Comparative antinociceptive and anti-inflammatory properties of the genetic variants of *Mirabilis jalapa*

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Abstract: Medicinal plants are in practice for the management of various ailments from ancient times. The mode and use of medicinal plants as medicaments for many diseases change day by day with new research and scientific approaches. Inflammation and its associated nociceptive pain are among the vital health challenges to the current researchers. In this research, we have used four genetic variants of *Mirabilis jalapa* for the possible management of inflammatory processes and associated nociceptive pain. The crude extract, chloroform and EtOAc fractions of the four variants (**MJ-1**, **MJ-2**, **MJ-3,** and **MJ-4**) have been used in this study. In the *in vitro* enzyme inhibition studies, all samples were evaluated against COX-2 (cyclooxygenase) and 5-LOX (lipoxygenase). The activity profile of the crude extract samples was encouraging and gave mediocre inhibitions against both COX-2 and 5-LOX. Among the crude samples, we noticed that Pink and light pink variants were comparatively more active against COX-2 and 5-LOX. In chloroform fractions, all four variants were poorly active. Similarly, the highest *in vitro* activity was shown by the ethyl acetate fraction of the pink variant giving IC_{50} values of 260 and 360.47 µg/ml against COX-2 and 5-LOX respectively. All selected variants were also tested safe in experimental animals for *in vivo* studies. In the carrageenan-induced method of inflammation, the pink variant was most active showing 56.7% inhibition during 3 h of observation. Similarly, in hot plate and tail immersion *in vivo* experiments, the ethyl acetate fractions of all four variants showed encouraging inhibitions. In the acetic acid writhing model, we also found ethyl acetate

fractions more active. Furthermore, both **MJ-4** and **MJ-3** were more active compared to other variants selected. It can be concluded from our current findings that among the four genetic variants, the pink variant was most potent on all the tested *in vitro* and *in vivo* targets of inflammation and nociception, which may be associated with relatively rich phenolic and flavonoid content of the variant. Furthermore, it was also obvious from our results that the ethyl acetate fraction was the most active and can be used as a targeted sample for further extended studies.

Keywords: *Mirabilis jalapa*; Inflammation; Nociception; COX; LOX; Carrageenan and Hot plate method

1. Introduction

Plant and plant-based remedies are long used as a remedy for various diseases [1-2]. The efficacy of such remedies is always associated with the presence of active metabolites in significant quantity [3]. To ensure optimum outcomes, standardization of such remedies is very important. Standardization of herbal medicines refers to prescribing a set of standards, constant parameters, and qualitative and quantitative values which ascertain quality, efficacy, safety, and reproducibility [4]. Hence standardization is simply a tool of the quality control process. Standardization or quality assessment of herbal remedies justifies their acceptance in the modern medicine system [5-6]. It has been reported that genetic, morphogenetic, ontogenetic, and environmental factors have significant effects on the biosynthesis and concentration of secondary metabolites in plants [7]. Change in any factor may alter the production of metabolites responsible for pharmacological response [8]. Similarly, the indiscriminate use of different genetic variants of the same plant in formulating herbal remedies is also common. Qualitative and quantitative variability of chemical constituents in different parts of a plant is well known [9-

10]. The existence of a strong relationship between ecological factors, genetic diversity, and variation in chemical constituents in the same plant has also been reported [11]. Hence mixing different plant parts and/or different genetic variants of a plant poses an important challenge to the best possible use of herbal remedies. *Mirabilis jalapa* (Nyctaginaceae) a Four O'clock plant is a bushy perennial herb with about 1m in height and swollen stem at the nodes. Leaves are pointed, ovate, and cordate on numerous branches with funnel-shaped fragrant flowers in different colors [12]. It is one of the extensively used plants in traditional remedies and has been reported with promising results in several health conditions. Juice of rhizome is taken orally as aphrodisiac, antidiarrheal, and remedy for colic [13], infusion and decoction are used in Brazil to treat inflammation and painful disorders [14], a paste of powder leaves is traditionally employed in uterine problems and gonorrhea in the western tribal belt of Pakistan [15], excellent anti-arthritic and antioxidant activity has been reported from flowering parts of the plant [16], crude alcoholic extract of the plant has been reported to possess hypoglycemic and hypolipidemic properties [17]. Other important activities like antimicrobial, antifungal, anti-asthmatic, and hepatoprotective properties have also been reported from the plant [18-21]. Apart from mirabilis antiviral protein, several important groups of constituents have been reported from the plant including, steroids, phenolic compounds, rotenoids, terpenoids, triterpenoids, carbohydrates, amine, and amino acids [22-28]. The anti-inflammatory and analgesic-like potential of the plant is well established [19, 29]. Several active polyphenols and flavonoids, including rutin, neochlorogenic acid, chlorogenic acid, etc having therapeutic importance have been reported from aerial parts of *M. jalapa* [30]. However, no attention has so far been paid to the possible variation in response among different variants of the plant providing an opportunity for non-standardized use. Such use of plants may increase unnecessary drug load associated with the use

of less effective variants. This study is focused on scientifically evaluating and comparing anti-inflammatory and antinociceptive potential among different genetic variants of *M. jalapa* using animal models. The study also involved *in vitro* evaluation of selected variants of the plant for COX-2 and 5-LOX inhibition. The findings are expected to provide scientific evidence for the folkloric use of the plant in pain and inflammation and to help traditional practitioners and herbal experts in selecting appropriate variants of the plant for the given health condition. Selection of appropriate and potent variants will help to obtain the desired level of response with a relatively small quantity of the drug which may avoid unnecessary drug load and side effects associated with the use of large quantity of no/less potent variants to obtain the desired level of response.

2. Materials and Methods

2.1 Animals

Male BALB/c mice (20 to 30g) and Sprague Dawley rats (200 to 300 g) were arranged from the National Institute of Health, Islamabad. Animals were housed at 25 ±1 °C temperature with a 12 h sleep-wake cycle, provided with standard chaw and water to keep them healthy and fit for the experimentation. All experimental protocols were formally approved by the Ethical Committee of the department via Ref. No. PHARM/AWKUM/10/215/2017.

2.2 Chemicals

Chemicals used in the study i.e. tramadol and diclofenac sodium, carrageenan, and acetic acid were procured from the local market.

2.3 Plant material

Aerial parts of *M. jalapa* Linn having yellow (**MJ-1**), white (**MJ-2**), light pink (**MJ-3**), and pink (**MJ-4**) flowers were separately collected during the blooming season, from District Abbottabad, positioned at 1225 m from sea level at 34.17436 longitude and 73.22702 latitude. All varieties

were collected from the same locality and during the same season to minimize the impact of changes in the environment and ecological factors. Plants were authenticated by a plant taxonomist at the botany department of the Abdul Wali Khan University, Mardan. Specimen of each variant was kept in the herbarium of the university, bearing voucher No. MJ001, MJ002, MJ003, and MJ004 for yellow, white, light pink, and pink variants, respectively. Collected plants were cleared of dirt, dried in the shade, and ground mechanically to powder form. Each powder was subjected to maceration by taking 2 kg of hydro ethanol for 4 weeks [31]. The menstruum obtained was filtered and evaporated under reduced pressure using a rotary evaporator (R-1001-LN, HT-UK) to get concentrated extract [32]. Different fractions of the crude extract including, hexane, chloroform, ethyl acetate, butanol, and water were obtained for all selected variants.

2.4 Gross Phytochemical Screening and Toxicity Study

Crude hydroalcoholic extract of all selected variants of *M. jalapa* was screened for the presence of important groups of natural products like alkaloids, flavonoids, glycosides, saponins, tannins, and phenols [33].

An acute toxicity study of crude hydroalcoholic extracts was carried out using a rat model to evaluate the toxicological potential of all selected variants [34]. Sprague Dawley female rats were given up to 2000 mg/kg of hydroalcoholic extract and animals were observed consecutively for 14 days to find out any change in behavior or deaths.

2.5 Total Flavonoid Contents (TFC)

Hydroalcoholic crude extracts were subjected to total flavonoid estimation using the aluminum chloride method with slight modification [35]. To 0.25 mL of plant extract 1.25 mL of distilled water was added along with 0.15 mL NaNO2 (5%) solution. The mixture was placed undisturbed

in the dark for 6 min and then 0.15 ml of 10% $AlCl_3$ was added. The resulting solution was incubated for another 6 min. Then 5 mL of NaOH (5%) solution was added to the reaction mixture. The final volume was adjusted to 5 mL with distilled water. Different concentrations of quercetin solution were prepared and used as standard. The absorbance of both standards and the test sample were determined at 510 nm using a spectrophotometer. The calibration curve was constructed to determine the total flavonoid content of the sample.

2.6 Determination of Total Phenolic Contents (TPC)

Folin–Ciocalteu method was employed for the estimation of the total phenolic contents of each extract [35]. The crude extract (1 mL) was mixed with Folin–Ciocalteu reagent followed by the addition of 2.0 mL of $NaCO_3$ (75%) solution after 5 min. The mixture was incubated at 50 °C for 10 min with occasional agitation. After cooling, absorbance was measured at 765 nm against blank. Phenolic contents were expressed as mg/g of gallic acid equivalents in milligrams per gram of dry extract.

2.7 Determination of Alkaloid Contents (TAC)

Each crude extract (I ml) was denatured in DMSO and 1 ml of HCl (1N) was added and filtered. This clear solution was put in a separating funnel and bromocresol green solution and phosphate buffer were added to 5 ml each. The resulting mixture was shaken vigorously with 1, 2, 3, and 4 mL of $CHCl_3$ and put in a 10 mL flask. Finally, volume was adjusted with chloroform and absorbance was measured at 470 nm. TAC was expressed as a milligram of AE/g of dry extract [36].

2.8 Anti-inflammatory Property

Carrageenan-induced paw edema model was used for an anti-inflammatory study using mice [37]. Animals were randomly grouped into five groups having 6 animals in each group. Group 1

was administered with normal saline (10 ml/kg), Group 2 diclofenac sodium (10 mg/kg), where Group 3-5 were given 100, 200, and 300 mg/kg doses of crude extractor 50, 100, and 150 mg/kg in case of fractions. Carrageenan 0.1 ml (1%) was injected into the sub-plantar tissue of the left hind paw of each mouse. Swelling induced by carrageenan was measured at 0, 1, 2, 3, 4, and 5 h using Plethysmometer (LE 7500, Panlab-Spain). Anti-inflammatory activity is reported as percent inhibition of edema.

$$Inhibition\ (\%) = 1 - \frac{B}{A} \times 100$$

Where A represents the edema volume of the control and B test group.

2.9 Antinociceptive property

2.9.1 Hot Plate Test

Eddy's hot plate method was employed for the evaluation of analgesic-like properties at the central level using a mice model [38]. Animals were selected 24 h before the experiment based on quick responses like jumping and withdrawal within 10 seconds to thermal stress. Animals were then divided into different groups with 6 mice in each. Group 1 was treated with normal saline, and groups 2, 3, and 4 were given crude extract in 100, 200, and 300 mg/kg or 50, 100, and 150 mg/kg of fraction. Positive control Group 5 was treated with tramadol (30 mg/kg). Mice of each group were placed on a hot plate at 55 ± 0.1 ºC with 10 s as the cut-off time to prevent physical injury to the animal. The behavior of each mouse was recorded before and after 30, 60, 90, and 120 minutes of the treatment.

The maximal possible effect in percent was determined for each mouse by-

$$MPE(\%) = \frac{Postdrug\ latency - Predrug\ latency}{10 - Predrug\ latency} \times 100$$

2.9.2 Tail immersion test

To determine nociceptive reaction towards thermal stimulant mice model was employed with 6 animals in each group. The animal's tail was put in water heated to 52 ± 2 °C, and the latency of response (reflexive withdrawal of the distal half of the tail) was measured for baseline latency response before injections of the drugs and at 15 min intervals for the next 60 min followed by measuring at 30 min intervals up to 180 min (post-treatment latency response) after injection. To prevent damage to the tail's skin a cut-off time of 15 s was set. To examine an anti-nociceptive effect crude extract (100, 200, and 300 mg/kg) and fractions (50, 100, and 150 mg/kg) were administered. The negative control received normal saline whereas the positive control was given tramadol [39] .

$$Analgesia\ (\%) = \frac{Test\ latency - Control\ latency}{15 - control\ latency} \times 100$$

2.9.3 Writhing test

For an analgesic-like effect at the peripheral level of acetic acid-induced writing, the method was employed using a mouse model [39]. Animals fasted overnight with free access to water were divided into five groups with 6 animals in each group. Group 1 received normal saline (10 ml/kg), Group 2 diclofenac sodium (10 mg/kg), and Group 3-5 were given 100, 200, and 300 mg/kg of crude extracts or 50, 100, and 150 mg/kg of the sub crude fractions. All groups were injected with acetic acid (0.6%) in a dose of 10 ml/kg (i.p) an hour after mice were given treatments. To assess the analgesic activity of the sample, the number of writhes was counted after 5 min for 30 min. Reduction in writhes count as compared to the control group is considered a function of the analgesic potential of the sample, and expressed as percent analgesic activity.

$$Analgesia\ (\%) = \frac{Mean\ writhing\ count\ (control - treated)}{mean\ writhing\ count\ (control)} \times 100$$

2.10 Cyclooxygenase (COX-2)/Lipoxygenase (5-LOX) inhibitory activity

The *in vitro* COX-2 inhibitory potential of selected variants was carried out using the reported method [40]. COX-2 enzyme solution was prepared at a concentration of 300 U/ml. The enzyme solution (10 µl) along with co-factor solution (50 µl) comprising N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) 0.24 mM, glutathione 0.9 mM, and 1 mM hematin in 0.1 M tris buffer pH 8, was activated on ice for 5 min. Test samples were added in 31.25-1000 µg/ml concentration to the resulting 60 µl enzyme-cofactor mixture and were kept at room temperature for 5 min. Afterward, 20 µl of arachidonic acid (30 mM) was added to initiate the required reaction. The resulting cocktail was incubated for 5 min at room temperature. Absorbance was recorded at 570 nm where percent inhibition of COX-2 was calculated using celecoxib as control.

In vitro, a 5-LOX inhibitory assay of crude extract and fractions of all selected variants was achieved using the reported method [40]. All experiments were performed in triplicate. Different concentrations i.e. 31.25–1000 µg/mL of sample were prepared. Similarly, a 10,000U/mL solution of 5-LOX enzyme and an 80 mM solution of Standard (linoleic acid) were also prepared. Sample solutions of different concentrations were solubilized in 250 µl of phosphate buffer (50 mM) having pH 6.3. About 250 µL of 5-LOX enzyme solution was added to each concentration of sample solution and incubated at room temperature for 5 min. Substrate solution (1000 µL) with 0.6 mM conc. was added with vigorous shaking. Absorbance was recorded at 234 nm using Zileuton as the positive control. Enzyme inhibition was calculated from absorbance data and reported as percent inhibition.

$$Inhibition(\%) = \frac{Control\ Abs - Sample\ Abs}{Control\ Abs} \times 100$$

IC_{50} values were determined by plotting percent inhibition against varying concentrations of the test sample.

2.9 Statistics Analysis

Data are reported as mean ± SEM, using TWO-WAY ANOVA followed by Bonferroni post-test. Graph pad prism software version 8.4.2 was used for the assessment of all the experimental analysis, *p< 0.05, **p< 0.01, and ***p< 0.001 was considered significant.

3. Results

3.1. Phytochemical tests

All selected variants of *M. Jalapa* were qualitatively evaluated for active phytochemical compounds presented in **Table 1**. The aerial parts of the plant showed the presence of different groups of compounds, including flavonoids, glycosides, alkaloids, phenols, and tannins. Quantitative estimation of different groups of compounds showed a considerable quantity of total flavonoids, phenolic, and alkaloids particularly in**MJ-4** variant. In the acute toxicity study, no signs of toxicity or mortalities were recorded for any variant during 14 days of observation after administering up to 2000 mg/kg of crude extract.

Table 1: Preliminary phytochemical screening of selected variants.

Phytochemical groups	**MJ-1**	**MJ-2**	**MJ-3**	**MJ-4**
Flavonoids	+	+	+	+
Glycosides	+	+	+	+
Saponins	+	+	+	+
Alkaloids	+	+	+	+
Phenolic comp.	+	+	+	+
Tannins	+	+	+	+
Total Flavonoids (mg/g)	3.02	2.64	4.21	4.62
Total Phen comp. (mg/g)	278	214	320	333
Total Alkaloids (mg/kg)	0.044	0.032	0.057	0.062

3.2. In vitro COX-2/5-LOX results of crude extract and sub-crude fractions

Initially, we evaluated the crude extracts, chloroform, and ethyl acetate fractions of selected genetic variants of *Mirabilis jalapa*, i.e. **MJ-1**, **MJ-2**, **MJ-3,** and **MJ-4** against the cyclooxygenase (COX-2) and lipoxygenase (5-LOX) enzymes as shown in **Tables 2**. **Table 2** is summarized form and contains the IC_{50} values only while the detailed information is provided in **Table S1-S3** of the supporting information. The COX-2 activity of the samples was compared with the standard celecoxib while for 5-LOX activity, zileuton was used as a comparative standard drug. All the samples including the standard drugs were evaluated in five different concentrations, i.e. 1000 to 62.5 µg/ml. Among the crude samples of the four plant varieties, the highest activity was shown by **MJ-4** samples against both COX-2 and 5-LOX enzymes as shown in **Table 2**. In the COX-2 assay, the **MJ-4** crude sample exhibited percent inhibitions of 81.41, 60.79, 44.29, 28.83, and 21.61% on concentrations 1000, 500, 250, 125, and 62.50 µg/ml respectively. The calculated and observed IC_{50} value of **MJ-4** was 281.26 µg/ml which was compared with celecoxib IC_{50} (97.23 µg/ml). Similarly, against the 5-LOX enzyme, **MJ-4** exhibited an IC_{50} value of 345.47 µg/ml in comparison to the zileuton IC_{50} 285.54 µg/ml. The remaining samples, i.e. **MJ-1**, **MJ-2,** and **MJ-3** exhibited IC_{50} values of 2000.43, 1325.32, and 370.27 µg/ml against the COX-2 enzyme. Similarly, the IC_{50} values against the 5-LOX enzyme were 755.82, 585.36, and 352.41 µg/ml for plant varieties **MJ-1**, **MJ-2,** and **MJ-3** respectively.

The results of chloroform fractions of four varieties **MJ-1**, **MJ-2**, **MJ-3,** and **MJ-4** are summarized in **Table 2,** were comparatively very low. This might be due to the absence of analgesic and anti-inflammatory phytochemicals in the chlorinated (chloroform) fraction. Among the chloroform samples, **MJ-4** exhibited IC_{50} values of 1750.46 and 482.98 µg/ml against the COX-2 and 5-LOX enzymes. The IC_{50} values for the remaining chloroform samples were not potent.

The results given for ethyl acetate fractions of four varieties **MJ-1**, **MJ-2**, **MJ-3,** and **MJ-4**revealed that the same fractions of the four varieties were potent compared to other solvent fractions. Among the four varieties, **MJ-3** and **MJ-4** were more potent against the COX-2 and 5-LOX enzymes. The remaining two varieties, i.e. **MJ-1** and **MJ-2** were less potent against the selected targets. The IC_{50} exhibited by **MJ-3** against the COX-2 and 5-LOX were 370 and 430.17 µg/ml respectively. Similarly, the most potent activity in our samples was given by **MJ-4** ethyl acetate fraction which was 260 and 360.47 µg/ml against the COX-2 and 5-LOX enzymes respectively.

Sample	**IC_{50} (µg/ml)/COX-2**			**IC_{50} (µg/ml)/5-LOX**		
	Crd. Ex	**Ch. Fr.**	**EtOAc Fr.**	**Crd. Ex**	**Ch. Fr.**	**EtOAc Fr.**
MJ-1	2000.43	>2000	1320.57	755.82	>2000	955.47
MJ-2	1325.32	>2000	1180.83	585.36	>2000	940.73
MJ-3	370.27	>2000	370.00	352.41	520.76	430.17
MJ-4	281.26	1750.46	260.00	345.47	482.98	360.47
Celecoxib	97.23			-		
Zileuton	-			285.54		

Table 2: COX/LOX inhibitory potential *of crude hydroalcoholic extract and sub-crude fractions of genetic variants of M. jalapa.*

All the values were expressed as mean ± SEM in comparison to standards celecoxib for COX (cyclooxygenase) and Zileuton for Lox (Lipooxygenase)

3.3.Carrageenan induced anti-inflammatory results

Subplantar injection (0.05 mL) of 1% carrageenan to the mice's hind paw induced a progressive increase in paw thickness reaching its maximum after 2 h in the negative control group. Anti-inflammatory property is expressed as % inhibition of carrageenan-induced edema. Significant dose-dependent edema inhibition was observed for a crude extract of variants compared to negative control with maximum effect at the higher dose used given in **Figure 1**. Among all

selected variants **MJ-4** at 300 mg/kg has demonstrated significant inhibition (56.7%) of carrageenan-induced edema formation during 3 h of observation. The inhibitory effect on edema continued till the end of the experiment. The same variant also showed a similar effect with a 200 mg/kg dose. **MJ-3** also demonstrated good inhibitory activity on edema formation at all doses used. A maximum inhibitory effect (45.9%) was observed during the last hour of observation at a higher dose. **MJ-1** also showed some inhibition of edema at higher doses, whereas **MJ-2** remained almost inactive. Ethyl acetate fraction of all variants proved to be the most potent fraction which inhibited paw edema formation in a dose-dependent pattern like corresponding crude extracts (**Figure 2**). A significant sustained inhibitory effect on induced paw edema was demonstrated by the EtOAc fraction of **MJ-4** in the later part of the experiment. Maximum inhibition on edema formation i.e. 54.7% was observed with 150 mg/kg at 5 h of the experiment. **MJ-3** also demonstrated a good inhibitory effect on edema formation during the later phase of the experiment in all three doses used. Some activity was also observed for the EtOAc fraction of the **MJ-1** variant of the plant where the same fraction of **MJ-2** showed poor or no inhibition on paw edema. Other fractions i.e. hexane, chloroform, butanol, and water fraction of all variants demonstrated poor or no activity against carrageenan-induced paw edema formation, showing that active components responsible for the inhibition of carrageenan-induced edema may have largely concentrated in ethyl acetate fraction of these variants. These findings revealed that among selected variants of *M. jalapa* the pink variant (**MJ-4**) got a relatively more inhibitory effect on carrageenan-induced edema formation.

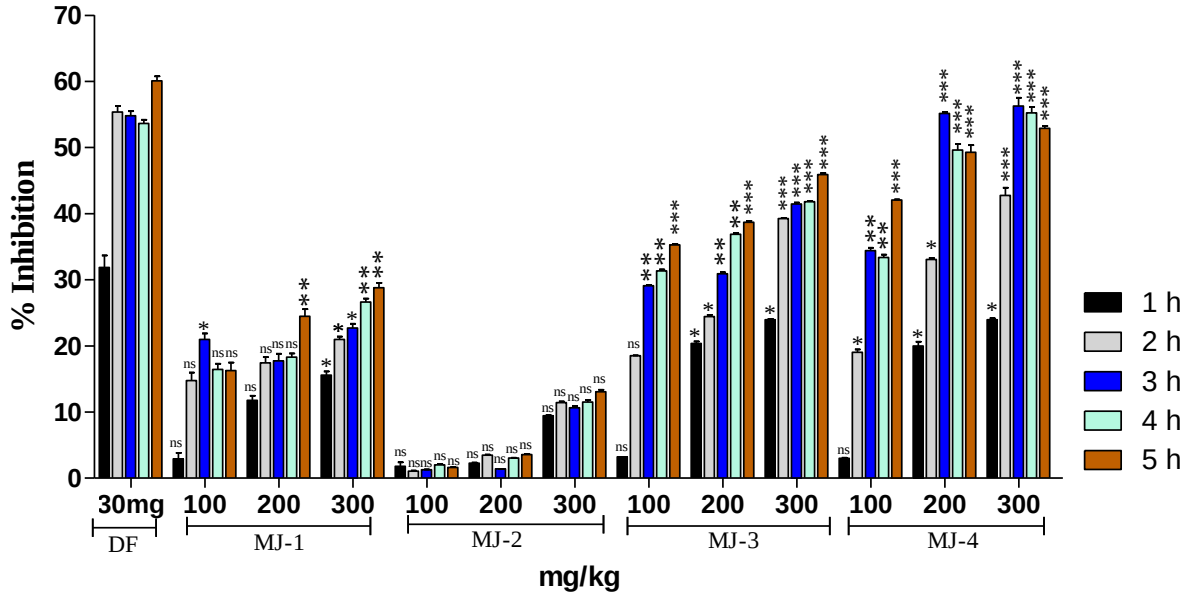

Figure 1: Inhibitory effect of crd. *M. Jalapa* on paw edema.
Values given as Mean ± SEM, ***p<0.001, **p<0.01, *p<0.05. Data was analyzed by TWO-WAY ANOVA followed by Bonferroni post-test.

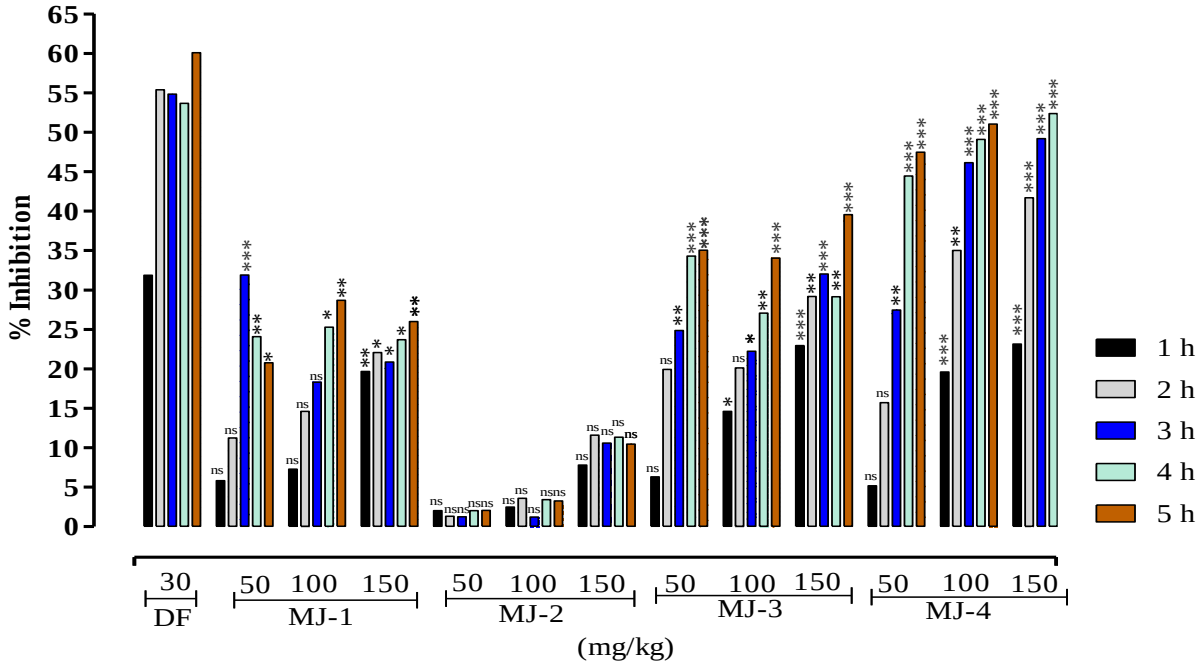

Figure 2: Inhibitory effect of EtOAc fraction on paw edema.

Values given as Mean ± SEM, ***p<0.001, **p<0.01, *p<0.05. Data was analyzed by TWO-WAY ANOVA followed by Bonferroni post-test.

3.4. Antinociceptive potentials

In vivo, antinociceptive/analgesic-like activity of crude extract and sub-crude fractions of selected variants of *M. Jalapa* was investigated at three different doses i.e. 100, 200, and 300 mg/kg (crude extract) and 50, 100, and 150 mg/kg (fractions). Hot plate and tail immersion tests were employed to assess the central, where the acetic acid-induced writhing method was used for determining the peripheral analgesic effect. Change is the reaction time in the presence and absence of standard/sample recorded in seconds. Values are expressed as Mean ± SEM (n=6).

On a hot plate during the first 30 mins of the experiment crude extracts of all the variants have demonstrated some analgesic activity reported as percent analgesic activity comparable to the standard drug used (**Figure 3**). A maximum effect (68.72%) was observed for **MJ-4** during the later phase of the experiment. Very small or no change was observed in the reaction time for small doses of chloroform fraction of all variants in the given time. However, the same fraction of variant **MJ-4** and **MJ-3** in higher doses has demonstrated good activity i.e. 65% and 57% respectively (**Figure 4**). Ethyl acetate fraction of all variants demonstrated a sustained analgesic activity at higher doses throughout the experiment, comparable to the standard drug used (**Figure 5**). Maximum percent inhibition i.e. 69%, 70%, 71%, and 66% was observed for **MJ-1**, **MJ-2**, **MJ-3,** and **MJ-4** respectively. The activities demonstrated by the ethyl acetate fraction of the variants suggested that the active ingredient responsible for an analgesic-like effect may be present in the same fraction making it the most potent fraction of all variants.

On tail immersion test response of variants towards pain given in Figure 6, was not too much different from what was observed with the hot plate model. Compared to 20.93% inhibition of the standard drug used the **MJ-4** and **MJ-3** variants at 300 mg/kg dose demonstrated an excellent

inhibitory effect i.e. 34.52% and 31.53.12% respectively. Ethyl acetate fractions of all selected variants have demonstrated good activity in the higher dose used (**Figure 7**). A maximum inhibitory effect of more than 35% was demonstrated by the **MJ-4** variant at 150 mg/kg dose.

On the acetic acid-induced writhing model for investigating the peripheral analgesic effect of these variants, the maximum inhibitory effect was observed for **MJ-4** i.e. 68.54% at the higher dose used (**Figure 8**). **MJ-3** variant also demonstrated a good inhibitory effect i.e. 66.93% at 300 mg/kg dose. Other varieties have also demonstrated some activity at higher doses only. Ethyl acetate fractions of all selected varieties have demonstrated good activity, especially at higher doses used (**Figure 9**). The maximum inhibitory effect was observed for **MJ-4** at 150 mg/kg dose, i.e. 70.16% compared to 82.25% of standard drug used. Other fractions of all selected varieties have demonstrated small or no inhibitory action on the number of acetic acid-induced writhing. The findings showed that among all selected genetic variants of *M. jalapa*, **MJ-4** possessed excellent inhibitory potential on the number of acetic acid-induced writhing, followed by **MJ-3**.

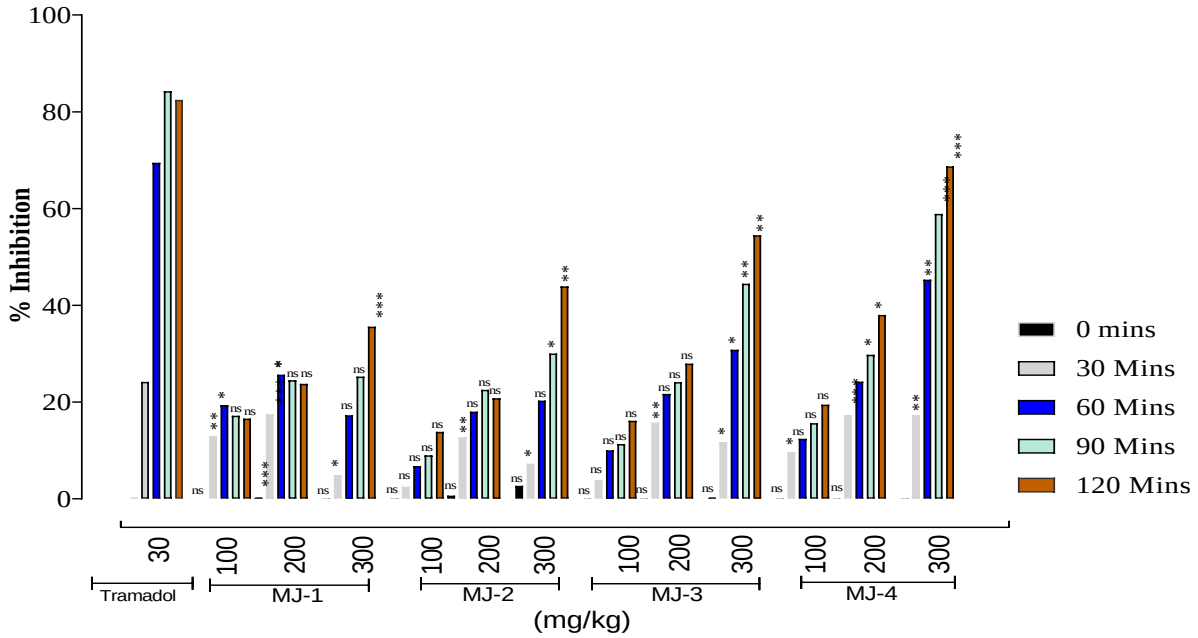

Figure 3: Analgesic-like effect *M. Jalapa* (crd) on hot plate model. Inhibitory effect of crd. *M. Jalapa* on paw edema.
Values given as Mean ± SEM, ***p<0.001, **p<0.01, *p<0.05. Data was analyzed by TWO-WAY ANOVA followed by Bonferroni post-test.

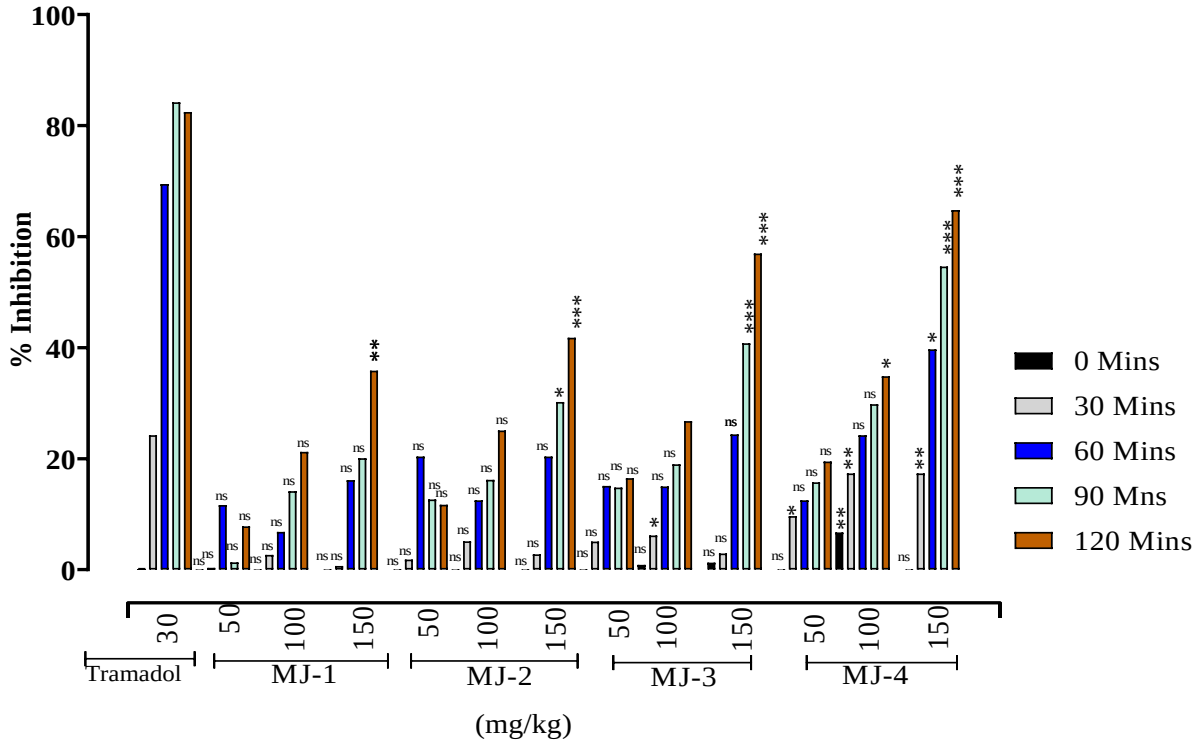

Figure 4: Analgesic-like activity of chloroform fractions on a hot plate.

Values given as Mean ± SEM, ***p<0.001, **p<0.01, *p<0.05. Data was analyzed by TWO-WAY ANOVA followed by Bonferroni post-test.

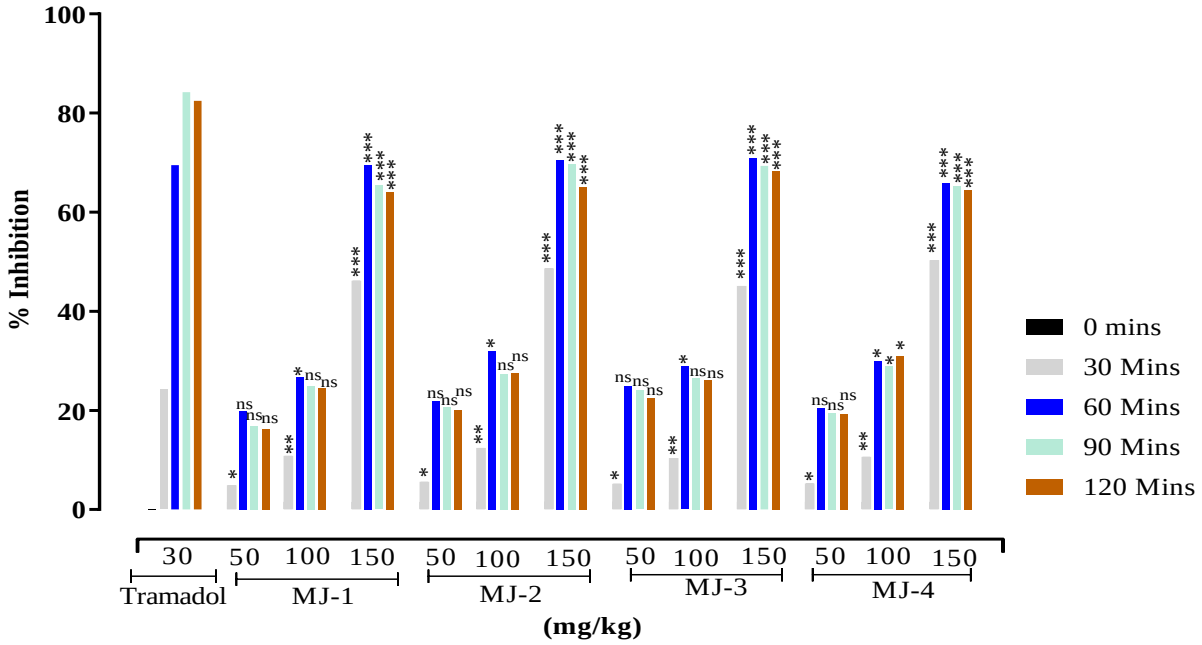

Figure 5: Analgesic-like activity of EtOAc fractions on a hot plate.
Values given as Mean ± SEM, ***p<0.001, **p<0.01, *p<0.05. Data was analyzed by TWO-WAY ANOVA followed by Bonferroni post-test.

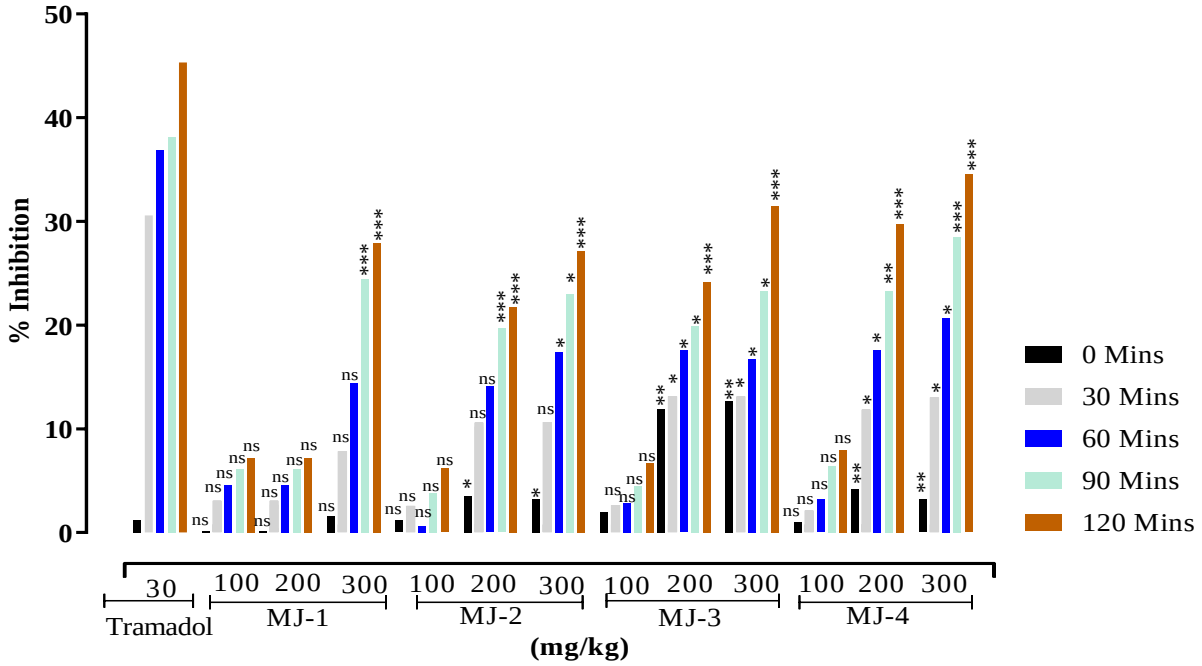

Figure 6: Analgesic-like activity of *M. jalapa* (crd) on tail immersion test.

Values given as Mean ± SEM, ***p<0.001, **p<0.01, *p<0.05. Data was analyzed by TWO-WAY ANOVA followed by Bonferroni post-test.

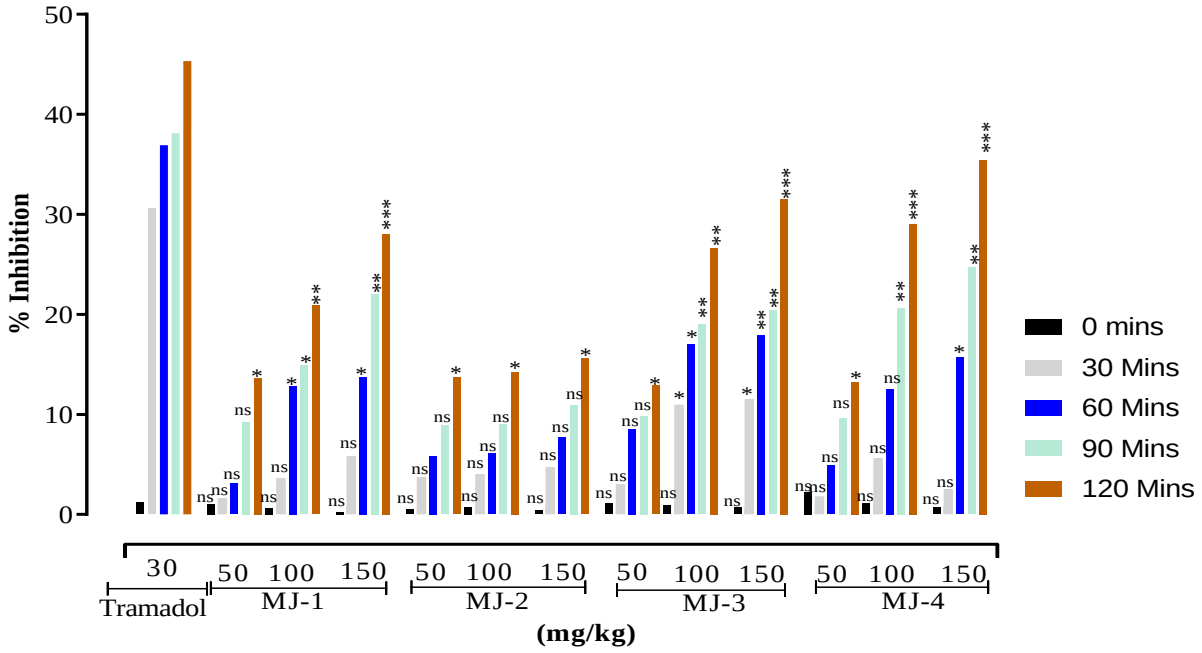

Figure 7: Analgesic-like activity of EtOAc fraction of selected variants of *M. jalapa* on tail immersion test.
Values given as Mean ± SEM, ***p<0.001, **p<0.01, *p<0.05. Data was analyzed by TWO-WAY ANOVA followed by Bonferroni post-test.

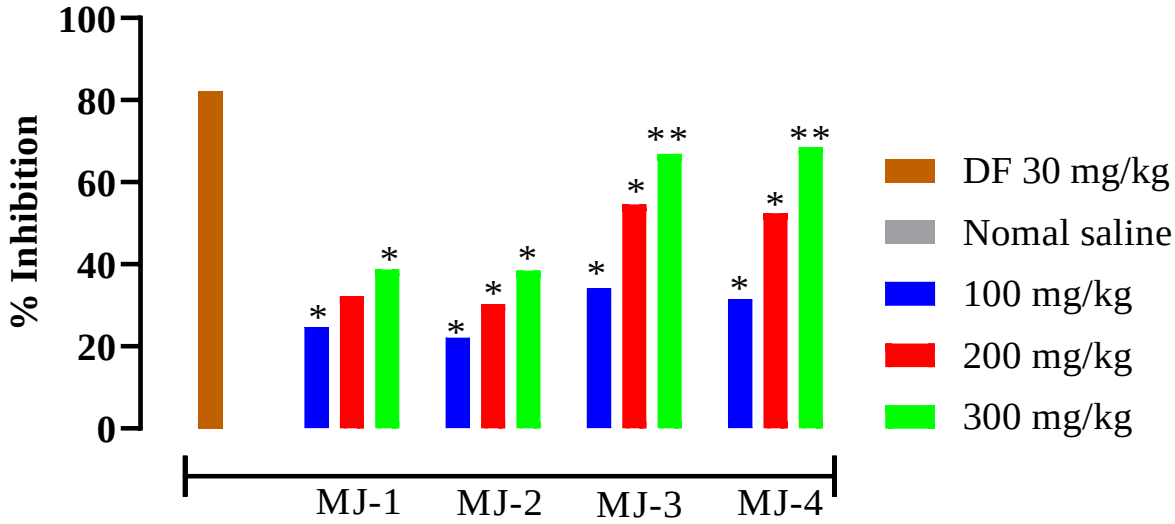

Figure 8: Analgesic-like activity of *M. jalapa* (crd) on the writhing test.
Values expressed as Mean ± SEM, *p<0.05, **p<0.01 and ***p<0.001, ns: not significant. TWO-WAY ANOVA followed by Bonferroni post-test used for data analysis.

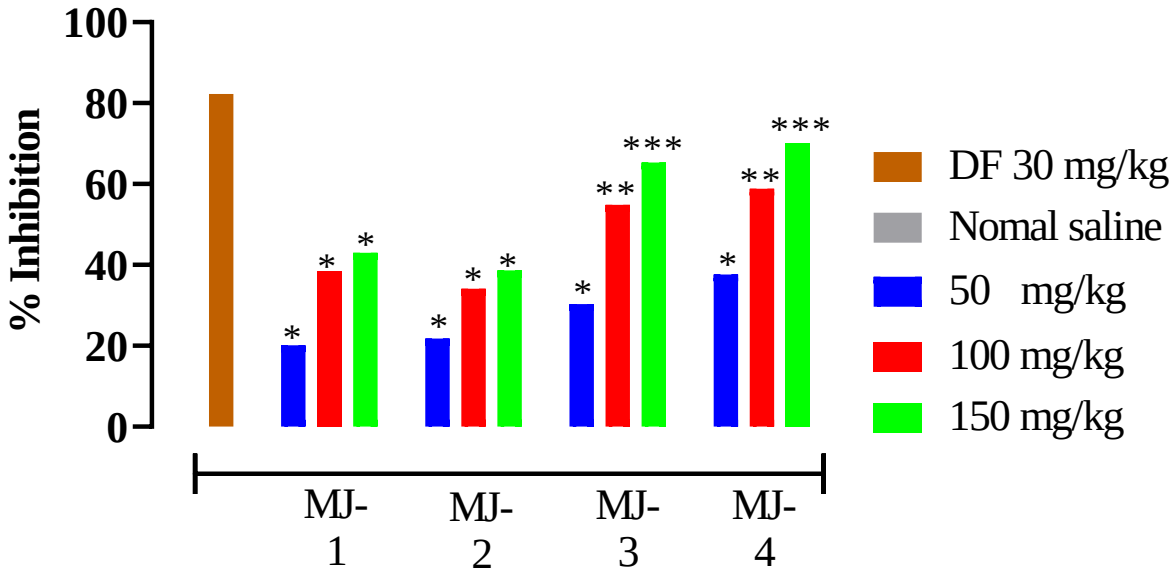

Figure 9: Analgesic-like activity of EtOAc fractions of *M. jalapa* on the writhing test.
Values given as Mean ± SEM, ***p<0.001, **p<0.01, *p<0.05. Data was analyzed by TWO-WAY ANOVA followed by Bonferroni post-test.

4. Discussion

Four variants of *M. jalapa* having yellow, white, light pink, and pink flowers collected from the same locality during the blooming season were investigated for anti-inflammatory and analgesic-like properties using *in vitro* COX-2/5-LOX inhibition and *in vivo* animal models. The pink variant proved to be the most potent inhibitor of post-carrageenan edema formation in mice. Both crude hydroalcoholic extract and ethyl acetate fraction of the same variant demonstrated dose-dependent and sustained inhibitory activity during 3 h which continued till termination of the experiment. On the hot plate model, good analgesic-like activity was observed for a crude extract of all variants at 300 mg/kg dose. Maximum activity was demonstrated by the pink variant at a higher dose used followed by the light pink variant. Chloroform fractions of the variants repeated almost the same behavior as that of corresponding crude extracts. An interesting pattern of analgesic-like activity was observed on the hot plate test for the higher dose of ethyl acetate fractions. All variants at 150 mg/kg dose similarly responded to thermal stress

having peak value at the first 60 min interval and with a slight decrease that remained sustained towards the end of the experiment. On the tail, the immersion test significant increase in reaction time observed at a higher dose of all variants. Maximum increase was demonstrated by hydroalcoholic extract of pink variant at 300 mg/kg dose i.e. 5.58% compared to 6.31% of tramadol and 3.25% of negative control. While comparing ethyl acetate fractions of selected variants, an excellent effect i.e. 5.64% was observed for the pink variant at 150 mg/kg followed by the light pink variant at 120 mins. Upon investigation for peripheral analgesic-like properties using acetic acid-induced writhing, the pink variant again showed the most potent inhibitory effect on the number of writes with 68.54% inhibition at 300 mg/kg dose followed by light pink with 66.93% inhibition at the same dose. Further increase in effect i.e. 70.15% was observed for ethyl acetate fraction of the same variant at 150 mg/kg.

The inflammation and its associated pain have been studied alone or in combination by various researchers [34, 41-42]. The approaches to control pain and inflammation can be both synthetic and also from natural products origins [34, 43]. Both of these have their advantages and disadvantages. However, natural-based drugs are considered to be safer compared to synthetic drug candidates in practice [44-45]. Comparing the total flavonoids and total phenolic contents of these variants it may be observed that the pink variant has a relatively large quantity of both flavonoids and phenolic-like compounds followed by the light pink variant. Several studies are available demonstrating excellent anti-inflammatory and analgesic-like properties of flavonoids and phenolic compounds. A study has reported that regulatory enzymes and transcription factors responsible for controlling mediators involved in inflammation are effectively inhibited by flavonoids [46-47]. Similarly, the phenolic content of plants has been reported to exhibit potent analgesic and anti-inflammatory properties [48]. Therapeutically important flavonoids have

recently been summarized with diverse properties including anti-inflammatory and analgesic–like activities [49]. Similarly, peril aldehyde is observed to be potent in anti-inflammatory and other infectious diseases [50] Promising results of preclinical studies to develop safe and effective natural polyphenol formulations as remedies for pain and inflammation have also been reported [51].

Looking at the findings and available information it may be suggested that the relative richness of flavonoids and phenolic contents of the pink variant may be the possible cause of an increased response towards carrageenan-induced edema and pain stimulants demonstrated by the same variant in different animal models.

5. Conclusions

Based on our current observations, we can claim that we have evaluated the anti-inflammatory and antinociceptive potentials of four genetic variants of *M. Jalapa*. Based on the *in vitro* COX-2 and 5-LOX results, it is obvious that among all the variants/extracts, the ethyl acetate fraction of the Pink variant was the most active. We also subjected all of our samples to experimental animals to confirm *in vivo* anti-inflammatory and antinociceptive potentials. The activity profile of all our samples was in a similar pattern in all *in vitro* and *in vivo* models. Based on the overall observations, we think that the ethyl acetate fraction of the Pink variant should be subjected to extended pharmacological studies for the discovery of new/potent drug molecules for the management of inflammation and its associated nociceptive pain.

Author Contributions: IA, IS and SK have identified and collected the plant sample. They have performed all the preliminary assays. AS helped in in-vitro assays. The whole project was under

the supervision of SK, and AS. All the authors have read and approved the final version of the manuscript for submission. The authors declare no conflict of interest.

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Data Availability Statement: The whole data is available within the manuscript and supporting information.

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