purpose and phytochemical tests were of laboratory grade.

Phytochemical screening: [12, 13, 14]

The plant may be considered as biosynthetic laboratory for the chemical compounds such as carbohydrates, protein, lipids, alkaloids, glycosides, tannins etc. The compounds that are responsible for therapeutic effect are usually the secondary metabolites. A systematic study of a crude drug embraces through consideration of both primary and secondary metabolites derived as result of plant metabolism. The plant material may be subjected to preliminary phytochemical screening for detection of various plant constituents.

Standard screening test of the extract was carried out for various plant constituents. The crude extract was screened for the presence or absence of secondary metabolites such as alkaloids, carbohydrate, phenolic compounds, flavonoids, saponins and tannins by using standard procedures.

Acute toxicity test: [15]

Acute oral toxicity study for the test extract of the plant was carried out using OECD/OCED guideline 425. The test procedure minimizes the number of animals required to estimate the oral acute toxicity. The test also allows the observation of signs of toxicity and can also be used to identify chemicals that are likely to have low toxicity.

Healthy, young adult albino Wistar rats of either sex (200 -250 g) were used for this study. Animals should be fasted prior to dosing (food but not water should be withheld Overnight). The fasted body weight of each animal is determined and the dose is calculated according to the body weight.

Limit Test at 2000 mg/kg: Dose 2000mg/kg body weight was administered orally to one animal. This first test animal survived. Since, four other animals were dosed (orally) sequentially, so that a total of five animals were tested. Animals are observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), and daily thereafter, for a total of 14 days. No animals were died. So the LD50 is greater than 2000 mg/kg.

Biological evaluation:

As per acute toxicity study, methanolic extract was found to be safe up to 2 gm/kg body weight. Hence the following expected activities were screened at 100, 200 and 400 mg/kg body weight orally. Mice or rats were divided into 5 groups consisting of 6 animals each. Group I received vehicle (10 ml/kg water) i.e. negative control, Group II, III, IV received per oral route methanolic extract of 100, 200 and 400 mg/kg respectively whereas Group V served as positive control i.e. Diclofenac (10 mg/kg, p. o.)

Analgesic activity: [16, 17, 18, 19]

Formalin induced paw licking in mice:

The methanolic extract (100, 200 and 400 mg/kg, p.o.) and diclofenac (10 mg/kg, p.o.) were administered 60 min, before formalin injection. Control animals received vehicle (10 ml/kg). 20 µl of 1 % v/v formalin was injected subcutaneously into the right hind paw of mice. The time (in seconds) spent in licking and biting responses of the injected paw was taken as an indicator of pain response. Responses were measured for 5 min after formalin injection (first phase) and 15–30 min after formalin injection (second phase). The early phase represents neurogenic pain while latter phase is of inflammatory pain.

Tail flick method in mice:

Basal reaction time of animals to radiant heat was recorded by placing the tip (last 1-2 cm) of the tail on the radiant heat source. The tail withdrawal from the heat (flicking response) is taken as the end point. The animals, which showed flicking response within 2-4 secs, were selected for the study. A cut off period of 15 secs is observed to avoid damage to the tail.

The measurements of withdrawal time using the tail flick apparatus (Analgesiometer) was conducted at 0, 30, 60 and 120 min after administration of drugs. The strength of current passing through naked nichrome wire was kept constant 55 ^{0}C.

Anti-inflammatory activity: **(20, 21, 2 2, 23)**

Carrageenan -induced rat paw oedema:
The animals were received vehicle/test drug or diclofenac orally and sixty minute later all the animals were challenged by injecting of 0.1 ml of 1% freshly prepared carrageenan suspension into the sub plantar region of the left hind paw. The paw was marked with ink at the level of the lateral malleolus and immersed in water up to this mark. The paw volume was measured by plythesmographic method before injection, immediately after injection (at 0 hour) and again at 1 & 3 hours after challenge with carrageenan. The percentage inhibition of paw volume was also calculated for each group recording as follows:
Percentage Inhibition = V_c-V_t / V_c * 100
Where-
V_c = Paw volume of control group
V_t = Paw volume of test or standard group

Histamine-induced rat paw oedema:
The paw oedema was produced by sub-plantar administration of 0.1% freshly prepared solution of histamine into the right hind paw of the rats. The animals were received vehicle/test drug or diclofenac orally. The paw volume was recorded immediately before administering the histamine injection (0 hour) and at 1 & 3 hours after the histamine injection. The drug and extracts were similarly administered 1 h before eliciting paw oedema. The anti-inflammatory effect of the extract was calculated using the formula given above.

Statistical analysis:
The results are expressed as mean ± SEM of 6 animals. Parametric data were assessed by the method of analysis of one- way ANOVA followed by Dunnett's test. $P< 0.05$ was considered as statistically significant.

Results

Phytochemical screening:
Phytochemical screening revealed the presence of flavonoids, saponins and carbohydrate, tannins and phenolic compounds in methanolic extract.

Acute toxicity:
Methanolic extract did not show any toxicity and mortality up to maximum dose of 2 gm/kg of body weight. Common side effects such as mild diarrhea, loss of weight and depression were not recorded.

Analgesic activity:

Formalin induced paw licking in mice: methanolic extract (100, 200 and 400 mg/kg, p.o.) reduced paw licking and biting responses counts significantly in dose dependent manner. (Table 1).

Tail flick method in mice: methanolic extract showed significant increase in latency time for thermal stimulation in tail flick test shown in Table 2 & Figure 1.

Table 1: Analgesic effect of methanolic extract in formalin induced pain in mice

Group(s)	Dose (mg/kg)	Total time in spent in licking (s) 0-5 min	Inhibition (%)	15-30 min	Inhibition (%)
Control	10 ml/kg	12.17±0.401	- - -	46.34±0.333	- - -
Extract	100	11.67±0.333^{NS}	4.108	45±0.2582*	2.89
	200	8.34±0.333**	31.47	32±0.3657**	30.97
	400	7±0.2582**	42.48	22.84±0.307**	50.71
Diclofenac	10	4.67±0.4216**	61.62	19±0.2582**	58.99

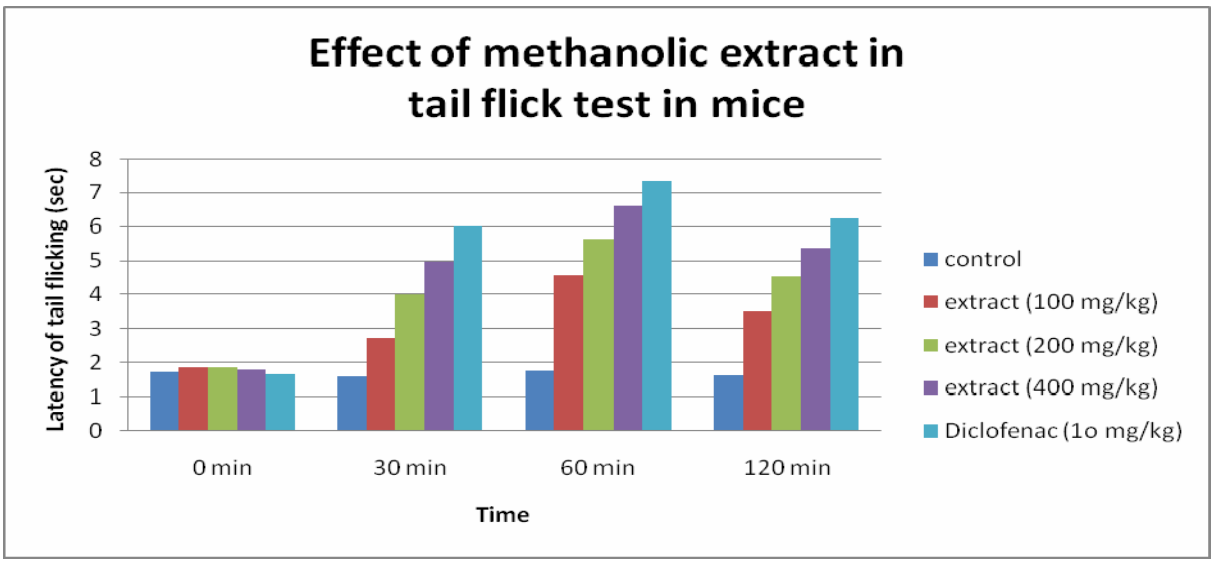
Values are expressed as mean±SEM; from 6 animals in each group. ^{NS}P>0.05, *P<0.05, **P<0.01.

Table 2: Analgesic effect of methanolic extract in tail flick latency in mice

Groups	Dose mg/kg	Mean latency time (seconds) at time post treatment 0 min	30 min	60 min	120 min
Control	10 ml/kg	1.73±0.16	1.6±0.23	1.76±0.28	1.64±0.18
Extract	100	1.88±0.20^{NS}	2.73±0.23*	4.56±0.48**	3.51±0.75*
	200	1.88±0.12^{NS}	4.01±0.23**	5.64±0.47**	4.54±0.34**
	400	1.80±0.41^{NS}	4.97±0.18**	6.60±0.46**	5.36±0.27**
Diclofenac	10	1.66±0.21^{NS}	6.0±0.45**	7.33±0.47**	6.25±0.18**

Values are expressed as mean±SEM; from 6 animals in each group. ^{NS}P>0.05, *P<0.05, **P<0.01.

Figure1. Effect of methanolic extract in tail flick latency in mice



Anti-inflammatory:

Carrageenan-induced paw oedema: Anti-inflammatory effect of methanolic extract against carrageenan-induced inflammation is shown in Table 3. Methanolic extract showed non-significant (NS) at low dose 100 mg/kg and significant ($p<0.01$) at higher dose 200 and 400 mg/kg so dose dependent anti-inflammatory activity were found in comparison to control group. The potential anti-inflammatory by methanolic extract was comparatively less than that of diclofenac (10 mg/kg *p.o.*).

Histamine-induced oedema: Table 4 shows that methanolic extract was also effective in histamine-induced inflammation. Histamine-induced paw oedema was inhibited by methanolic extract significantly ($P <0.05$ and $P <0.01$) as compared to the control rats.

Table 3:Anti-inflammatory effect of methanolic extract in carrageenan-induced paw oedema in rat

Group(s)	Dose (mg/kg)	Paw volume after 1 h (ml)	Inhibition (%)	Paw volume after 3 h (ml)	Inhibition (%)
Control	10 ml/kg	0.19±0.024	- - -	0.21±0.025	- - -
Extract	100	0.15±0.021^{NS}	21.05	0.17±0.02^{NS}	18.26
	200	0.11±0.012**	42.10	0.08±0.013**	58.65
	400	0.09±0.01**	52.63	0.068±0.01**	67.30
Diclofenac	10	0.06±0.007**	68.42	0.04±0.007**	76.92

Values are expressed as mean±SEM; from 6 animals in each group. $^{NS}P>0.05$, $^{**}P<0.01$.

Table 4: Anti-inflammatory effect of methanolic extract in histamine-induced paw oedema in rat

Group(s)	Dose (mg/kg)	Paw volume after 1 h (ml)	Inhibition (%)	Paw volume after 3 h (ml)	Inhibition (%)
Control	10 ml/kg	0.21±0.02	- - -	0.30±0.03	- - -
Extract	100	0.15±0.01*	30.5	0.16±0.01**	46
	200	0.12±0.11**	41.17	0.10±0.012**	66.66
	400	0.09±0.01**	54.67	0.07±0.01**	76
Diclofenac	10	0.08±0.006**	61.06	0.06±0.01**	80

Values are expressed as mean±SEM; from 6 animals in each group. $^{*}P<0.05$, $^{**}P<0.01$.

Discussion

In this work, we have demonstrated the effect of methanolic extract (100, 200, & 400 mg/kg; p.o.) on formalin induced paw licking in mice and tail flick method in mice. The methods for investigating analgesic effects of methanolic extract were selected such that both centrally and peripherally mediated effects were investigated. Two different animal's models (i.e. histamine-induced paw edema in rat and carrageenan--induced hind paw edema in rat) were employed to investigate the potential anti-inflammatory activity of the methanolic extract of *Vitis vinifera* in this study.

The purpose of the present study was to establish scientific evidences for the usage of this plant in inflammatory conditions. Preliminary phytochemical screening of *Vitis vinifera* showed presence of flavonoids, saponins and carbohydrate, tannins and phenolic compounds in methanolic extract.

According to acute toxicity study it found that methanolic extract of *Vitis vinifera* did not cause any mortality up to 2gm/kg of body weight and was considered as safe.

Formalin test has advantage that it involves biphasic pain, with an early pain representing neurogenic and late phase of inflammatory reaction. In the present study inhibition of both these phases was found in formalin induced licking.

Drugs that act centrally inhibit pain produced by thermal stimuli [(24)]. The methanolic extract produced anti-nociceptive effect against thermal induced pain stimuli in mice in tail flick method at various points indicates that it might be centrally acting. In the present study, diclofenac also inhibited the pain produced by tail flick method. Although, this model is specific for centrally inhibited pain, there are certain evidences that support; NSAID's also inhibit the pain induced by thermal stimuli [(25)]. The observations from formalin test and tail flick model suggests that methanolic extract inhibited the pain induced by chemical and thermal stimuli.

Anti-inflammatory activity of flavonoids, triterpenoids and phytosterols have been reported by several researchers [(26, 27)] so it might possible that particular these phytoconstituents from methanolic extract of *Vitis vinifera* could have contributed to this activity. Further studies intended to confirm these activities, as well as the isolation of active biomolecules responsible, are being conducted.
Inflammation comprises of three phases, namely acute inflammation, the immune response and chronic inflammation. In acute inflammation, due to the changes in small blood vessel, fluid and granulocytic cells accumulate at the site of injury. This reaction often triggers a systemic response such as fever, leucocytosis, protein catabolism and altered hepatic system synthesis of acute phase proteins such as C-reactive protein. Chronic inflammation characterized by tissue infiltration by macrophages and lymphocytes. [(29)]

There are two phases of carrageenan-induced inflammatory reaction: early or first phase and later or second phase. It has been proposed that early phase results from histamine, serotonin and bradykinin liberation while late phase is associated with the release of prostaglandin [(30)]

Histamine is one of the important inflammation mediators and it is a potent vasodilator substance and increases the vascular permeability [(31)]. This study showed that all the doses of methanolic extract effectively inhibited the oedema produced by the carrageenan and histamine, which indicates that the extract exhibit its anti-inflammatory action by means of either inhibiting the synthesis, re-lease or action of inflammatory mediators viz. hista-mine, serotonin and prostaglandin might be involved in inflammation.

From these results, it suggested that anti-oedematogenic effects of the methanolic extract on carrageenan and histamine induced oedema may be related to inhibition of inflammation mediator formation. Further investigations are required to understand its mechanism of action.

Conclusion

The results of present study revealed anti-inflammatory and analgesic activity of methanolic extract of *Vitis vinifera*. Thus it substantiates the traditionally proven effectiveness of this plant in painful and inflammation conditions. Further investigations are required to understand its influence on various pain and inflammatory mediators.

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