Antinociceptive and Anti-Inflammatory Activities of *Wulfenia Amherstiana*

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Abstract

In order to search for new resources of antinociceptive and anti-inflammatory agents, the study was to investigate the antinociceptive and anti-inflammatory potential of ethanolic extract and fractions of *Wulfenia amherstiana* (*W.amherstiana*) belonging to family Scrophulariaceae. Phytochemical screening was performed through standard procedures indicated the presence of flavonoids, steroids, saponin, phenols, tannins, glycosides and triterpenoids. Antinociceptive activity of the plant was evaluated by chemically induced writhing behavior and hot plate thermal nociception in mice model.Ethanolic extract and different fractions of this plant were also subjected to assess its anti-inflammatory potential at these doses in carrageenan paw edema model in mice. The ethanolic extract at 200 mg/kg and chloroform fraction at 300 mg/kg doses demonstrated significant antinociceptive response (reduction in the number of writhing induced by acetic acid) i.e.54 and 56.30 %, respectively,compared to diclofenac sodium (at a dose of 50mg/Kg) as 62 % ($P < 0.001$). Similarly, an increase in the hot plate latency time observed in case of ethanolic extract was at doses of 200 mg/Kg (15.40 ± 0.75) & 300 mg/Kg (16.50 ± 1.23) revealing its antinociceptive potential greater than its fractions. The results demonstrated a significant anti-inflammatory potential associated with this plant. Ethanolic extract, chloroform and n-hexane fractions at 200 and 300 mg/kg doses produced significant anti-inflammatory responses at h 1-5 ($P < 0.01$, $P < 0.001$), respectively. The study imply that ethanolic extract along with the chloroform and n-hexane fractions might be containing the active molecules which are responsible for the biological activities.

Key words :*Wulfenia amherstiana*, Antinociceptive, Antioxidant, Phytochemical screening, Hot plate

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INTRODUCTION

Higher plants have been the source of medicinal agents since earliest times, and today they continue to play a dominant role in the primary health care of about 80% of the world population [1]. Natural products, and medicinal agents derived there from, are also an essential feature in the health care system of the remaining 20% of the population residing mainly in developed countries, with more than 50% of all drugs in clinical use have a natural product origin [2]. Of the world's 25 best-selling pharmaceutical agents, 12 are natural product derived [3]. Natural products continue to play an important role in drug discovery programs of the pharmaceutical industry and other research organizations [4]. Research into the chemical and biological properties of natural products over the past two centuries has not yielded drugs for the treatment of human ailments, but has provided the stimulus for the development of modern synthetic organic chemistry, and the emergence of medicinal chemistry as a major route for the discovery of novel and more effective therapeutic agents. Numerous phytomedicines are registered and extensively used in Europe, and more than 600 botanical items have been recognized in various editions of the United State Pharmacopoeia , in spite of legislative ban on some of the marketable items as drugs [5]. The family Scrophulariaceae is mostly herbs or small shrubs comprising about 190 genera and 4,000 predominately temperate species. Different species of the said family are found in different parts of Pakistan. One of such specie is *W.amherstiana* or *W. amherstiana* [6, 7] found in Pakistan found in Pakistan and no systematic scientific work has been done on this specie. Though literature reports a significant research on other species of this family like *Wulfenia carinthiaca* [8]. The methanolic extract of *Wulfenia carinthiaca* has been found to demonstrate antinociceptive, anti edematogenic and anti-inflammatory effects due to the glycosides present in it [9]. In another study an iridoid known as 10-O-cinnamoyl asystasioside E isolated from *Wulfenia baladaccii* which was the major factor for its antinociceptive activity [10]. Inflammation is the natural alarming system and physiological defense mechanism that warns against infections, potential trauma, burns, allergens, autoimmune responses and toxic chemicals and makes it possible to preclude the threat in the initial stage. If it is left untreated, it may prelude to many other chronic diseases [11]. Currently, the inflammatory conditions and the associated pain conditions are treated by either opioids or non-opioids like salicylates and corticosteroids. However, These synthetic drugs are linked with a number of side effects [12]. Furthermore, these synthetic medicines are either too expensive or toxic and most often these are not available to the rural population (considerable part of the world population), making it more necessary to develop safe and effective therapy for inflammation and pain. Plants have been an excellent source for cheap and effective medicines since ancient times [13]. Based on the reported antinociceptive and anti-inflammatory potential of the other species of this family, *W. amherstiana* was evaluated for the said prospective activities.

EXPERIMENTAL

Materials

Various solvents such as ethanol, chloroform, ethyl acetate, n-butanol and n-hexane used were used (Merck Germany). The diclofenac sodium, ascorbic acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich.

Plant Collection and Identification

The collection of plant *W. amherstiana* was carried out from Baragali, Khyber Pakhtunkhwa during the month of August. Plant was identified by Dr. Muhammad Ibrar (a taxonomist) and a voucher specimen (ID No. WA450987) was submitted to the herbarium at Department of Botany, University of Peshawar, KPK, Pakistan, for future reference.

Preparation of Plant extract

About 15 kg of plant was shade dried and the dried plant was sliced in to small pieces, ground to powder and soaked in ethanol (4 liters) for about one and half week with on and off shaking. To concentrate the extract, the solvent was evaporated by employing rotary apparatus and about 175 g gummy residue was obtained. Multiple solvents like chloroform, n-butanol, n-hexane, and ethyl acetate were used for getting sub-fractions of the ethanolic extract.

Animals

Swiss albino mice of either sex weighing 20-24 g were provided by Pakistan Council of Scientific & Industrial Research (PCSIR) laboratories, Peshawar, KPK, Pakistan. The mice were kept under standardized housing conditions (25 ± 2 ^{o}C and 50% relative humidity) [14, 15].

Preliminary Phytochemical screening

Mayer's Test was performed for the confirmation of alkaloids and Liebermann-Burchard test for steroids and terpenoids. The flavonoids, saponins, tannins and phenols were screened through sodium hydroxide (NaOH), foam tests and ferric chloride ($FeCl_3$) tests respectively. While Keller-Kiliani Test was used to confirm the presence of glycosides.

Antinociceptive Activity

Antinociceptive activity of crude ethanolic extract and its fractions were evaluated by using acetic acid induced writhing and hot plate models.

Acetic acid Induced Writhing Assay

Mice were divided into 7 groups, each consisting of 6 mice. Group I was given normal saline at dose of 10 ml/kg. Group II was given diclofenac sodium 50 mg/kg. Group III, IV, V, VI and VII were injected (i.p) with plant ethanolic extract and its fractions n-hexane, chloroform, n-butanol and ethyl acetate at doses of 200 and 300 mg/kg. Acetic acid was administered at 10 ml/kg (i.p)

to all mice except the saline group, 30 minutes after the administration of the different treatments. Leaving the initial 5 minutes after acetic acid injection, the number of writhes was counted over a period of 20 minutes [16-18].

Inhibition (%) = [(Wc-Wt) × 100]/Wc

Where, W_c = average writhing reflex in the control group

W_t = average writhing reflex in the test group.

Hot plate test

Animals were divided into 7 groups, each consisting of 6 mice. Group I was given was given normal saline at dose of 10 ml/kg. Group II was injected with diclofenac sodium 50 mg/kg (i.p). Group III, IV, V, VI and VII were injected with ethanolic extract and fractions n-hexane, chloroform, n-butanol and ethyl acetate at the doses of 200 and 300 mg/kg (i.p). Animals were acclimatized to the laboratory conditions 2 hours before the start of experiment and were deprived of food with *ad libitum* access to water. Hot plate was maintained at a temperature of 55° ± 0.5 °C. After placing the animal on the hot plate, the latency time was noted at 0, 30, and 60 minutes. A cut off time of 30 s was selected [19, 20].

Anti-inflammatory Assay

Carrageenan Induced Paw Edema

Carrageenan-induced paw edema model for anti-inflammatory activity assessment was used. Mice of either sex (25-35 g) were fasted overnight and randomized into the different groups. Group I and II received vehicle and diclofenac sodium 50 mg/kg; i.p, while groups III, IV, V, VI, and VII received ethanolic extract, n-hexane, chloroform, n-butanol, and ethyl acetate, 200 and 300 mg/kg, i.p respectively.

Thirty minutes prior to carrageenan injection, the vehicle, diclofenac sodium (50 mg/kg) or the treatments were administered to the respective group of animals. After thirty min, 0.05 mL of 1% carrageenan was administered into the sub-planter region of the hind paw of each animal [21]. The edema (in mL) induced by carrageenan injection was measured with digital plethysmometer and the paw volume was determined at 1st , 3rd and 5th hours after treatment to evaluate the anti-inflammatory potential of the test drug.

Disposal Method for Animals

After experiment, the animals were killed by cervical dislocation and buried in soil [22].

Ethics approval and consent to participate

Ethical Board of the Abasyn University, Peshawar, KPK, Pakistan accorded approval of the study protocols via approval No. Dir/ABASYN/EB/IV/004456 which is in compliance with with "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) and/or the declaration of Helsinki promulgated in 1964 as amended in 1996.

Statistical analysis

All the experimental results were presented as standard error of mean ± SEM and analyzed through ANOVA by using Statistical Package for Social Sciences (SPSS) version.19, IBM Inc (USA). The values having $P < 0.05$ were considered significant.

RESULTS

Phytochemical Screening

The phytochemical screening was carried out by the standard procedures as mentioned in material and methods. These tests confirmed the presence of flavonoids, steroids, saponin, phenols, terpenoids, tannins and glycosides while alkaloids found absent (Table 1).

Table 1: Phytochemical Screening Results

Phenols	Flavonoids	Alkaloids	Steroids	Saponins	Terpenoids	Tannins	Glycosides
+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve

ANTINOCICEPTIVE ACTIVITY

ACETIC ACID INDUCED WRITHING METHOD

A significant decline in the number of acetic acid induced writhes was inflicted by crude ethanolic extract and its sub-fractions (Table 2). Crude ethanolic extract at doses of 200 and 300 mg/kg resulted in a significant inhibition (54% and 56.30 %) of chemically induced nociceptive response compared to the standard treatment-diclofenac sodium 50 mg/kg (62%) ($P < 0.001$). A dose dependent nociceptive inhibitory response was induced by its fractions, chloroform 50.5% and 53.7% ($P < 0.001$) , n-hexane 44.63% and 41% ($P < 0.01$), ethyl acetate 33.3% and 35.50%, $P < 0.05$ and n-butanol fractions showed 28% and 32%, $P < 0.05$.

Table 2: Antinociceptive activity by acetic acid induced writhing method

Treatment	Dose	Number of writhings	% Reduction in writhing
Saline	10 ml/kg	32.60 ± 1.36	----------
Diclofenac	50 mg/kg	12.40 ± 0.96***	62.00
Ethanolic Extract	200 mg/kg	15.00 ± 0.76***	54.00
	300 mg/kg	14.25 ± 0.84***	56.30
Chloroform	200 mg/kg	16.21 ± 0.82***	50.20
	300 mg/kg	15.10 ± 1.05***	53.70
n-hexane	200 mg/kg	19.25 ± 1.21**	41.00
	300 mg/kg	18.05 ± 1.03**	44.63

n-butanol	200 mg/kg	23.51 ± 0.67*	28.00
	300 mg/kg	22.20 ± 0.76*	32.00
Ethyl acetate	200 mg/kg	21.75 ± 0.32*	33.30
	300 mg/kg	21.01 ± 0.81*	35.50

The data is shown as mean ± SEM. $P < 0.05$ = *, $P < 0.01$ = **, $P < 0.001$= *** in comparison to the group given normal saline ($n = 6$).

Hot plate Method

Hot plate latency time was significantly enhanced after the administration of either the extract or the fractions compared to the normal control animals (Table 3). Crude ethanolic extract and its fractions at doses of 200 and 300 mg/kg produced a statistically significant inhibition of thermally induced nociceptive response as 15.40 ± 0.75 and 16.50 ± 1.23, chloroform (14.25 ±.78 and 15.00 ± 0.84) ($P < 0.001$), n-hexane (2.05 ± 0.90 and 13.50 ± 0.58) $P < 0.01$, n-butanol (10.25 ± 0.86 & 11.51 ± 1.32) $P < 0.05$, and ethyl acetate (10.05 ± 1.05 & 11.60 ± 0.96) $P < 0.05$ respectively. The results as shown in the (Table.3) clearly indicated that antinociceptive activity of plant extracts is dose-time dependent. The antinociceptive potential of ethanolic extract was found greater than its fractions. While among the fractions, chloroform was found most effective.

Table 4: Antinociceptive Activity by Hot Plate Method

Group	Treatment	Dose	latency time(seconds)		
		mg/kg	0 min	30 min	60 min
I	Normal Saline (control)	10ml/kg	6.35±0.32	6.53±0.42	6.62±0.51
II	Diclofenac sodium (standard)	50	8.35 0.24	16.40 ±1.32	19.26±1.08***
III	Ethanol	200	7.05±1.21	12.65±1.03	15.40±0.75***
		300	8.05±0.26	13.45±0.62	16.50±1.23***
IV	Chloroform	200	7.10±0.65	11.21±0.45	14.25±.78***
		300	7.94±0.78	12.40±0.60	15.00±0.84***
V	n-hexane	200	6.95±0.24	9.52±1.12	12.05±0.90**
		300	7.65±0.28	10.29±0.43	13.50±0.58**
VI	n-butanol	200	6.25±0.47	8.46±0.66	10.25±0.86*
		300	7.42±0.47	9.36±0.61	11.51±1.32*
VII	Ethyl acetate	200	6.35±0.21	8.09±0.88	10.05±1.05*
		300	7.54±0.46	9.50± 0.92	11.60±0.96*

The data is shown as mean ± SEM. $P < 0.05$ = *, $P < 0.01$ = **, $P < 0.001$= *** in comparison to the group given normal saline ($n = 6$)

Anti-inflammatory Activity

The anti-inflammatory potential of ethanolic extract and its various fractions was evaluated through a sub-planter injection of carrageenan in mice. The data at different time intervals (1, 3 and 5 hr) after the administration of various treatments compared to the standard and normal saline treatment show a significant decline in the carrageenan induced paw edema (Figure 1). Ethanolic extract at 200 mg/kg dose induced a significant decrease in the paw edema at 1 hr ($P < 0.05$), with more significant results at 3rd and 5th hr ($P < 0.01$). At 300 mg/kg dose, a more potent anti-edema response was observed throughout the observation period of 1 to 5 hrs ($P < 0.01$). Ethyl acetate fraction at doses of 200 and 300 mg/kg demonstrated a significant edema lowering effect ($P < 0.05$) after its administration at hr 3-5. At doses of 200 mg/kg, n-hexane showed appreciable antiedema effects at hr 1 ($P < 0.05$), hr 2 and 3 ($P < 0.001$). Similarly, at 300 mg/kg, its effect was more significant through the entire duration of experiment (hr 1-5) ($P < 0.001$).

Chloroform fraction at 200 and 300 mg/kg demonstrated a lesser activity at h 1 ($P > 0.05$) while at h 3-5, a more robust activity ($P < 0.001$) was observed. Paw edema was not significantly lowered by 200 mg/g while 300 mg/kg dose of n–butanol fraction ($P < 0.05$) through the entire duration of experiment. The standard drug, diclofenac sodium explicitly imparted a decline in the inflammatory response at all the testing periods ($P < 0.001$). n-butanol fraction did not show any declining effect on the carrageenan induced paw edema at 200 mg/kg dose ($P > 0.05$) but it did impart a significant effect at a dose of 300 mg/kg through hr 1 to 5 ($P < 0.01$).

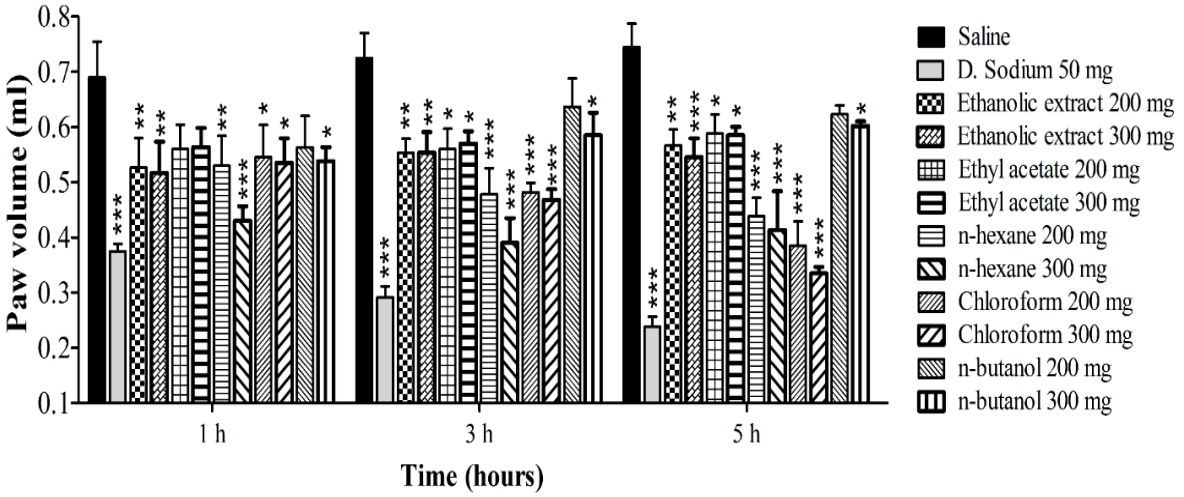

Figure 1: Effects of ethanolic extract and its various fractions on paw edema in mice after 1, 3, and 5 hrs of the of sub-plantar injection of carrageenan. Data expressed as mean percent inhibition ± SEM. Two-way RM ANOVA followed by Bonferroni's post hoc test. $P < 0.05$, $P < 0.01$, $P < 0.001$, compared to vehicle control. (n=6 mice each group).

DISCUSSION

Presence of a huge number of bioactive molecules in the ethanolic extract of *W. amherstiana*, as evident from the phytochemical screening, ensures its potential to be used as a potent therapeutic option for various disease conditions.Seeds of *Telfairia occidentalis* were found to have significant antinociceptive and antioxidant activities. The phytochemical screening of methanolic plant seed extract revealed the presence of various biological compounds such as flavonoids, phenols, alkaloids, anthraquinones, tannins and saponins.The antinociceptive activity was assumed to be due to the flavonoid content of the plant [23]. Acetic acid induced writhing and hot plate assays revealed that the ethanolic extract and its sub-fractions of *W. amherstiana* plant possessed highly significant antinociceptive potential. Methanolic extract of *Russelia equisetiformis* belonging to the same family, showed antinociceptive activity in acetic acid induced writhing and tail flick tests at doses of 10, 20, and 40 mg/kg [24]. Phytochemical screening revealed that the plant contained saponins and cardiac glycosides that may be associated with its antinociceptive activity. Research work on other plants like *Scoparia dulcis* showed antinociceptive activities tested in acetic acid induced writhing and hot plate models at 100 and 200 mg/kg (p.o) [25]. Acetic acid administration induces the pain via release of prostaglandins leading to increased capillary permeability and augmented pain sensation [26, 27]. Hot plate model for the assessment of nociception has long been a tool for the behavioral pharmacologist to analyze the pain alleviating potential of various natural and synthetic products [28]. Ethanolic extract from *Scoparia dulcis*, a plant from the same family, and revealed fascinating antinociceptive results in hot plate model, where a dose dependent response was observed [29]. The antinociceptive activity possessed by *Scoparia dulcis* was mainly due to the two terpenoids such as glutionl and scoparinol present in it [29]. Another study has held responsible flavonoids along with glutinol for the said activity through both central and peripheral mechanisms [30].

It is well known that a number of active components exert their anti-inflammatory effects via inhibition of cytokine production [31]. Crude extracts and fractions of *W. amherstiana* have shown pronounced anti-inflammatory activity in mouse paw edema assay. The antiedematogenic response may, probably, be due to the inhibition of some steps of arachidonic acid pathway, like to the mechanism of action of diclofenac sodium[32]. The Flavonoids (also known as nature's tender drugs) hold various biological/pharmacological properties including anti-cancer, anti-microbial, anti-viral, anti-inflammatory, immunomodulatory, and anti-thrombotic activities[33]. The role of flavonoids, as important constituents of the ethanolic extract and its fractions, in diminishing the inflammatory response cannot be overlooked. Flavonoids have been reported to exhibit antinociceptive and anti-inflammatory effects. They show anti-inflammatory activity both in vitro and in vivo, with several proposed mechanisms. One of the well-known mechanisms is the inhibition of eicosanoid producing enzymes (phospholipase A_2, cyclooxygenases, and lipoxygenases) to a reduced production of prostanoids and leukotrienes. Furthermore, many of the recent studies have shown that specific flavonoids (especially flavones) demonstrate their anti-inflammatory effect by modulation of pro-inflammatory gene expression

such as Inos (inducible nitric oxide synthase), cyclooxygenase-II, and a number of other key cytokines. Due to these unique action mechanisms and significant in vivo activity, flavonoids are considered to be reasonable candidates for new anti-inflammatory drugs [32].

A study was conducted to evaluate the anti-inflammatory activity of Picrorhiza kurroa extract (belonging to same family, Scrophulariaceae) in the arthritis induced mice through formaldehyde. The results revaled that it has significant anti-inflamatory activy in arthritis may by inhibiting Cytokines, Angiogenesis and by attenuation of degrading enzymes such as MMPs [34].The anti-inflammatory activity was found associated with various iridoid glycosides present in it [35]. In the present syudy *W. amherstiana* has also found to contain glycosides revealed by phytochrmical screening. Diclofenac sodium, like most of the non-steroidal anti-inflammatory drugs, impedes the biosynthesis of prostaglandins and this effect might elucidate its anti-inflammatory activity in carrageenan-induced mice paw edema [36].

CONCLUSION

The present study implied that *W.amherstiana* ethanolic extract, chloroform and n-hexane fractions showed significant antinociceptive effect and anti-inflammatory activities. Further studies are required for the isolation of compounds responsible for these activities of this plant, structure elucidation and mechanism of these compounds.

DECLARATION

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Conflicts of interest Statement

The authors declare no conflicts of interest

Author's contributions

Maria Kakar and Muhammad Usman Amin conceived and designed the experiments; Maria Kakar and Nisar Ahmad performed the experiments; Muhammad Usman Amin analyzed the data and contributed reagents and materials. All the authors contributed in paper write up.

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