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Pharmacological activities and gas chromatography–mass spectrometry analysis for the identification of bioactive compounds from *Justicia adhatoda* L.

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The current study aimed to assess the pharmacological potential of *Justicia adhatoda* by evaluating the presence of biologically active compounds using the gas chromatography–mass spectrometry approach and to undertake biological activities for the effectiveness of the present compounds using standard tests. A total of 21 compounds were identified in the gas chromatography–mass spectrometry analysis of the ethyl acetate fraction in which 14 of the identified compounds are recognized for their pharmacological potential in the literature. In total, four fractions (ethyl acetate, chloroform, n-hexane, and aqueous) were evaluated for pharmacological activities. In carrageenan-induced inflammation, the chloroform fraction exhibited high anti-inflammatory activity (46.51%). Similarly, the analgesic potential of ethyl acetate fraction was the most effective (300 mg/kg) in the acetic acid-induced test. Similarly, in the formalin test, ethyl acetate fraction exhibited maximum inhibition in both early (74.35%) and late phases (88.38). Maximum inhibition of pyrexia (77.98%) was recorded for the ethyl acetate fraction (300 mg/kg). In DPPH assay, the ethyl acetate fraction revealed the highest scavenging potential among other fractions (50 µg/ml resulted in 50.40% and 100 µg/ml resulted in 66.74% scavenging).

KEYWORDS

antioxidant, analgesic, antispasmodic, anti-inflammatory, antibacterial, medicinal plants

Introduction

Plants have been used by humans for the treatment of various diseases, and this practice date back to ancient civilizations. Furthermore, plants and/or their products have played an immensely important role in the development of pharmaceutical industries due to the presence of unique bioactive compounds (Sundur et al., 2014). Over the last few decades, a number of pharmacologically important compounds have been isolated from plants, and even today the use of medicinal plants in pharmaceutical industries is extensive. It is widely acknowledged that some 80% of the world population of the developing countries of Africa, Asia, and elsewhere still rely on plants as sources of their medications (Khan et al., 2021). New plant taxa have been added to the Flora of Pakistan having great medicinal importance (Ali et al., 2017). Worldwide interest in traditional medicines is rising; this is evident from the increasing number of plant-based commercial companies as well as the international legislation and treaties that allow judicious and sustainable utilization of medicinal plants or their products (Bashir et al., 2011; Khan et al., 2017).

Nonetheless, in folklore, plants have been used in the form of powder, decoctions, infusions, or tablets to treat a variety of human illnesses with little or no information on the safe dosages. Furthermore, the mode of administration and dosage taken varies with culture and traditional beliefs (Khan et al., 2017). Thus, with no known intrinsic standards, low or higher dosages of medicinal plants (also referred to as ethnomedicines) are often associated with complications (Irfan et al., 2022; Ullah et al., 2022). To overcome these limitations, one of the leading and reliable approaches in pharmacology is the use of a model organism to check the efficacy of a specific plant extract and/or dosage applied against disorder/s (Jan and Khan, 2016; Ullah et al., 2019).

There are worldwide growing interests in the identification of new as well as unique plant-based formulations that could be applied for treating inflammations, as antioxidants, and relieving pain and pyrexia, etc. (Simmons, 2006; Bhowmick et al., 2014: Ji et al., 2016; Jan and Khan, 2016; Shah et al., 2017; Ullah et al., 2019).

Justicia adhatoda L. belongs to the Acanthaceae family, and it is locally referred to as Vasaka and Malabar nut. The plant is a perennial, green shrub scattered over wide ranges of Southeast Asian tropical regions (Kaur et al., 2013). Its leaves are used for the treatment of diarrhea (Ahmad et al., 2016); leaves and roots are used in treating diabetes and vomiting (Irfan et al., 2017); leaves and flowers are used against cough, wound healing, and dysentery (Irfan et al., 2018a); leaves are used in treating bronchitis and cough and prevent loose motion (Irfan et al., 2018b); leaf extract is used for the treatment of rheumatism and asthma (Irfan et al., 2018c); decoction of leaves is used against dysentery and for the treatment of scabies (Irfan et al., 2018d; Irfan et al., 2018e); the extract of leaves is used as expectorant and antispasmodic and as antipyretic agent (Irfan et al., 2018f). A literature survey revealed reports of *Justicia adhatoda* being used for biological activities, i.e., anti-tubercular, bronchodilator, antibacterial, and anti-asthmatic potential (Latha et al., 2018). However, to the best of our knowledge, no report was found regarding the anti-inflammatory potential of *Justicia adhatoda*. Therefore, the current study was designed to integrate the folklore use of *Justicia adhatoda* with a gas chromatography–mass spectrometry approach to identify biologically active compounds and then investigate the potency of different fractions of *Justicia adhatoda* in pharmacological bioassays using animal models.

Materials and methods

Plant collection

Justicia adhatoda L. was collected from Charsadda District, Khyber Pakhtunkhwa, Pakistan, in May 2021. The plant was identified with the help of the relevant literature (Malik and Ghafoor, 1988), and a voucher specimen (AWK0518) was deposited in the Herbarium, Department of Botany, Abdul Wali Khan University Mardan, Pakistan.

Extraction

Leaves were manually separated from branches and washed with tap water for 10 min before leaves were shade-dried for 20 days. These dried leaves were ground to a coarse powder using a grinder. For extraction, 6 kg of leaf powder was soaked in 23 L methanol (80%) for 18 days. The filtrate was mixed and condensed through a rotary evaporator, and finally 400 g of crude methanol extract was obtained (Sharifi-Rad et al., 2020a).

Fractionation

The crude methanolic extract of *Justicia adhatoda* L. was shifted into a separating funnel and diluted with 500 ml distilled water followed by the addition of 500 ml. The mixture was kept until it formed the upper and lower layers. The n-hexane layer was isolated, and this procedure was repeated three times, adding 500 ml n-hexane each time. For the final fraction, all of the n-hexane layers were combined in a rotary evaporator to the final concentrated n-hexane fraction of 20 g. The same process was performed to obtain chloroform and ethyl acetate fractions weighing 27 and 80 g, respectively. Finally, a dry water fraction (120 g) was also obtained (Zeb et al., 2017; Sharifi-Rad and Pohl, 2020).

Experimental animals

The whole set of experiments was monitored in albino mice of mixed sexes that were obtained from the Veterinary Research Institute, Peshawar, Khyber Pakhtunkhwa, Pakistan. All experimentation followed stringent biosafety protocols and

bioethical procedures as approved by the Biosafety and Bioethics Committee of the Department of Botany, AWKUM.

Acute toxicity bioassay

Two major groups consisting of control and test (treatments) were made, each comprised four test models. The fractions were orally administered using different dosages, i.e., 150–1800 mg/kg. Tween-80 was used as a solvent in preparation for the dosages. Mice were examined for the next 72 h for decreased allergic symptoms and any abnormal behavior after receiving the dose/s (Zeb et al., 2016).

Anti-inflammatory activity

Carrageen-induced inflammatory test

The carrageenan-induced paw edema test was carried out following Winter et al. (1962). Albino mice were grouped, and initial paw volume was measured, and then carrageenan solution was injected in the hind paw of mice, i.e., subcutaneously injected at 0.05 ml (1%). A standard drug (diclofenac) was injected, and different fractions such as ethyl acetate, n-hexane, chloroform, and aqueous were injected at doses of 150 and 300 mg/kg to the respective groups. The procedure of the plethysmometer (Ugo Basil 7150) method was followed after the first, second, third, and fourth hour of injections of standard drug and fraction (Sharifi-Rad et al., 2021).

Analgesic activity

Acetic acid-induced writhing test

For analgesic potential, the acetic acid writhing test was carried out on *Justicia adhatoda* L. The mice were divided into different groups, while oral dosages at 150 mg/kg and 300 mg/kg of ethyl acetate, n-hexane, chloroform, and aqueous fractions were administered, consequently, after 30 min, and 10 ml/kg of acetic acid (0.6%) was injected intraperitoneally to the model mice. Group I control 0.5% was administered with Tween-80 (3 ml/kg), and Group II was considered standard and administered with the standard drug (10 mg/kg). The number of writhes (contraction of the abdomen extension of body and limbs, twisting of the mice trunk, and elongation) was counted from 5, 15, 30, and 60 min after the injection of acetic acid (Franzotti et al., 2000).

Analgesic activity

Formalin-induced licking paw test

The formalin-induced licking paw test was carried out for the assessment of analgesic ability of *Justicia adhatoda* (Santos and Calixto, 1997). Mice were categorized into groups, where group I received 0.5 percent Tween-80 (3 ml/kg) of negative regulation and group II received standard drug morphine (5 mg/kg), while other groups received ethyl acetate, n-hexane, and chloroform fractions of *Justicia adhatoda* with doses of 150 mg/kg and 300 mg/kg divided into respective groups, while 2.5% formalin (20 µl) was subcutaneously injected into the plantar surface of the mice's hind paw after 30 min. Formalin-induced paw licking was recorded as an important signal for understanding the harmful sexual behavior. The behavioral responses to the sensation of nociception were properly noted like, the leakage and bite of the injected paw, respectively. Total time taken was 30 min, where the first 15 min were considered the early stage of the nociceptive reaction and the later 15 min were considered the late stage of the nociceptive reaction (Sharifi-Rad et al., 2020b).

Analgesic activity

Tail immersion test

Tail immersion potential was evaluated by the method of Imam and Sumi, (2014). Ethyl acetate, n-hexane, chloroform, and aqueous fractions were administered using doses of 150 mg/kg and 300 mg/kg and morphine (10 mg/kg), respectively, before 30 min of the experiment. Then, 15 min ahead of the trial, 1 cm to 2 cm of mice tail was submerged in warm water and held at 52 ± 1°C stable. The response time was the time the mice needed to bounce the tail. The latency time of tail removal response was taken as the ant nociception index (Sharifi-Rad et al., 2022).

Antipyretic activity

Brewer's yeast-induced pyrexia method

The antipyretic activity was evaluated for *Justicia adhatoda* L. using the method of Muhammad et al. (2012). The albino mice of both sexes were used, and each test contained four mice. At the beginning of the experiment, normal mice's body temperature was taken *via* a digital thermometer, and pyrexia was then induced in all mice by injecting 20% brewer's yeast. Mice were fasted overnight but permitted free access to drinking water, and the rectal temperature of each mouse was recorded after 24 h. Group I was injected with normal saline (10 ml/kg) as a negative regulation and Group II received paracetamol (10 mg/kg), while ethyl-acetate, n-hexane, chloroform, and aqueous fractions of *Justicia adhatoda* at the concentration of 150 mg/kg and 300 mg/kg were administrated to other groups.

Antioxidant activity

DPPH method

The scavenging effect of *Justicia adhatoda* was evaluated following Feghhi-Najafabadi et al. (2019). Fractions with the

concentration of 50 and 100 μL/ml were tested. DPPH methanol solution was applied to various plant extracts at concentration levels of 50 and 100 μg/ml. DPPH solution was prepared, and the mixture of fraction and solution (2 ml of DPPH methanol solution and 50 and 100 μg/ml) was gently mixed, and the absorbance was measured at 517 nm using a spectrophotometer after 60 min of incubation in dark. For the calculation of % inhibition, the following formula was followed:

$$\text{Inhibition}\,(\%) = [(A^{\circ} - A1)/A^{\circ}]\times 100\,,$$

where A° represents the absorbance of the control and A1 represents the absorbance of the sample.

Antispasmodic activity by normal intestinal transit

Albino mice were divided into groups of four animals each. The first group was considered control and saline solution was administrated (10 ml/kg). Other groups were treated with aqueous, ethyl acetate, chloroform, and n-hexane fractions of *Justicia adhatoda* at different doses, while one group in each was considered the standard group. After thirty minutes, a regular charcoal meal (0.2 ml/mouse of 10% charcoal suspension in 5% gum acacia) was given to the mice orally (Hsu, 1982). On charcoal administration in mice meal, the tested animals were slaughtered in 30 min, and the small intestine was immediately removed. Similarly, the peristaltic index of each mouse was monitored by subtracting the distance traveled by the charcoal meal in the intestine from the total length of the small intestine (Than et al., 1989).

Gas chromatography–mass spectrometry analysis of the extract and identification of the phytocompounds

For the identification of bioactive phytochemicals in the ethyl acetate fraction of *Justicia adhatoda*, gas chromatography–mass spectrometry (Thermo Scientific Co.) was used. Identification of active phytochemicals was as per the 'National Institute of Standards and Technology 2008' (NIST-2008) database that contained over 62,000 patterns used for interpreting gas chromatography–mass spectrometry mass spectra. A comparison of the spectrum of an unknown component with the spectrum of the known component in the NIST library was performed (Sher et al., 2022).

Statistical analysis

Data were recorded in the form of triplicate and expressed as mean ± standard error of the mean (SEM). The data were then quantified for normality and homogeneity, and the statistical investigations were carried out by means of one-way analysis of variance (ANOVA), followed by multiple Duncan's range test using statistical software SPSS, V 20.0 (SPSS, Chicago, IL, United States). As compared to control/standard, significant stimulatory/inhibitory effects were monitored using the following formula, and significant differences were considered by means of various statistical bars at $p < 0.05$.

(1) Reduction in pyrexia was evaluated by the following formula used by Muhammad et al. (2012):

$$\text{Percent reduction} = \text{B} - \text{Cn/B} - \text{A}\times 100,$$

where B represents the temperature after pyrexia induction, Cn represents the temperature after 1, 2, 3, 4, and 5 h, and A represents the normal body temperature.

(2) The % inhibition of inflammatory effect of different fractions was calculated using the formula of Hossain et al. (2016):

$$\text{Percentage inhibition of inflammation} = [(\text{Vc} - \text{Vt})/\text{Vc}]\times 100,$$

where Vc is the average inflammation of the control group and Vt is the average degree of inflammation by the test group.

(3)The percent inhibition of inflammation was calculated at different time intervals using the following formula (Shah and Shah 2015):

$$\text{Percent inhibition} = \text{A} - \text{T}/\text{A}\times 100,$$

where A is the average inflammation of control and T is the paw volume of the test group.

(4) The following standard formula (Than et al., 1989) was used to calculate the initial transit percentage (percent) of antispasmodic action:

$$\text{Intestinal Transit}\,(\%) = \text{D/L}\times 100,$$

where D = charcoal meal length (cm) and L = total intestinal length (cm).

Results

Anti-inflammatory activity

The effect of *Justicia adhatoda* on carrageenan-induced hind paw edema is shown in Figure 1. The mice paw becomes edematous after injection of carrageenan. It was noted that the reference drug (diclofenac) inhibited paw edema up to 47.67%, while the administration of chloroform fraction at a higher concentration (300 mg/kg) showed significant anti-inflammatory activity at fourth hour with a paw edema inhibition rate of 46.51%. Moreover, the

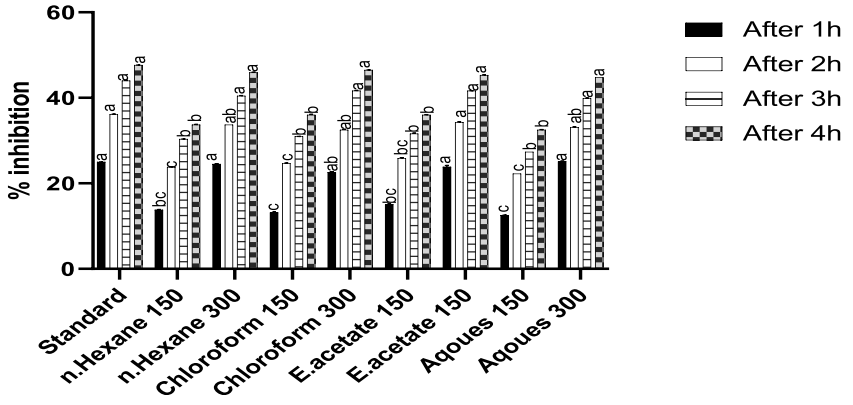

FIGURE 1
Anti-inflammatory activity of different fractions of *Justicia adhatoda* at doses of 150 and 300 mg/kg in carrageenan-induced paw edema in Swiss albino mice after 1, 2, 3, and 4 h. Various bars represent statistical difference at $p < 0.05$.

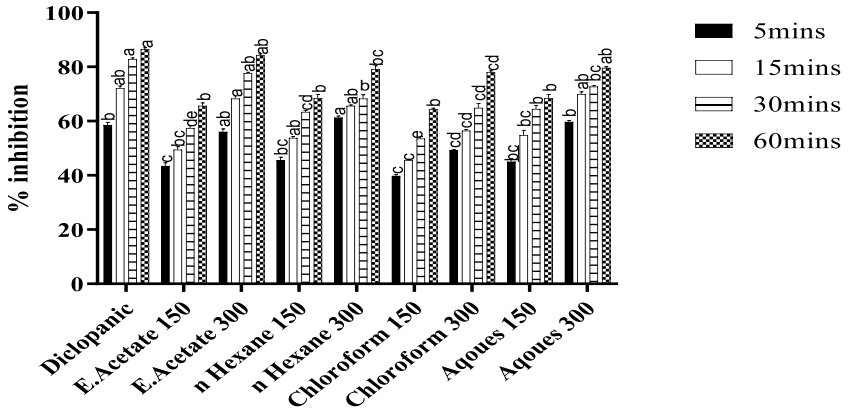

FIGURE 2
Analgesic activity of *Justicia adhatoda*'s different fractions was monitored at the dose of 150 and 300 mg/kg in acetic acid-induced Swiss albino mice. Different statistical bars represent statistical differences at $p < 0.05$.

other fractions, namely, n-hexane, ethyl acetate, and aqueous at higher extract dose also showed inhibition on fourth hour, i.e., 45.93%, 45.34%, and 44.76%, respectively.

Writhing test

The isolated fractions of *Justicia adhatoda* were checked for analgesic activity using the writhing test (Figure 2). As compared to the standard diclofenac sodium (10 mg/kg) that significantly inhibited the writhing (86.44%), the ethyl-acetate fraction also caused significant inhibition (84.18%). Similarly, the other fractions, i.e., chloroform, n-hexane, and aqueous at a higher dose of 300 mg/kg also inhibited writhing after 6 min by 77.96, 79.09, and 79.66%, respectively.

Formalin test

Two concentrations of each fraction (150 and 300 mg/kg) obtained from *Justicia adhatoda* were orally administered and that significantly inhibited the formalin-induced paw licking at early and late phases of the test (Table 1; Figure 3). As compared to the standard, i.e., morphine (86.06% in the late phase), the ethyl acetate fraction was found effective at a higher dose of 300 mg/kg that significantly reduced the paw licking up to 74.35 and 88.38% in the early and late phases, respectively. Moreover, the chloroform, aqueous, and n-hexane fractions were also effective at higher concentrations (300 mg/kg) and inhibited the induced paw licking in the early phase by 61.71, 71.58, and 69.23% as well as in the late phase by 87.55, 85.06, and 87.55%, respectively.

TABLE 1 Effect of *Justicia adhatoda* in different fractions on formalin-induced pain in mice.

Treatment	Dose	Early phase	% Inhibition at the early phase	Late phase	% Inhibition at the late phase
Negative control (tween-80)	3 ml/kg (0.50%)	48.75 ± 2.2^{e}	. . .	60.25 ± 0.70^{d}	. . .
Morphine	5 mg/kg	8.25 ± 0.62^{a}	83.07	4.25.47^{a}	92.94
Ethyl acetate	150 mg/kg	25 ± 0.91^{c}	48.71	16.75 ± 2.3^{c}	72.19
	300 mg/kg	12.5 ±1^{b}	74.35	7 ± 0.91^{ab}	88.38
n-Hexane	150 mg/kg	27.75 ± 1.3^{cd}	44.1	17.5 ± 1^{c}	70.4
	300 mg/kg	15 ± 1.2^{b}	69.23	10.5 ± 0.95^{b}	82.57
Chloroform	150 mg/kg	30.75 ± 0.85^{d}	36.92	18 ± 0.4^{c}	70.12
	300 mg/kg	11.5 ± 0.64^{ab}	61.71	7.5 ± 0.28^{b}	87.55
Aqueous	150 mg/kg	30.5 ± 1.3^{d}	37.43	17.5 ± 0.64^{c}	70.95
	300 mg/kg	13.25 ± 0.85^{b}	71.58	9 ± 0.7^{b}	85.06

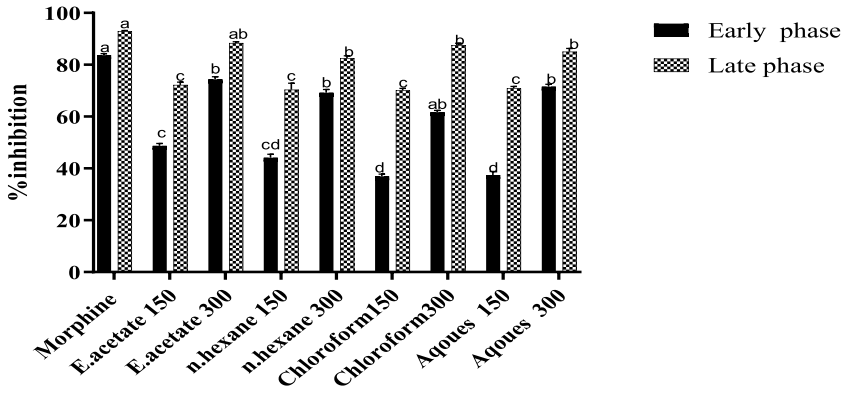

FIGURE 3
Effect of various fractions of *Justicia adhatoda* at doses of 150 and 300 mg/kg in the formalin-induced licking paw test in Swiss albino mice.

Tail immersion test in mice

The reflex time for tail withdrawal after administration of different fractions increased in a dose-dependent manner (Figure 4). Chloroform and aqueous fractions showed preferred results as compared to the reference drug (morphine).

Antipyretic test

The effect of different fractions of *Justicia adhatoda* on pyrexia induced by brewery yeast is shown in Figure 5. The pyrexia inhibition was dose-dependent and significantly related to a higher dose (300 mg/kg). As compared to the standard (85.71%), maximum inhibition (77.98%) was shown at 300 mg/kg of ethyl acetate fraction, while the other fractions, viz., aqueous (77.03%), followed by n-hexane (75.82%) and chloroform (75.70%) also showed considerable inhibition rates.

2, 2′-Diphenyl-1-picrylhydrazyl free radical-scavenging activity

The antioxidant activity was assessed by DPPH free radical-scavenging activity (Figure 6). As compared to the standard, ascorbic acid showed 76.49% and 82.33% inhibition at concentrations of 50 and 100 µg/ml, while the ethyl acetate fraction showed a scavenging effect of 50.40% at 50 µg/ml and 66.74% at 100 µg/ml. Similarly, the aqueous fractions were followed by n-hexane and

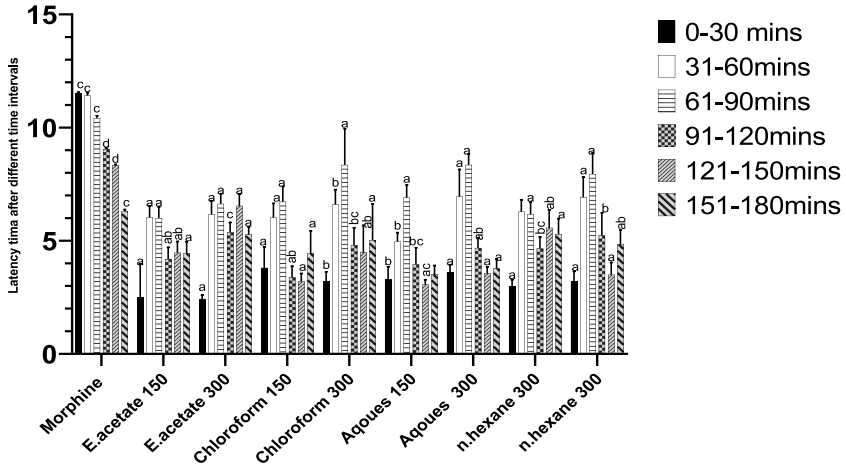

FIGURE 4
Effect of *Justicia adhatoda*'s fractions at different time intervals in the tail immersion test in Swiss albino mice.

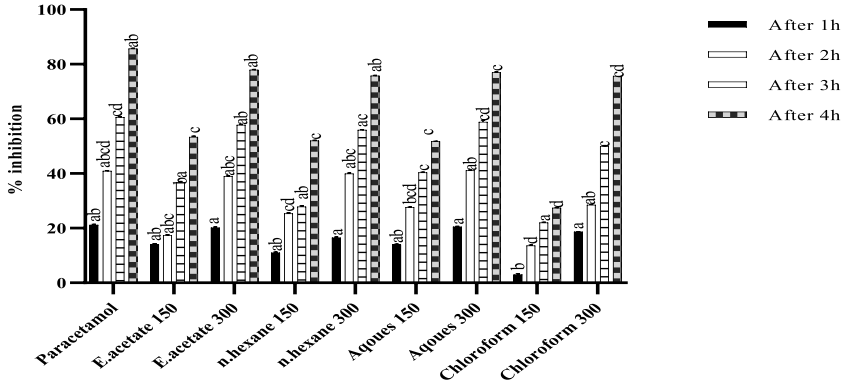

FIGURE 5
Antipyretic activity of various fractions of *Justicia adhatoda* at doses of 150 and 300 mg/kg by brewer's yeast-induced pyrexia in Swiss albino mice after 1, 2, 3, and 4 h.

chloroform with inhibition rates of 77.03, 75.82, and 75.70, respectively.

Antispasmodic activity

The antispasmodic activity of *Justicia adhatoda* fractions was assessed using charcoal-induced intestinal spam in mice, i.e., 150 and 300 mg/kg (Table 2). As compared to the standard drug, i.e., atropine sulfate, the intestinal transit was 94.57%, and significant % inhibition of the n-hexane fraction at 300 mg/kg was 72.75%. The other fractions also revealed inhibition at a higher concentration of dose (300 mg/kg), i.e., chloroform (71.55%), followed by ethyl acetate (71.47%) and aqueous (67.94%), respectively.

Gas chromatography–mass spectrometry analysis of the ethyl acetate fraction

The gas chromatography–mass spectrometry analysis of *Justicia adhatoda* ethyl acetate fraction was carried out using the NIST (National Institute Standard and Technology) library of known compounds of approximately 62,000 patterns. Our gas chromatography–mass spectrometry analysis revealed the presence of 21 compounds (secondary metabolites) that could

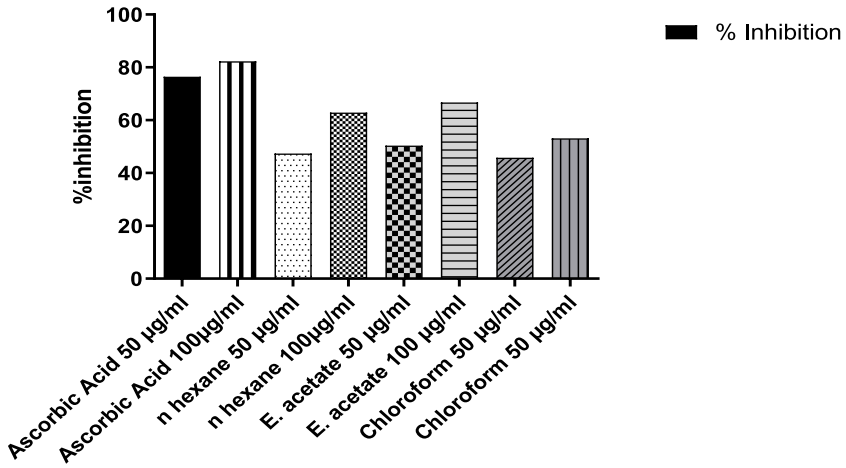

FIGURE 6
Percent inhibition of DPPH free radical-scavenging activity of *Justicia adhatoda* at different concentrations.

TABLE 2 Effect of different fractions of *Justicia adhatoda* on intestinal transit in mice.

Treatment	Dose	Total intestine length	Charcoal meal length	% Inhibition
Atropin sulfate	10 mg/kg	51.675 ± 1.4^{a}	48.85 ± 1.83^{d}	94.54
Chloroform	150 mg/kg	50.775 ± 2.2^{a}	26.4750 ± 2.09^{a}	52.13
	300 mg/kg	50.1 ± 3.5^{a}	35.85 ± 3.8^{b}	71.55
Ethyl acetate	150 mg/kg	49.275 ± 2.5^{a}	26.2750 ± 3.39^{a}	53.31
	300 mg/kg	47.575 ± 1.6^{a}	34 ± 1.3^{ab}	71.47
n-Hexane	150 mg/kg	51.25 ± 2.5^{a}	30.2250 ± 3.89^{ab}	58.96
	300 mg/kg	50.1 ± 0.70^{a}	36.45± 0.5^{b}	72.75
Aqueous	150 mg/kg	48.72 ± 2.4^{a}	22.3250 ± 1.36^{a}	45.81
	300 mg/kg	50 ± 2.8^{a}	33.975 ± 3.0^{ab}	67.94

possibly contribute to the medicinal properties of the plant. The identifications of these phytochemicals were confirmed based on peak area, molecular weight, and retention time (Table 3; Figure 7).

Discussion

Plants have been recognized as rich sources of medicines, colors, flavors, food, cosmetics, and fuel since the dawn of human civilization. However, compared to the other uses, medicinal plants have been widely used for the treatment of different disorders due to the presence of active phytochemicals (Jan and Khan, 2016; Ullah et al., 2018; Iftikhar et al., 2019; Irfan et al., 2019). With the tremendous technological advancements over the years, isolation and identification of novel phytochemicals from plants has gained more interest and attention, particularly *via* various pharmacological bioassays (Ibrahim et al., 2018; Khan et al., 2021). *Justicia adhatoda* is a well-known medicinal plant and has been widely used for treating a variety of infectious diseases, including asthma, tuberculosis, bronchitis, antibacterial, bronchodilator, anti-asthmatic, anti-tubercular, and anti-inflammatory potential. For scientific validation as well as search for novel compound isolation and identification, different pharmacological activities were undertaken to evaluate the anti-inflammatory activities of *Justicia adhatoda*.

Inflammation and its secondary forms like fever and pain are recognized because of the high level of interleukins, TNF- α, and prostaglandins (Muhammad, et al., 2012). For the assessment of anti-inflammatory effect of *J. adhatoda*'s different fractions, carrageenan-induced paw edema was considered (Linardi et al., 2000). In the carrageenan-induced paw edema test, the fractions exhibited significant anti-inflammatory effects in a dose-dependent manner. Among other fractions, the

TABLE 3 List of phytochemicals identified in the ethyl acetate fraction of *Justicia adhatoda* through the gas chromatography–mass spectrometry approach.

S. no.	Compound	Area (%)	Rt	Probability	Chemical formula
1	Phenol, 2-methyl-5-(1-methylethyl)-	0.06	10.51	53.88	$C_{12}H_{18}O$
2	Cyclotetradecane	0.01	11.80	5.60	$C_{14}H_{28}$
3	Cyclohexene, 1-methyl-4-hexenyl)-, (S)-	0.01	13.63	11.52	$C_{10}H_{16}$
4	1-Hexadecene	0.01	15.03	12.03	$C_{16}H_{32}$
5	10-Heneicosene (c,t)	0.01	4.70	18.01	$C_{21}H_{42}$
6	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.16	18.48	37.42	$C_{20}H_{40}O$
7	10-Heneicosene (c,t)	0.01	18.01	4.70	$C_{21}H_{42}$
8	Z-(13,14-Epoxy)tetradec-11-enol acetate	0.01	8.24	8.24	$C_{16}H_{28}O_3$
9	Isophytol	0.00	19.41	43.63	$C_{20}H_{40}O$
10	Hexadecanoic acid, ethyl ester	0.06	19.76	72.13	$C_{18}H_{36}O_2$
11	Phytol	0.29	20.62	78.03	$C_{20}H_{40}O$
12	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	0.05	20.96	18.79	$C_{19}H_{32}O_2$
13	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.01	21.25	6.92	$C_{20}H_{40}O$
14	Thiophene, 3-methyl-2-pentadecyl-	0.00	22.04	22.45	$C_{20}H_{36}S$
15	Pentacosane	0.00	22.80	13.86	$C_{25}H52$
16	1,2-Benzenedicarboxylic acid, diisooctyl ester	2.14	23.14	34.29	$C_{24}H_{38}O_4$
17	1-Monolinoleoylglycerol trimethylsilyl ether	0.01	23.77	36.37	$C_{27}H56\ O_4\ Si_2$
18	Tetratetracontane	0.01	24.71	7.64	$C_{44}H_{90}$
19	Oleanolic acid	0.00	25.40	18.41	$C_{30}H48\ O_3$
20	Stigmasta-5,22-dien-3-ol, acetate, (3á)-	0.01	25.67	13.06	$C_{31}H_{50}\ O_2$
21	á-Sitosterol	0.01	27.09	45.96	$C_{29}H_{50}\ O$

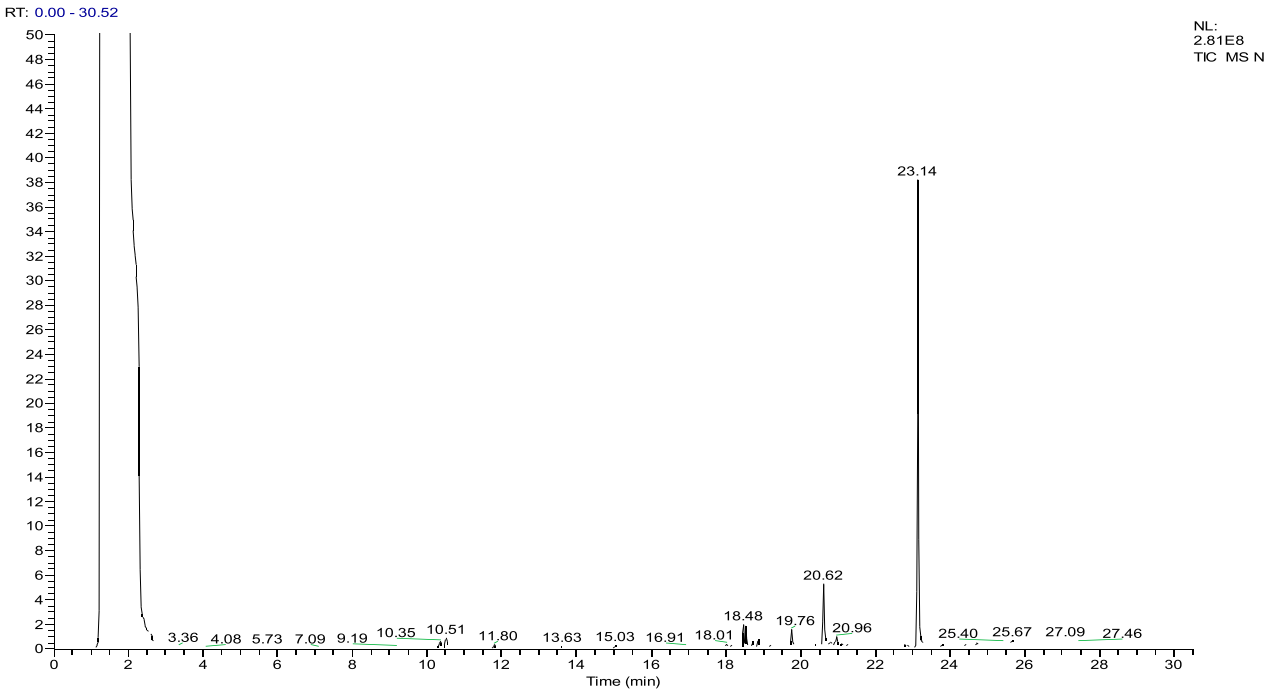

FIGURE 7
Chromatogram of the ethyl acetate fraction. Identification of phytochemical is based on peak area, molecular weight, and retention time.

chloroform fraction of 300 mg/kg was found more effective (Yam et al., 2010; Pournamdari et al., 2018) in 1–4 h, which caused 46.51% inhibition. Our results also showed a number of compounds *via* gas chromatography–mass spectrometry analysis as shown in Table 3.

Anti-nociceptive activities of different fractions of *Justicia adhatoda* were tested. Three different models were chosen to investigate the peripheral-mediated influence of *Justicia adhatoda*'s fractions. In the current study, four fractions of *Justicia adhatoda* in two concentrations, i.e, 150 and 300 mg/kg decreased the writhing, and specifically, the ethyl acetate fraction resulted in the highest reduction of writhing (84.18%). Our results are in alignment with previous findings (Abdul-Wahab et al., 2012). Similarly, the current result revealed that a higher dose of the ethyl acetate fraction is much effective against acetic acid-induced peripheral pain (Figure 2). The writhing (induced by acetic acid) model in mice is a useful test for the evaluation of the analgesic effects of therapeutic drugs (Gou et al., 2017). However, writhing caused by acetic acid affects the peripheral nervous system. The abdominal writhing procedure caused by acetic acid is a type of acute chronic nociception and a common model for intense pain in which acetic acid is used as a congenic agent (Feng et al., 2003). When injected intraperitoneally, acetic acid causes acute pain in animals by activating primary afferent sensory Aɗ and C nerve fibers 16, and the procedure is typically common in peripheral analgesic agent identification (Azi et al., 2014).

The formalin test is a reliable predictor for acute tonic pain, which has the advantage of detecting pain in central and peripheral mechanisms. Currently, both phases of the formalin paw licking test of *Justicia adhatoda* showed a significant anti-nociceptive effect in a dose-dependent manner. Ethyl acetate fractions at doses of 150 and 300 mg/kg significantly reduced the formalin-induced paw licking (88.38 and 77.24%, respectively) in the late and early phases. Furthermore, the result revealed that ethyl acetate fractions of *Justicia adhatoda* are effective in both phases, while other fractions showed minimum potential as compared to ethyl acetate. Previously, it was concluded that formalin-induced persistent nociception in mice paws provided a marked response to biphasic licking (Hunskaar and Hole., 1987; Bukhari et al., 2010).

The tail immersion model was used for the evaluation of acute pain. In our study, mice increase in latency time was noted, and the thermal pain threshold was inhibited. The dose of 300 mg/kg of *Justicia adhatoda* had a potent anti-nociceptive effect. *Justicia adhatoda's* chloroform and aqueous fractions have shown significant analgesic effects in acetic acid-induced pain, as well as in the late phase of formalin and tail immersion tests. Similar results have been reported earlier (Saha et al., 2013). The tail withdrawal response of mice is mainly considered to be selective for centrally acting analgesics, while the peripherally acting drugs are known to be inactive on such heat-induced pain responses (Imam and Sumi 2014). This approach is established on the finding that morphine-like medications extend the tail withdrawal time from hot water in mice (Moniruzzaman and Imam, 2014).

Antipyretic effectiveness of the *Justicia adhatoda* fractions was assessed by subcutaneous injection of brewer's yeast-induced pyrexia in animal models. Prostaglandin synthesis was elevated during this process, and the inhibition capability of plant-based medicine on prostaglandin synthesis was used as a test for antipyretic capacity (Shah et al., 2017). Here, the injection of ethyl acetate, n-hexane, chloroform, and aqueous fractions of *Justicia adhatoda* significantly decreased the rectal temperature of yeast-induced febrile mice (Figure 4). Among these fractions, the ethyl acetate fraction at 300 mg/kg had the most efficient antipyretic effect in yeast-produced temperature by mitigation of rectal temperature as well as normal body temperature in mice. Ullah et al. (2016) used the hydro-ethanolic extract from *Monotheca boxfolia* and concluded the presence of an active antipyretic compound oleanolic acid as well as phytol (Islam et al., 2020). Notably, phytol and oleanolic acid were identified in the current gas chromatography–mass spectrometry analysis (Table 3). The strong antipyretic potential of ethyl acetate could be the possible effect of oleanolic acid and phytol (Kashyap et al., 2016). Oleanolic acid is a pentacyclic triterpenoid compound that is known to have the properties of downregulation of many intracellular and extracellular molecular targets that are linked directly or indirectly with the disease progression (Castellano et al., 2013; Xu et al., 2021). However, the major anti-inflammatory properties of oleanolic acid and phytol have been reported to be involved in the inactivation of STATE3/6, NF, and Akt/mTOR pathways (Kashyap et al., 2016).

The ability of plant-based products to donate electrons can be evaluated by bleaching 2, 2′-diphenyl-1-picrylhydrazyl radical (DPPH) assay. The process is based on DPPH scavenging by adding a free radical-donating species or any sort of antioxidants in order to decolorize the DPPH solution. The degree of change in the color is directly linked with the antioxidant potential (Saeed et al., 2012). The ethyl acetate fraction was found to have a potent scavenging activity at 50 µg/ml with 50.40%, while at 100 µg/ml it showed 66.74%. The reason for the ethyl acetate fraction performing better might be due to its high polarity that solubilizes chemical components better than aqueous, ethanolic, and methanolic fractions (Zhang et al., 2011). However, most of the diseases due to free radicals are neurodegenerative diseases. Similarly, plant-derived antioxidants are much better for the treatment of serious diseases like cancer because of their scavenging potential (Veeru et al., 2009). The search for potent natural

TABLE 4 List of biological activities of compounds of *Justicia adhatoda* identified through gas chromatography–mass spectrometry.

S. no.	Compound	Biological activities	References
1	Phenol, 2-methyl-5-(1-methylethyl)-	Antioxidant, anti-inflammatory, and analgesic	Majid et al. (2015)
2	1-Hexadecene	Antimicrobial and antioxidant, analgesic, and anti-inflammatory	Mou et al. (2013)
3	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Anti-inflammatory and antioxidant and analgesic	Chansiw et al. (2019), Majid et al. (2015)
4	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	Antioxidant, antipyretic anti-inflammatory, and analgesic	Chetia and Phukan, (2014), Shaaganti and Amareshwari, (2019)
5	Hexadecanoic acid, ethyl ester	Antioxidant activities and anti-inflammatory	Kim et al. (2020), Guerrero et al. (2017)
6	Phytol	Anti-nociceptive, antioxidant, anti-inflammatory, and antipyretic	Santos et al. (2013), Islam et al. (2020)
8	Isophytol	Anti-inflammatory and antioxidant	Keawsa-Ard et al. (2012), Elsharkawy et al. (2013), Sanseera et al. (2012)
9	9,12,15-Octadecatrienoic acid ethyl ester, (Z,Z,Z)-	Anti-inflammatory and antioxidant	Guerrero et al. (2017), Tian et al. (2018)
10	Pentacosane	Antioxidant	Marrufo et al. (2013)
11	1,2-Benzenedicarboxylic acid, diisooctyl ester	Antioxidant	Sivasubramanian and Brindha, (2013)
12	1-Monolinoleoylglycerol trimethylsilyl ether	Antioxidant and anti-inflammatory	Majumder et al. (2019), Mary and Giri, (2016)
13	Tetratetracontane	Antioxidant	Rhetso et al. (2020)
14	Oleanolic acid	Anti-inflammatory, anti-nociceptive, and antipyretic	Singh et al. (1992), Ullah et al. (2016)

antioxidants is a high priority because of the adverse effects associated with synthetic antioxidants (Kumar et al., 2012).

Diarrhea is the release of excessive liquids through the gastrointestinal tract, and it may lead to motility (Kumpf, 2014). Based on ethnomedicinal uses of *Justicia adhatoda* in folklore, the antispasmodic potential was also evaluated by charcoal meal intestinal transit (Table 2). High inhibition (72.75%) was observed at 300 mg/kg of n-hexane fraction, which might be due to the presence of a variety of alkaloids in the form of deoxyvasicine, vasicine, and vasicinine, and these are previously reported to be excellent antispasmodic agents (Rashmi et al., 2012).

Gas chromatography–mass spectrometry analysis of the current study revealed the presence of different anti-inflammatory compounds in *Justicia adhatoda* which are active against inflammation. The gas chromatography–mass spectrometry approach of *Justicia adhatoda* revealed various biologically active compounds that possess a number of pharmacological activities. Of the 21 compounds identified by GCMS analysis, 14 are bioactive compounds and are known for their excellent anti-inflammatory, anti-nociceptive, antipyretic, antioxidant, and other pharmacological activities (Tables 3, 4; Figure 7), while no activity has been reported for some compounds, i.e., cyclotetradecane, cxyclohexene, 1-methyl-4-hexenyl)-, (S)-, 10-heneicosene (c,t), thiophene, 3-methyl-2-pentadecyl-, stigmasta-5,22-dien-3-ol, acetate, and (3á)-, á-sitosterol.

Conclusion

The potential of *Justicia adhatoda* fractions was confirmed in different pharmacological activities. Furthermore, the gas chromatography–mass spectrometry analysis also confirmed a number of biological compounds that are already acknowledged for their anti-nociceptive, analgesic, anti-inflammatory, antipyretic, antispasmodic, and antioxidant potential. Taken as a whole, *Justicia adhatoda* plant has immense potential to be used for such bioassays in clinical trials. These fractions identified here could offer better sources for the isolation and identification of different biologically active compounds that may lead to novel plant-based drugs. However, additional studies are required for purification, characterization, and structural elucidation of these bioactive compounds.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Biosafety and Bioethics Committee of the Department of Botany, Abdul Wali Khan University Mardan, Pakistan.

Author contributions

MM and GJ designed the project and performed the experiments; FJ, MH, and MI collected the data and wrote the very first draft of the manuscript, and NA helped in reviewing the manuscript; and AR, AA, MA, and HS helped in funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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