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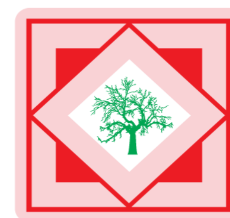

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Antinociceptive activity of methanolic extract of leaves of *Vitis vinifera*

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ABSTRACT

Vitis vinifera (Vitaceae) is traditionally used to treat some painful conditions. Purpose of the present study was to evaluate antinociceptive activity of methanolic extract of leaves of Vitis vinifera (MELV) and also its possible mechanism of action. Antinociceptive activity of MELV was evaluated in writhing, hot plate, tail flick and formalin models in mice (n = 6). To elucidate the mechanism of action, animals were pre-treated with opioid antagonist, naloxone (5 mg/kg) and muscarinic antagonist, atropine (5mg/kg) and subjected to hot plate test. Oral administration of MELV exhibited significant (p<0.05) antinociceptive activity in a dose dependent manner in writhing, hot plate, tail flick, and formalin models. Pre-treatment with atropine did not cause any change in antinociceptive activity of MELV, while, pre-treatment with naloxone significantly (p < 0.05) diminished the MELV antinociceptive activity in hot plate test. The MELV exhibits opioid-mediated antinociceptive activity at the peripheral and central levels, which supported the traditional use of the Vitis vinifera in the treatment of some painful diseases.

Keywords*:* Pain; Mice; *Vitis vinifera*; Vitaceae; Mechanism of action.

INTRODUCTION

Over the years, natural products have contributed to the development of important therapeutic drugs used currently in modern medicine. The study of plants that have been traditionally used as pain killers should still be seen as a fruitful and logical research strategy, in the search for new analgesic drugs and pain mechanisms [1].

Vitis vinifera (Vitaceae) is a perennial woody vine native to Asia which was introduced in Europe and other continents [2]. It is commonly known as grape and draksha. The ripe fruit is 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**.** The flowers are expectorant and haematinic, and are useful in bronchitis**.** In Iran, grape leaves are used in a traditional food and for treatment of diarrhea and bleeding [2].

The chemical analysis has shown the presence of procyanidins, anthocyanins, Flavanoids, hydroxylcinnamic acid derivatives, triterpenes, sterols, tannins, polysaccharides, monosaccharide's and nonalkaloid nitrogen containing compounds**.** The procyanidins are the most important constituents of grape [3]. The stilbene groups, as resveratrol and viniferins, have also been isolated from leaves [4]**.** 3-oxo-a-ionol, vomifoliol and dehydrovomifoliol were identified for the first time in fruits from *Vitis vinifera* [5].

Several scientific studies have been carried out on the various parts of the *Vitis vinifera* for various pharmacological properties including antibacterial activity [6], epimerase activity [7] and spasmolytic activity [8]. Grape seed extract has been reported to reduce blood lipids in hyperlipidimic rabbits [9] and its procyanidins have demonstrated vasorelaxant effects in human aorta [10]. Grape leaves with antioxidant activity [11] have been reported to treat chronic venous insufficiency in human [12] and nephrotoxicosis induced by citrinin [13]. Anti-inflammatory and anti-pyretic activities [14] were also studied using *Vitis vinifera* leaves extract, but still today no scientific study was carried out to find antinociceptive activity of *Vitis vinifera.* Hence, the aim of the present study was to investigate the antinociceptive activity of methanolic extract of leaves *Vitis vinifera.*

MATERIALS AND METHODS

Plant material and Extraction

Vitis vinifera leaves were collected in Balaji nursery, India and authenticated from Department of Botany, Kakatiya University, Warangal, India, where a voucher specimen was deposited (number KUH - 1887). Leaves were dried in shade for 15 days. Then dried leaves were crushed to a coarse powder. The coarse powder (25 g) was successively extracted with 250 ml of methanol (at 40 ˚C) using soxhlet extractor for 8 h. The methanolic extract was filtered through Whatman filter paper (No. 1) and filtrate was evaporated in vacuum oven at 100 mm of Hg at 40 ˚C. The percentage yield obtained from the extraction of coarse powder of *Vitis vinifera* leaves was 16%. The extract was stored at -40 ˚C in a desiccant until required.

Animals

All experiments were conducted using adult Swiss albino mice (20–30 g), males or females, at about 6–8 weeks of age, and distributed in groups of 6 animals per treatment (n = 6). All animals were procured from Mahaveer enterprises Pvt Ltd, Hyderabad. The animals were maintained with free access to food and water and kept at 22 ± 2 ˚C under a controlled 12 h light/dark cycle. Twelve hours before each experiment animals received only water, in order to avoid food interference with substances absorption. The care and maintenance of the animals were carried out as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi. The research protocols were approved by the Institutional Animal Ethical Committee (IAEC) (number: 1016/SPIPS/Wgl/IAEC/2010).

Drugs and Chemicals
Naloxone and tween 80 were purchased from Sigma (USA), acetic acid, indomethacin and formaldehyde were purchased from Merck Inc., tramadol was purchased from Aurobindo (Hyderabad), atropine was purchased from Biopharmacule speciality chemicals Pvt Ltd (Hyderabad). A solution of formalin (2.5%) was prepared by dissolving 0.92% formaldehyde in saline solution (NaCl 0.9%). The MELV and indomethacin were used as suspensions in tween 80 (2%) solution.

Acute toxicity [15]
The acute toxicity of *MELV* was assessed using the up-and-down method as described by Bruce (1985). After the administration of one single dose of *MELV* (10, 30, 100, 300 and 1000 mg/kg, p.o.), the survival of animals was observed during 24 h. If an animal survived at any given dose, the dose for the next animal was logarithmically increased; if it died, the dose was decreased.

Antinociceptive activity

Writhing test [16]
The total number of writhings following intraperitoneal administration of 0.7% (v/v) acetic acid at a dose of 10 ml/kg was recorded over a period of 15 min, starting 5min after acetic acid injection. Mice were pre-treated with MELV (100, 200 and 400 mg/kg, p.o.) or reference drug indomethacin (5 mg/kg, p.o.), 60 min before administration of acetic acid.

Tail flick test [17]
Briefly, one-third of mice tails were immersed in a water bath set at temperature of 50 ± 1 °C. The time necessary for the mice to withdrawal the tail, in seconds (named reaction time) was registered at 20, 40, 60, 80, 100 and 120 min after administration of MELV (100, 200 and 400 mg/kg, p.o.) or reference drug tramadol (50 mg/kg, p.o.). Baseline was considered as the mean of reaction time obtained at 40 and 20 min before administration of MELV or tramadol and was defined as normal reaction of animal to the temperature. Increase in baseline (in %) was calculated by the formula: (reaction time×100)/baseline) −100.

Hot plate test [17]
Mice were individually placed on a hot plate set at 55 ± 1 °C. Reaction time was recorded when the mice licked their fore- and hind-paws and jumped at 30, 60, 90, 120, 150 and 180 min after administration of MELV (100, 200 and 400 mg/kg, p.o.) or reference drug tramadol (50 mg/kg, p.o.). Baseline was considered as the mean of reaction time obtained at 60 and 30 min before administration of MELV or tramadol and was defined as normal reaction of animal to the temperature. Increase in baseline (in %) was calculated by the formula: (reaction time×100)/baseline) −100.

Formalin test [18]
Animals received the injection of 20 µl of formalin (2.5% v/v) into dorsal surface of the left hind paw. Immediately, the time that the animal spent licking the injected paw was recorded. The nociceptive response develops in two phases: first 5 min after formalin injection (first phase, neurogenic pain response) and 15–30 min after formalin injection (second phase, inflammatory

pain response). The animals were pre-treated with vehicle, MELV (100, 200 and 400 mg/kg, p.o.) or tramadol (50 mg/kg, p.o.), 60 min before administration of formalin.

Evaluation of the mechanism of action of MELV [19, 20]

To evaluate the mechanism of action, a group of mice were pre-challenged with naloxone (5 mg/kg, i.p.) [19] and another group of mice were pre-challenged with atropine (5 mg/kg, i.p.) [20]. After 10 min, both groups were orally injected with MELV (400 mg/kg) and subjected to the hot plate test.

Statistical analysis

All experimental groups were composed by six animals. The results were presented as the mean ± SEM. Statistical analysis was done by ANOVA followed by Bonferroni's test. $p < 0.05$ was considered as significant.

RESULTS

Acute toxicity

In toxicity studies, no mortality for all the doses of MELV (10-1000 mg/kg, p.o.) was observed in mice during the observation periods of 24 h. Hence, we supposed that MELV at the doses of 100, 200 and 400 mg/kg, p.o. injected to mice was safe.

Acetic acid-induced abdominal writhing

Intraperitoneal injection of acetic acid (0.7%) induced 46 writhings in a period of 15 min in control group. When mice were pre-treated with crescent doses (100, 200 and 400 mg/kg) of MELV, a significant ($p < 0.05$) inhibition of total writhing was observed compared to control group in a dose dependent manner and the percentage inhibitions were calculated as 33.32 % (30.67 writhings), 45.28 % (25.17 writhings), 55.06 % (20.67 writhings), respectively. While, pre-tretment with indomethacin resulted in 60.15 % (18.33 writhings) inhibition ($p < 0.05$) compared to control group (Fig. 1).

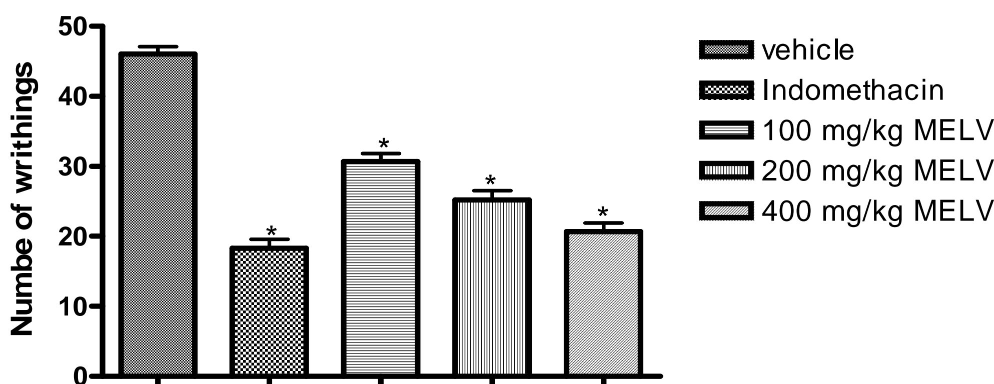

Fig. 1. Antinociceptive effect of MELV on acetic acid-induced writhings in mice

*Each column represents a mean ± S.E.M. of 6 animals. Statistical differences between the treated and the control groups were evaluated by ANOVA followed by Bonferroni's test and the asterisks denote the levels of significance in comparison with control groups, *p < 0.05.*

Tail flick test

Table 1 shows the anti-nociceptive profile of MELV assessed using the tail flick test. Significant ($p < 0.05$) increase in baseline was observed after oral administration of crescent doses (100, 200 and 400 mg/kg) of MELV in a dose dependent manner.

The onset of anti-nociceptive activity of the MELV, regardless of the dose used, was seen at the interval of 20 min following the extract administration. Furthermore, this anti-nociceptive activity was observed until the end of the experiment (interval of 120 min).

The peak of antinociceptive activity for MELV was seen 80 min after administration compared to tramadol (50 mg/kg, p.o.), which also exerted its peak of activity at the interval of 80 min.

Table 1: Anti-nociceptive activity of the MELV in tail flick test in mice

Treatment group (n=6)	Increase in baseline (%)					
	20min	40 min	60 min	80 min	100 min	120 min
1. Vehicle	1.71 ± 0.18	3.01 ± 0.33	1.93 ± 0.42	0.79 ± 0.2	2.68 ± 0.52	1.92 ± 0.55
2. 50 mg/kg Tramadol	17.1 ± 0.93*	42.06 ± 1.81*	61.57 ± 1.49*	76.89 ± 1.52*	46.44 ± 1.32*	21.39 ± 1.71*
3. 100 mg/kg MELV	7.7 ± 0.28*	19.79 ± 0.56*	26.35 ± 0.91*	37.11 ± 0.36*	34.19 ± 0.65*	29.08 ± 0.77*
4. 200 mg/kg MELV	9.41 ± 0.66*	28.19 ± 0.38*	42.27 ± 0.25*	57.43 ± 0.46*	50.24 ± 0.98*	28.68 ± 0.84*
5. 400 mg/kg MELV	14.93 ±0.37*	35.77 ± 0.59*	64.95 ± 0.75*	70.36 ± 1.35*	66.77 ± 0.28*	37.04 ± 0.34*

*Increase in baseline (%) was expressed as mean ± S.E.M, *p < 0.05 compared with vehicle-treated group.*

Hot plate test

Due to the spinal antinociceptive effect observed in the tail flick model, we decided to test MELV on the supraspinal model, the hot plate test. Significant ($p < 0.05$) increase in baseline was observed after oral administration of crescent doses (100, 200 and 400 mg/kg) of MELV in a dose dependent manner. The onset of anti-nociceptive activity of the MELV was seen at the interval of 20 min at the dose of 100 mg/kg, 40 min at the dose of 200 mg/kg and 60 min at the dose of 400 mg/kg, following the extract administration.
The peak of anti-nociceptive activity for the 100, 200 and 400 mg/kg MELV was seen at the interval of 120, 90 and 120 min, respectively compared to 50 mg/kg tramadol, which exerted its peak of activity at the interval of 120 min (Table 2).

Table 2: Anti-nociceptive activity of the MELV in hot plate test in mice

Treatment group (n=6)	Increase in baseline (%)					
	30min	60 min	90 min	120 min	150 min	180 min
1. Vehicle	0.76 ± 0.22	0.78 ± 0.26	1.17 ± 0.23	0.7 ± 0.2	1.26 ± 0.49	0.47 ± 0.17
2. 50 mg/kg Tramadol	4.03 ± 1.74*	15.38 ± 0.87*	28.36 ± 1.23*	49.21 ± 3.82*	39.74 ± 1.14*	27.88 ± 1.28*
3. 100 mg/kg MELV	2.03 ± 0.28	4.99 ± 0.29	22.67 ± 1.67*	31.58 ± 1.51*	29.1 ± 1.39*	19.14 ± 1.46*
4. 200 mg/kg MELV	2.23 ± 0.66	14.24 ± 1.25*	40.72 ± 1.72*	34.71 ± 1.64*	22.18 ± 1.14*	16.7 ± 0.85*
5. 400 mg/kg MELV	3.03 ± 0.37*	16.38 ± 1.12*	39.19 ± 3.36*	53.51 ± 2.76*	31.83 ± 1.85*	12.93 ± 1.13*
6. Nlx + 400 mg/kg MELV	1.03 ± 0.23#	2.1 ± 0.19#	3.25 ± 0.22#	3.79 ± 0.15#	5.6 ± 0.62#	4.34 ± 0.19#
7. Atr + 400 mg/kg MELV	2.49 ± 0.19	17.92 ± 1.65	37.09 ± 1.36	45.97 ± 1.83	35.58 ± 1.54	18.91 ± 0.7#

*Increase in baseline (%) was expressed as mean ± S.E.M, *p < 0.05 compared with vehicle-treated group, #p < 0.05 compared with 400 mg/kg MELV-treated group.*

Formalin test

When MELV was tested on formalin test, it was observed that MELV significantly ($p < 0.05$) reduced both the first and second phases of the formalin test at the doses of 100, 200 and 400 mg/kg, compared to control group. Fig. 2 shows that MELV significantly reduced the time that the animal spent on licking of the injected paw. In order to compare the antinociceptive potency of MELV, a group of mice was injected with tramadol. This drug also significantly ($p < 0.05$) inhibited the time that the animal spent licking in both phases after formalin injection.

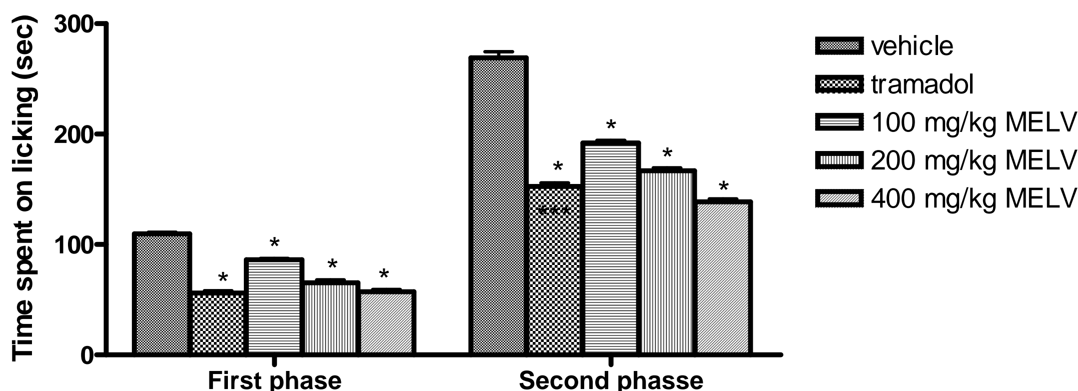

Fig. 2. Antinociceptive effect of MELV in formalin test in mice

*Each column represents a mean ± S.E.M. of 6 animals. Statistical differences between the treated and the control groups were evaluated by ANOVA followed by Bonferroni's test and the asterisks denote the levels of significance in comparison with control groups, *p < 0.05.*

Evaluation of mechanism of action

In order to elucidate the mechanism by which MELV induce antinociceptive effect, animals were pre-treated with the opioid antagonist, naloxone or muscarinic antagonist, atropine. Treatment with atropine (5mg/kg) did not change the antinociceptive effect of MELV. On the other hand, pre-treatment with Naloxone (5 mg/kg) completely prevented the antinociceptive effect of MELV (Table. 2).

DISCUSSION

In the present study antinociceptive activity of methanolic extract of leaves of *Vitis vinifera* was evaluated in different experimental models of pain. The models for investigating antinociception were selected based on their capacity to investigate both centrally and peripherally mediated effects. The abdominal constriction induced by acetic acid investigates the peripheral activity. The hot plate and tail flick methods investigate the central activity, while the formalin test investigates both.

The acetic acid-induced writhing is a visceral pain model. Algesic effects of acetic acid are due to liberation of several mediators such as histamine, serotonin, cytokines, and eicosanoids with an increase in peritoneal fluid levels of these mediators [21]. In this test, our results indicate that antinociceptive activity of MELV may be due to blockade of liberation or receptors of those inflammatory mediators. Another possible mechanism could be the blockade in the eicosanoid system by blocking cyclooxygenases (COX-1and/or COX-2).

The tail flick (spinal analgesia) and hot plate (supraspinal analgesia) models are central models in which opioid agents exert their analgesic effects via spinal and supra spinal receptors, respectively [22]. These models are used to test the central antinociceptive activity of MELV. Experimental evidence obtained in this study suggest that the MELV shows antinociceptive property by acting on both supra spinal and spinal receptors.

The formalin test is a model constituted of two distinct phases. The first transient phase, corresponds to acute neurogenic pain, is caused by the direct effect of formalin on sensory C-fibers, and the second prolonged phase is associated with the development of an inflammatory response and the release of nociceptive mediators [23, 24]. It was reported that substance P and bradykinin participate in the appearance of the first phase responses, and histamine, serotonin, prostaglandin and bradykinin are involved in the second phase responses [25]. Substances that act primarily as central analgesics inhibit both phases while peripherally acting drugs inhibit only the second phase [26]. In this test, our results show that MELV has both central and peripheral antinociceptive activity and reinforce results obtained in peripheral and central models.

The anti-nociceptive activity of MELV demonstrated in the abdominal constriction, formalin, tail flick and hot-plate tests suggested the extract potential in inhibiting chemically- and thermally induced noxious stimuli. Moreover, the ability to inhibit both types of stimuli also indicates that the extract possesses a characteristic of strong analgesic with centrally mediated activity [27, 28].

Naloxone, an opioid antagonist, showed influence on the antinociceptive action of MELV (400 mg/kg). This suggests the participation of the opioid system in the modulation of pain provoked by the compound. Muscarinic receptors involvement is however unlikely to be operative since atropine, a muscarinic antagonist was failed to inhibit the MELV induced-antinociceptive activity. Although *Vitis vinifera* contains several chemical constituents, further studies must be carried out to investigate the exact chemical substance present in the MELV that exerts antinociceptive activity.

CONCLUSION

In Conclusion, this study has shown that MELV has central and peripheral antinociceptive activities and these results support the traditional use of *Vitis vinifera* in some painful conditions. The mechanism through which MELV exerts antinociceptive activity seems to be mediated, at least in part, by acting on opioid receptors and not by acting on cholinergic receptors.

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