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UNVEILING THE THERAPEUTIC POTENTIAL: ISOLATION AND ANALGESIC ACTIVITY OF *JUSTICIA ADHATODA VASICA (JAV)*

Shivendra Pratap Singh, *Alok Kumar Dash

Department of Pharmacy, Veer Bahadur Singh Purvanchal University, Jaunpur-222003, India

*Corresponding author

Dr.Alok Kumar Dash

Assistant Professor

Institute of Pharmacy,VBSPurvanchal University,Jaunpur

mail id: dr.alokdash@gmail.com

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ABSTRACT

This study investigates the medicinal potential of *Justicia Adhatoda Vasica (JAV)* by focusing on the isolation, phytochemical analysis, and in-vivo analgesic activity of its bioactive compound. Then obtained crude extract underwent isolation process. The in-vivo analgesic activity of the compound was assessed, shedding light on their potential in pain relieving effect. In the Preliminary TLC of *JAV* Methanolic extract was performed and Rf values were found to be 0.51 and 0.51 of *JAV* and Std. Flavonoid. Advanced spectroscopic techniques like UV spectroscopy, FT-IR spectroscopy, Mass spectroscopy, NMR were employed for phytochemical analysis to identify and characterize the isolated compound. The study recorded UV spectra of isolated fraction of *JAV*, determining λmax and wavelength of 292 nm. Physical, chemical, and spectral investigation confirmed the presence of 5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one in *JAV* Methanolic extract fraction (J). In-vivo analgesic study found that oral administration of both extract and isolated compound at 200 and 40 mg/kg and standard drug significantly decreased the number of licking induced by formalin compared to the control group, with significant effects observed at all experimental doses. Hence, this research provides valuable insights into the pharmacological properties of *Justicia Adhatoda Vasica*, laying the groundwork for further exploration of its therapeutic applications.

Keywords: *Justica Adhatoda Vasica*, Spectroscopy, In-vivo activity, Isolation.

1. INTRODUCTION

A key medicinal plant in traditional South Asian medicine is *Justicia Adhatoda L*. Syn. *Adhatoda Vasica*. The use of it for respiratory conditions is well documented. For physicians practicing Indian traditional medicine, this herb is like a mother. For this reason, the plant is called Vaidyamata Singhee in Sanskrit **(Anshutz, 1996).** A common medicinal plant called *Justicia adhatoda L*. is advised for the treatment of a variety of illnesses, including bronchitis, fever, jaundice, arthritis, muscle soreness, and ulcer diseases **(Mandal and Laxminarayna, 2014; Maurya and Singh, 2010a).** It is an evergreen shrub that ranges in height from 1.0 to 2.5 meters and is a member of the Acanthaceae family. It is also known by a number of common names, including *Vasaka, Baker,* and *Malabar Nut* **(Pham *et al.*, 2008).** It has a disagreeable fragrance and a bitter flavour **(Mishra *et al.*, 2011).** Numerous research investigations have shown the application of leaves and blossoms as expectorants, antispasmodics, cough and cold remedies. An in vivo investigation using rats shown that it may stop carbon tetrachloride-induced oxidative damage **(Maurya and Singh, 2010b).** *A.vasica* contains phenolic compounds that have been shown to have the best antioxidant activity and to scavenge free radicals **(Rajurkar *et al.*, 2012).** *Adhatoda Vasica* is a promising drug discovery target due to its medicinal properties. Its phytochemicals and active components can help prevent the damage caused by free radicals, which are highly reactive oxygen species that can damage biomolecules and lead to diseases like cancer and degenerative diseases like arthritis, cirrhosis, atherosclerosis, and emphysema **(Atitegeb Abera *et al.*, 2015, J Mishra et al., 2013).** Many biological activities of plant-derived compounds, such as those related to cancer, inflammation, arthritis, immunomodulation, etc., have been studied **(Nelson *et al.*, 2020; Talhouk *et al.*, 2007; Ganju *et al.*, 2003)**. It was discovered through the use of in vitro and in vivo experiments that the analgesic and anti-inflammatory effects of extract were studied. It was found that the plant extract significantly decreased inflammation and, consequently, pain perception by lowering inflammatory mediators like IL-1β and TNF-α and eliciting an anti-inflammatory response **(Ganju *et al.*, 2003; Hasan *et al.*, 2014; Anwikar and Bhitre, 2010).** Based on the previously reported studies, in this investigation we isolated the bioactive substance from *Justicia Adhatoda Vasica* whole plant and evaluated the characteristics of specified compound, In-vivo analgesic activity is analyzed by formalin induction method.

2. MATERIAL METHOD

2.1 Plant material

The whole plant of *Justicia Adhatoda Vasica* was collected from local area of VBSPurvanchal University,Jaunpur Uttarpradesh. The sample was identified by Arti Garg, Scientist-E/Head of Office Botanical Survey of India, CRC, 10 Chatham line, Allahabad-211002. The specimen Accession no. of *Justicia Adhatoda Vasica is* 104530.

2.2 Chemicals and reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

2.3 Cold Maceration:

Plant material was extracted by using cold maceration method. Extraction yield of extract was calculated using the following equation below:

$$\textbf{Percentage Yield} = \frac{\textbf{Actual yield}}{\textbf{Theoretical yield}} \times 100$$

2.4 Preliminary Thin layer chromatography:-

Thin-layer chromatography is a "solid-liquid adsorption" chromatography. In this method stationary phase was TLC plates of silica gel 60 F_{254} pre coated with layer thickness of 0.2 mm using different solvent system. In this method, the mobile phase travels upward through the stationary phase. Spots were applied manually using capillary tube, plates were air dried using and TLC chamber were developed at room temperature with respective solvent system. The solvent travels up the thin plate soaked with the solvent by means of capillary action. During this procedure, it also drives the mixture priorly dropped on the lower parts of the plate with a pipette upwards with different flow rates. Thus the separation of analytes was achieved. This upward travelling rate depends on the polarity of the material, solid phase, and of the solvent **(Ozlem *et al.*, 2016).**

$$\textbf{R}_f \textbf{ Value} = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

Solvent system developed in preliminary TLC for *JAV* methanolic extract in which the maximum spots were visible in Toluene: Ethyl acetate: Acetic acid (6:4:0.4) mobile phase with std. Flavonoid. So that Toluene: Ethyl acetate: Acetic acid (6:4:0.4) solvent was taken as mobile phase for column chromatography.

2.5 Column chromatography

Methanolic extract was subjected to silicagel column chromatography for isolation of Flavonoid from *Justicia Adhatoda Vasica* extract. A vertical glass column made of borosilicate material was used for chromatography. The column was rinsed with the acetone and was completely dried before packing. Column was packed using wet packing technique using silica gel (60-120) as the adsorbent. Slurry was prepared using toluene and was poured in to the column. 1gm of extract was added over the top of the column. Gradient elusion technique was followed for column chromatography. The column was eluted with Toluene: Ethyl Acetate: Acetic acid (6:4:04) number of elutes were collected. The fractions/elutes collected were concentrated and TLC was performed to identify the presence of single compound **(Srivastava *et al.*, 2021).**

2.6 Spectroscopic characterization:-

2.6.1 UV-visible Spectroscopy

The isolated fraction (J) of *JAV* Extract was scanned from 200 to 800 nm wavelength using UV-Visible Spectrophotometer (Shimadzu UV-1700) and the characteristic peaks were

detected and recorded **(Patel *et al.,* 2022)**.

2.6.2 FT-IR

To establish the presence of the functional groups in the isolated fraction (J) of *JAV* Extract, FT-IR spectroscopy was performed using Perkin Spectrum BX spectrophotometer. The sample was dried and ground with KBr pellets and analyzed on Thermo Nicolet model 6700 spectrum instrument. A disk of 200 mg of KBr was prepared with a mixture of 2% finely dried sample and then examined under IR-spectrometer. Infrared spectra were recorded in the region of 400 - 4,000 cm-1 **(Luciene *et al.,* 2008).**

2.6.3 NMR Spectroscopy

NMR spectroscopy was performed for the isolated fraction (J) of *JAV* Extract to identify the structure of the compound present in the isolated fraction. JEOL RESONANCE NMR spectroscopy for this purpose was Fourier Transform Nuclear Magnetic Resonance spectroscopy **(Zia *et al.,* 2019).**

2.6.4 Mass Spectroscopy

Mass spectrometry converts molecules into ions and according to their mass and charge the ions can be separated and sorted. The mass spectrometer used for the identification of the molecular weight of isolated fraction (J) of *JAV* Extract was recorded on mass spectrometer instrument micrOTOF-MS **(Wiley *et al.,* 1995).**

2.7 In-vivo Analgesic activity

2.7.1 Animals

The animals Swiss albino mice (procured from in-house animal facility of PBRI) was kept in standard large spacious hygienic polypropylene cages and maintained at 22 ± 2 ◦C temperature with 12/12-h light and dark cycle. All the animals were fed with commercially available rat normal pellet diet (NPD) purchased from Keval Sales Corporation, Vadodara and water ad libitum was provided up to the end of the study.

2.7.2 Experiment

Mice fasted overnight with the provision of water. Then, the overnight fasted mice were randomly selected and assigned into groups of four, each group with six mice. Group I receiving DW (5 mL/ kg) was assigned as the negative control. Group II receiving 5 mg/kg morphine (positive control). The remaining groups (III to IV) were given the test extract JAV at a dose of 200 mg/kg and isolated compound ICJF 40 mg/kg respectively. Two phases of nociception, namely the early and late phase, were observed during the course of the experiment. The first phase was recorded by taking the time of the animals spent licking their paw for 0–5 min after the injection of formalin. The second phase was recorded by taking the time the animal spent licking its paw for 15–30 min after formalin injection. (**AK Dash et al., 2014, D Saha et al., 2011**). The percent inhibition of nociception for the two phases was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Control mean - Test mean}}{\text{Control mean}} \times 100$$

Recording of nociceptive behaviors began immediately following formalin injections (time 0) and was continued for 60 minutes. The first 5 minutes was considered as early phase and minutes 15 to 30 were considered as the late phase of formalin test.

3. RESULTS

3.1 Preliminary TLC preparation for the estimation of active constitutes – TLC of *Justicia Adhatoda Vasica* Methanolic extract

For Flavonoid:-

Mobile Phase- Toluene: Ethyl acetate: Acetic acid (6: 4: 0.4)

Figure 1 TLC of *JAV*

Short-UV (254 nm)

Long-UV (365 nm)

Visible Light

Figure 1 TLC estimation by UV lamp for *JAV* with Std. Flavonoid

(Std. = Standard, *JAV* = *Justicia Adhatoda Vasica*)

Table 1 TLC of *Justicia Adhatoda Vasica* Methanolic extract

S. No.	Solvent system	No. of spots	Colour of spots at Wavelength (254 & 365nm)	Rf value (Extract)	Rf value (Std. Flavonoid)
`1.	Toluene: Ethyl Acetate: Acetic acid (6:4:04)	10	Light Blue (Std 365) Green (Std 254) Florescence (JAV 365) Florescence Light Pink Light Purple Light Blue Dark Pink Purple Pink Purple Light Green (JAV 365) Light Green Light Green Green Green	- - 0.12 0.24 0.47 0.49 0.51 0.58 0.61 0.67 0.74 0.12 0.24 0.47 0.49 0.51	0.51

			Light Green Light Green	0.58 0.61	

3.2 Column Chromatography

3.2.1Column Chromatography of *JAV* Methanolic extract –

Table 2 Fraction collected from Column Chromatography of *JAV* Methanolic extract

Sr. No.	Eluent composition	Fraction collected	Remarks
1	Toluene: Ethyl Acetate: Acetic acid (6:4:04)	01 (A)	White creamy coloured mixture of compound
2		02 (B)	Dark Yellowish coloured mixture of compound
3		03 I	Light Yellowish coloured mixture of compound
4		04 (D)	White creamy coloured mixture of compound
5		05 (E)	Light Greenish coloured mixture of compound
6		06 (F)	Green Brownish coloured mixture of compound
7		07 (G)	Dark Green Brownish coloured mixture of compound
8		08 (H)	Greenish coloured mixture of compound
9		09 (I)	Light Greenish coloured mixture of compound
10		10-12 (J) (J1, J2, J3)	Very Light Greenish coloured mixture of compound
11		13 (K)	Dark Green Brownish coloured mixture of compound
12		14 (L)	Dark Greenish coloured mixture of compound
13		15 (M)	Green Brownish coloured mixture of compound
14		16-17 (N) (N1, N2)	Light Greenish coloured mixture of compound
15		18 (O)	White creamy coloured mixture of compound

3.2.2 TLC of all collected fractions-

A) TLC of all collected fractions of *JAV* Methanolic extract -

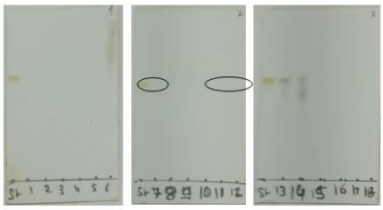
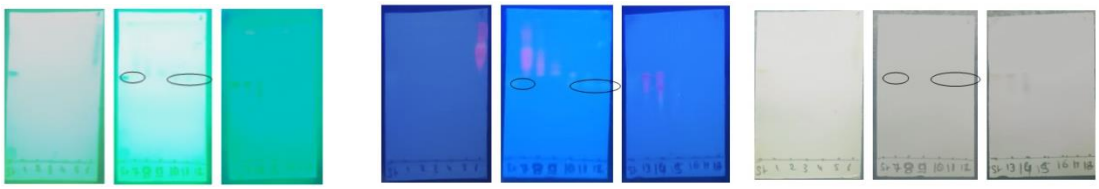


Figure 2 TLC estimation for *JAV* fractions after column chromatography with Std. Flavonoid.



(a) Short-UV (254 nm) (b) Long-UV (365 nm) (c) Visible Light

Figure 3 TLC estimation by UV lamp for *JAV* fractions after column chromatography with Std. Flavonoid.

3.3 TLC of fractions (A, B, C, D, E, F, G, H, I, J, K, L, M, N & O) of *JAV* Methanolic extract -

Table 3 Rf values of all collected fractions of *JAV* after column chromatography

Sr. No.	Fraction	Solvent system	No. of spots	Colour of spots at Wavelenth (365nm)	Rf value (Extract)	Rf value (Std. Flavonoid)
1.	A	Toluene: Ethyl Acetate: Acetic acid (6:4:04)	-	-	-	0.52
2.	B		-	-	-	
3.	C		01	Fluorescence	0.41	
4.	D		01	Fluorescence	0.41	
5	E		01	Fluorescence	0.41	
6	F		04	Pink Purple Fluorescence Purple	0.67 0.71 0.74 0.81	
7	G		03	Pink Fluorescence Purple	0.68 0.73 0.80	
8	H		03	Pink Light Pink Fluorescence	0.65 0.68 0.80	
9	I		02	Light Pink Light Purple	0.65 0.68	
10	J1		02	Light Blue Light Pink	0.52 0.57	
11	J2		02	Light Blue Light Pink	0.52 0.57	
12	J3		02	Light Blue Light Pink	0.52 0.57	
13	K		02	Light Purple Dark Purple	0.49 0.54	
14	L		03	Fluorescence Dark Purple Light Purple	0.46 0.49 0.54	
15	M		01	Fluorescence	0.46	
16	N1		01	Fluorescence	0.46	
17	N2		-	-	-	
18	O		-	-	-	

3.4 Spectroscopic characterization:-

3.4.1 Active constitutes estimation By UV-Spectroscopy-

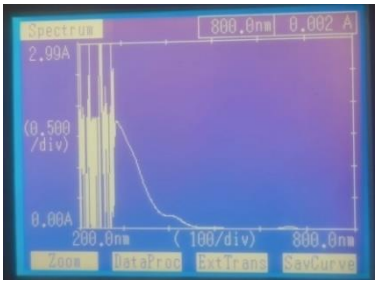


Figure 4 Active constitutes estimation By UV- Spectra of J fraction of *JAV* Methanolic extract after column chromatography

3.4.2 Active constitutes estimation By FTIR – Spectroscopy

(A) IR spectra of the isolated fraction (J) of JAV Methanolic extract

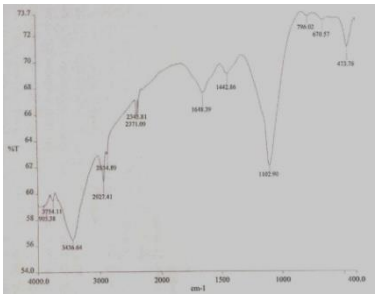


Figure 5 IR spectra of the isolated fraction (J) of *JAV* Methanolic extract

Table 4 FTIR- Spectrum Frequency Range of the isolated fraction (J) of *JAV* Methanolic extract

Sr. No.	Fraction	Frequency Range	Group Absorption (cm^{-1})	Appearance	Group	Compound Class
1	J	3550-3200 (cm^{-1})	3436.64	Strong, Broad	O-H stretching	Hydroxyl Group
		3000-2840 (cm^{-1})	2927.41	Medium	C-H stretching	Alkane
		3000-2840 (cm^{-1})	2854.89	Medium	C-H stretching	Alkane
		2400- 2000 (cm^{-1})	2371.09	Strong	C-H stretching	Alkane
		2000- 1600 (cm^{-1})	1648.39	Medium	C-O stretching	Carbonyl group
		1600-1400 (cm^{-1})	1442.86	Strong	C=C stretching	Benzene Ring
		1400- 1100 (cm^{-1})	1102.90	Weak	C-C stretching	Alkane
		840-790 (cm^{-1})	796.02	Medium	C=C bending	Alkene
		730-665 (cm^{-1})	670.57	Strong	C=C bending	disubstituted

3.4.3 ^{1}H NMR - Spectroscopy-

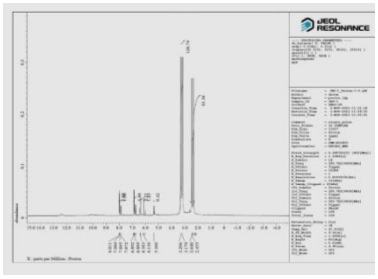
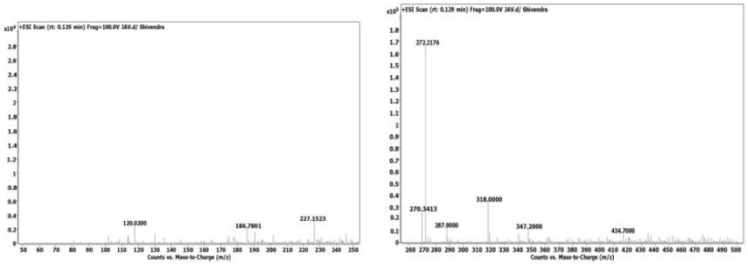


Figure 6 ^{l}H-NMR spectra of the isolated Fraction (J) of *JAV* Methanolic extract

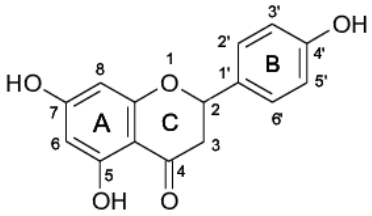
3.4.4 Mass – Spectroscopy-

(A) Mass spectra of the isolated Fraction (J) of JAV Methanolic extract -



(a) **(b)**

Figure 7 Mass spectra (a & b) of the isolated Fraction (J) of *JAV* Methanolic extract



5, 7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one

3.5 In-vivo Analgesic activity

Table 5 Formalin induced lick test

Treatment group	**Dose**	**Mean lick time (sec) ± SD**	
		Early Phase	Late phase
Vehicle (DW)	5 ml/kg	67.16 ± 6.853	74.83 ± 3.763
Standard (Morphine)	5 mg/kg	3.5 ± 1.378	2.33 ± 1.505*
Extract JAV	200 mg/kg	42.16 ± 4.167	30.83 ± 4.167*
Isolated compound ICFJ	40 mg/kg	29.83 ± 4.070	18.33 ± 2.160*

Results are provided as Mean ± SD (n=6). Results were analyzed statistically using one-way analysis of variance (ANOVA) followed by Bonferroni t-test. $P < 0.05$*

Table 6 Percent protection

Treatment group	**Dose**	**Percent inhibition**
Vehicle (DW)	5 ml/kg	-
Standard (Morphine)	5 mg/kg	96.88
Extract JAV	200 mg/kg	58.79
Isolated compound ICFJ	40 mg/kg	75.50

4. DISCUSSION

In the Preliminary TLC of *Justicia Adhatoda Vasica* Methanolic extract was performed on different solvent systems (solvent system was selected on the basis of literature survey). TLC performed in Toluene: Ethyl acetate: Acetic acid (6:4:0.4) with Std. Flavonoid that were clearly visible bands in *JAV* Methanolic extract**.** The Rf value were found to be 0.51 and 0.51 of *JAV* and Std. Flavonoid **Fig 1, Table 1**. So that Toluene: Ethyl acetate: Acetic acid (6:4:0.4) was taken as mobile phase for column chromatography. Active constitutes are isolated from column chromatography with the mobile phase of Toluene: Ethyl acetate: Acetic acid (6:4:0.4) for *JAV* to obtained Fractions 01 (A), 02 (B), 03 (C), 04 (D), 05 (E), 06 (F), 07 (G), 08 (H), 09 (I), 10-12 (J1, J2 & J3), 13 (K), 14 (L), 15 (M), 16-17 (N1 & N2) and 18 (O) **Table 2**. Rf value Resulted after performing the TLC estimation is also done for the confirmation of active constituents in fractions J of *JAV* with mobile phase Toluene: Ethyl acetate: Acetic acid (6:4:0.4) by comparing with Std. Flavonoid **Fig 2 & 3, Table 3**. The collected Fractions were taken properly and performed the UV spectrum. UV spectra of the isolated fractions (J) of *JAV* was recorded over a scanning range of 200-800 nm, λmax was also determined and the wavelength of *JAV*, J fraction was found to be 292 nm **Fig 4**. The IR Spectra of isolated fraction (J) of *JAV* Methanolic extract showed that -OH group Strong, Broad peak appeared at 3436.64 cm-1, C-H stretching peaks of Alkane at 2927.41, 2854.89 & 2371.09 cm-1. The Carbonyl group C-O stretching peak at 1648.39 cm-1, C=C Stretching peak of Benzene Ring at 1442.86 cm-1, C-C stretching peak of Alkane at 1102.90 cm-1 and C=C bending peak of Alkene at 796.02 cm-1. The C=C stretching peak of disubstituted at 670.57 cm-1 **Fig 5, Table 4.** In ^{1}H NMR spectra of isolated fraction (J) of *JAV* Methanolic extract showed that ^{l}H-1 proton appeared at 2.45 (dd) ppm, ^{l}H-1 proton appeared at 2.66 (dd) ppm, ^{l}H-2 protons appeared at 3.15-3.30 (3.17 (dd) ppm, 3.29 (dd) ppm), ^{l}H-2 protons appeared at 5.36 (dd) ppm, ^{l}H-2 protons appeared at 6.10-6.40 (6.13 (d) ppm, 6.38 (d) ppm), ^{l}H-2 protons appeared at 6.85-6.90 (6.86 (ddd) ppm, 6.89 (ddd) ppm) and ^{l}H-2 protons appeared at 7.85-7.99 (7.89 (ddd) ppm, 7.98 (ddd) ppm) **Fig 6**. A mass spectrum of isolated Fraction (J) of *JAV* Methanolic extract was recorded on Mass Spectroscopy. Mass spectra of isolated Fraction (J) of *JAV* Methanolic extract showed molecular ion [M^{+}] peaks at mlz 272.2175 which obtained 5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one compound in which presence of carbons (C_{15}), Hydrogens (H_{12}) and Oxygen (O_5). Finally the molecular formula of isolated Fraction (J) of *JAV* Methanolic extract was found to be $C_{15}H_{12}O_5$ according to their fragments (**Fig. 7**). From this physical, chemical and spectral investigation were confirmed the presence of 5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one in Fraction (J) of *JAV* Methanolic extract. In-vivo analgesic activity represents that isolated compound caused inhibition of the licking number in both phases of the formalin test **Table 5,6**. The effect is noteworthy with all of the experimental doses, where 29.83± 4.070 of licking in the first phase and 18.33 ± 2.160 in second phase were observed with the dose of 40 mg/kg of isolated compound. Similarly, the effect is significant with all of the experimental doses, whereas 42.16± 4.167 of licking in the first phase and 30.83 ± 4.167 in second phase were observed with the dose of 200 mg/kg of Extract. Therefore, Oral administration of both extract and isolated compound at 200 and 40 mg/kg and standard drug caused a significant decrease in the number of licking induced by formalin compared to the control group.

5. CONCLUSION

In conclusion, the study on *Justicia Adhatoda Vasica* focused on isolation, phytochemical analysis, and in-vivo analgesic activity. The research has successfully identified active compound that exhibit significant analgesic effect. These findings contribute to the growing body of knowledge surrounding the potential of *Justicia adhatoda* in pain management and support its use in traditional medicine. Furthermore, from the physical, chemical and spectral investigation of the *JAV* Methanolic extract plant of *Justicia Adhatoda Vasica* belonging to the family *Acanthaceae* confirmed the presence of **5, 7-dihydroxy-2-(4-hydroxyphenyl)-2, 3-dihydrochromen-4-one** in Fraction (J) of *JAV* Methanolic extract. Overall, the findings from this research enhance our knowledge of the plant's bioactive constituents and reinforce its significance in traditional medicine and pharmaceutical research.

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