Advances in Bioresearch
Adv. Biores., Vol 10 (4) July 2019: 124-131

Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.10.4.124131

Advances in Bioresearch

ORIGINAL ARTICLE

Evaluation of The Screening and Isolation of Total Flavonoids of *Sambucus wightiana* (TFSW) for Its Analgesic Activity in Albino Mice

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ABSTRACT
The purpose of this study is to isolate total flavonoids from aerial parts of Sambucus wightiana (TFSW). and to carry out acute toxicity studies for TFSW to determine a safe dose range. In addition to this analgesic activity has also been determined .Safe dose range of TFSW was identified through Lorke method of acute toxicity. Analgesic and antipyretic properties of TFSW were observed in mice models, receiving 50, 100, 150 mg/kg of TFSW. Analgesic activity of TFSW was confirmed through the aceticacid-induced writhing model and hotplate induced nociception while antipyretic activity was studied through Brewer's yeast induced mice. TFSW was confirmed to be safe up to 500mg/kg body weight. Different test doses of TFSW (50, 100, 150 mg/kg) was observed for their analgesic and antipyretic activity. TFSW showed analgesic and antipyretic activity in a significant dose-dependent manner. The maximum analgesic effect of 56.10%,22.22% was observed respectively in acetic acid-induced writhing and hot plate model at a dose of 150mg/kg dose while diclofenac sodium(50mg/kg) and Tramadol (30mg/kg) showed a maximum effect of 78.65%. and 88.14% respectively. TFSW analgesic activity was reversed by naloxone (0.5mg/kg) in hot plate pain induced nociception model. The finding of our study concluded that the S. wightiana contains flavonoids which are non-toxic up to 500mg/kg and possess analgesic and antipyretic activity.
Keywords*: Sambucus wightiana, Analgesic. Antipyretic, Brewer's yeast, Hot plate.*

Received 07.01.2019 Revised 18.03.2019 Accepted 13.05.2019

How to cite this article:
N Muhammad, S A Tariq, A A Mangi, F Khuda, M Qibtia, M A Shahzad, S Parveen ,A Said, ,A Ali, T A Chano. Evaluation of The Screening and Isolation of Total Flavonoids of *Sambucus wightiana* (TFSW) for Its Analgesic Activity in Albino Mice. Adv. Biores., Vol 10 [4] July 2019.124-131.

INTRODUCTION

Man's well-being is among the basic needs and priorities from the very first day of human in this world. For this purpose, mankind has been in a continuous struggle to conduct trials and experiments on natural products that are available nearby easily [1]. Around 8,000 therapeutic plants are utilized as part of South Asia for different infirmities and 19% of these are found in Pakistan [2]. In Pakistan, about 600-100-different plant species are being used utilized to treat different disease by 40,000 registered and unregistered Hakims/Tabibs [3]. Traditional health treatment is not on the scientific base. It is practice base knowledge, inherited by ancestors to their breeds [4]. Sambucus a sort of blooming plants belongs to family Adoxaceae. The different species of this family are normally named as elder or elderberry [5]. Sambucus species shows various pharmacological activities such as lipid profile and serum antioxidant

capacity in humans [6], antiulcerogenic [7], diuretic, diaphoretic, purgative, hemostatic properties [8] and antidiabetic properties [9]. *S.wightiana* is a Perennial shrub up to 2 meters tall, it cultivates in wet and dry soils on roadsides, slope inclines and water banks. *S.wightiana* is widely allocated in cold mountain pathways of Himalayas mainly in Kashmir, Afghanistan, Pakistan and India at an altitude of 2000 to 2500 meters [10]. In Pakistan, it is found in the hilly areas of Miandm, Kalam and Azad Jammu Kashmir [11].*S.wightiana*is used for various dermatological, gastrointestinal and various skeleton muscular problems. It also has anti-inflammatory, hypotensive, diuretic, diaphoretic and expectorant properties. In Kashmir the fruit juice of *S. wightiana* is utilized to initiate vomiting for ousting unpalatable sustenance from the stomach. Paste from dried leaves and flowers are used to relieve symptoms of burn and rheumatism [10, 11]**.** *S.wightiana* is a rich source of steroids, tannins, phenolics, saponins, alkaloids, glycosides and glycosides, anthocyanins like sambucina or sambucianina, esters of fatty acids, flavonoids as quercetin, organic acids, sterols, triterpenes, iridoids, polyphenols and ursolic acid [12].The point of our examination was to give logical confirmation that the TFSW possess analgesic and antipyretic activity.

MATERIAL AND METHODS

Chemicals

Diclofenac sodium, Acetic acid, Brewer's yeast, Paracetamol, Lit, Naloxone, Tramadol.

Experimental animals

Swiss albino mice of either male/female were buyed from the animal house of National Institute of health, Islamabad, Pakistan. Animals were feed with standard food and proper light/dark cycles and air ventilation was provided. The ethical committee of the Institute of basic medical sciences, Khyber Medical University Peshawar, Pakistan approved the experimental protocols.

Plant material

Collection and extraction

S. wightiana (13 kg) was gathered from the valleys of Swat (Beha and Kalam valleys) KPK, Pakistan, in July 2017. The plant was identified by charge Herbarium, Department of the Botany, University of Peshawar. Plant material was dried under shade for around one month. The dried plant (6kg) was then ground to fine powder by specially designed grinder of medicinal plants in PCSIR lab. Maceration of powder was done with methanol at room temperature for 14 days with intermittent shaking. The methanolic extract became then filtered and focused through rotary evaporator followed with recirculation chiller (Neslab gadgets) and a warming showerat 40oc, as a result dark brown semisolid mass was obtained. (0.8 kg).

Fractionation and Isolation

Sequential fractionation was done with n-hexane, chloroform, and ethyl acetate. Ethyl acetate fraction was then dissolved in water and was shaken with chloroform in a separating funnel. A drop wise addition of 10% Sodium chloride solution results in separation of tannins. The tannins werethen removed by separating superficial layer of the aqueous solution and was the again shaken with ethyl acetate in a separating funnel and solvent was evaporated through rotary evaporator yields a fraction of total flavonoids [13].

Phytochemical status

Different standard phytochemical assays were carried out on the concentrated methanol extract and on its subsequently obtained solvent fractions separately .Ferric chloride(FecCl3) was added drop wise to test extracts separately, green precipitate on shaking confirmed the presence of tannins. Test extracts were heated separately, the formation of foam confirmed the presence of sapponins. Solution of test extract and 10% lead acetate solution, the yellow precipitate was formed that confirmed the presence of flavonoids. Extract solution with 1% HCl was treated with Dragendorff's reagent, Mayer's reagent gave a red and buff colored precipitate which confirmed the presence of alkaloids. Sodiumnitroprussides and sodium hydroxide solutions were added to test extract and HCL solution. The dark red color solution formation confirmed the presence of glycosides. Benedict's reagent was added dropwise to test extract solutions separately and was heated gently. Reddish-brown precipitate was formed which confirmed the, reducing sugar.

Quantitative phytochemical study of total flavonoid content

Various subsequent fractions of *S. wightiana*methanol extract were quantified for its flavonoid content through Aluminum chloride colorimetric method(14). 0.5 ml solution of*S. wightiana* subsequent solvent fractions in methanol (1:10 g/ml) were individually mixed with 1.5 ml of methanol, 0.1ml of 10% Aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8ml of distilled water. After keeping the solutions for 30 min at 10-28C°,absorbance of each solution was observed at 415nm, quercetin was taken as standard.

Acute toxicity

Lorke method was used to find out a safe dosage range of TFSW in Swiss albino mice models containing either sex (male/female) ranging from 25-35 gm. (15). Seven groups of mice each containing three animals.

Group 1 was given with 0.9% Normal saline (10 ml/kg) while six groups were given with test doses (0.1, 1, 10, 100, 500, 1000 mg/kg) of TFSW. Animals were constantly observed 2 hours for any behavior changes and were kept for 24 hours for morbidities and any mortalities if occur.

Analgesic activity

Hot plate method

Hot plate digital analgesiometer was used in this method for induction of pain and reading of latency time in test animals. Test animals were adapted to laboratory condition just before an hour before activity. Animals were kept for 24 hours without food while having water to drink. Five groups each containing six mice were grouped. Group-I animals were administered with saline (10 ml/kg). Group-II was administered with Tramadol (30 mg/kg i.p). Groups III - V mice were dosed with different doses (50, 100, and 150 mg/kg) of TFSW i.p. Pain was induced after 30 min of injecting, throughhotplate analgesiometer that was set at temperature of 55 ºc. Response to nociception like unusual movements, licking of the hind paw and jumping was observed in each animal. The latency time was measured in seconds.

Each animal was placed for maximum 30 seconds, to prevent tissue damage. Latency time was countdown in seconds at a period of 30, 60, 90 and 120 min (3, 16). Calculation was done through the formula

$$\% \text{ Analgesic effect} = \frac{(\text{test latency} - \text{control latency})}{(\text{cut off time} - \text{control latency})} \times 100$$

Analgesic activity with Naloxone

Naloxone (0.5 mg/kg) was given to find out the central analgesic activity of TFSW. Test animals were grouped into four each containing six mice. Naloxone (0.5 mg/kg) was injected toall groups. After 10 min Group I animals was administered Tramadol (30 mg/kg), group II, III, IV were dosed with (50, 100 and 150 mg/kg) of TFSW. Latency time was countdown in seconds at a period of 30, 60, 90 and 120 min and calculation of percent analgesia was made through formula.

$$\% \text{ Analgesic effect} = \frac{(\text{test latency} - \text{control latency})}{(\text{cut off time} - \text{control latency})} \times 100$$

Acetic acid induced writhing

Five groups each containing six mice were kept without food for 24h just before the activity. Group-I animals was dosed with 0.9%saline (10 ml/kg) as a control. Group-II was administered with Diclofenac sodium (50 mg/ kg) as a positive control and III – V was administered with different doses of TFSW(50, 100 and 150 mg /kg). Writhing was induced through injecting 1% acetic acid. Writhing were counted for each mousefor 10 minutes after 5 minutes of administering acetic acid. Percent analgesic effect of TFSW and diclofenac sodium (50 mg/ kg) was calculated through formula:

$$\% \text{ Inhibition of writhing} = \frac{\text{writhing in control group} - \text{writhing in the test group}}{\text{writhing in the control group}} \times 100$$

Brewer's yeast induced pyrexia.

Five groups each containing six mice were kept without food for 24h just before the activity. Group-I was administered with saline (10 ml/kg), Group-II animals were administered with Paracetamol (50 mg/kg) as a positive control. The groups III - V were administered with different doses (50, 100, 150 mg/kg) of TFSW. Normal basaltemperature of all groups was recorded through inserting digital thermometer into rectum of mice and then temperature was elevated by injecting 20% aqueous suspension of Brewer's yeast to all groups. Animals were kept without food while having water to drink overnight. Temperature was again recorded after 24hr. Animals that show rise in temperature greater than 0.5°Cwere included. Different test doses (50, 100, and 150 mg/kg) of TFSW, standard drug were administered to pyretic animals and after it, the rectal temperature was recorded for 1,2,3,4 and 5 hours.

RESULTS

Fractionation

Sequential fractionation was performed on 500 g of concentrated methanol extract of *S. wightiana*.

Table 1: List of subsequent fractions of S. wightiana methanol extract with their % yield.

Fractions	Weight (g)	Yield (%)
Total plant	6000 gm	----------
Methanol extract	500 gm	8.33 %
n-hexane fraction	112 gm	22.4 %
Chloroform fraction	61 gm	12.2 %
Ethyl acetate	45 gm	9 %
Aqueous fraction	260 gm	52 %

Phytochemical status

Various preliminary phytochemical tests were carried out on the methanol extract and on its consequent solvent fractions.

Table 2: List of preliminary phytochemical assays of crude methanolic extract and subsequent solvent fractions of S. wightiana.

Chemical	Test	Meth	n-hex	Chlo	Eth-acet	Aq
Tannins	Ferric chloride test	+	+	+	+	-
Saponins	Frothing test	+	-	+	-	-
Flavonoids		+	-	+	+	-
Alkaloids	Dragendorff's reagent and	+	-	+	+	-
	Mayer's reagent.	+	-	+	+	-
Carbohydrates	Benedict' test	+	+	+	+	+
Glycoside	Nitroprusside test	+	-	+	+	_

(Meth= methanolic, Hex= n-hexane, Ch= chloroform, Eth-acet=ethyl acetate and Aq= aqueous)

Quantitative phytochemical assay of flavonoids:

Flavonoids were quantified in methanol extract and different consequent solvent fractions

Table 3 Quantification of total flavonoids of *S. wightiana*.

Concentrate Fraction	Quantity (mg/g)	Percent quantity (%)
Methanol	27.65	2.765
n-hexane	10.40	1.04
Chloroform	69.35	6.935
Ethyl acetate	75.30	7.53
Aqueous	3.10	0.31

Acute toxicity

Different doses of TFSW were administered to find out acute toxicity dose. A minimum dose of 0.1mg/kg and a maximum dose of 750 mg/kg were given to different groups. There were no mortalities found up to 500 mg/kg while 750 mg/kg showed 20 % mortalities.

Analgesic activity

Acetic acid induced writhing test

All the test doses of TFSW (50, 100 and 150 mg/kg) showed a significant analgesic activity in asdose-dependent manner. A test dose of 150 mg/kg showed maximum inhibition of writhing 56.10%. Positive standard group of diclofenac sodium (50 mg/kg) showed the most potent analgesic activity, having percent inhibition of writhing 78.65% shown in **Fig-1.**

Table 4 Effect of TFSW 50, 100, 150mg/kg in acetic acid inducedwrithing test.

Treatment	Dose (mg/kg)	Number of writhing	% Inhibition of writhing
Saline (G-1)	10ml/kg	54.667	-------
Diclofenac (G-2)	50	11.6667	78.65%
Test groups (G-3, 4, 5)	50	47.67± 3.559	12.804%
	100	36.33±3.327	33.54/%
	150	24.00±6.229	56.10%

Mean ±S.E.M for a group of six animals. Statistic was done through ANOVA and then Dunnett's test was applied.

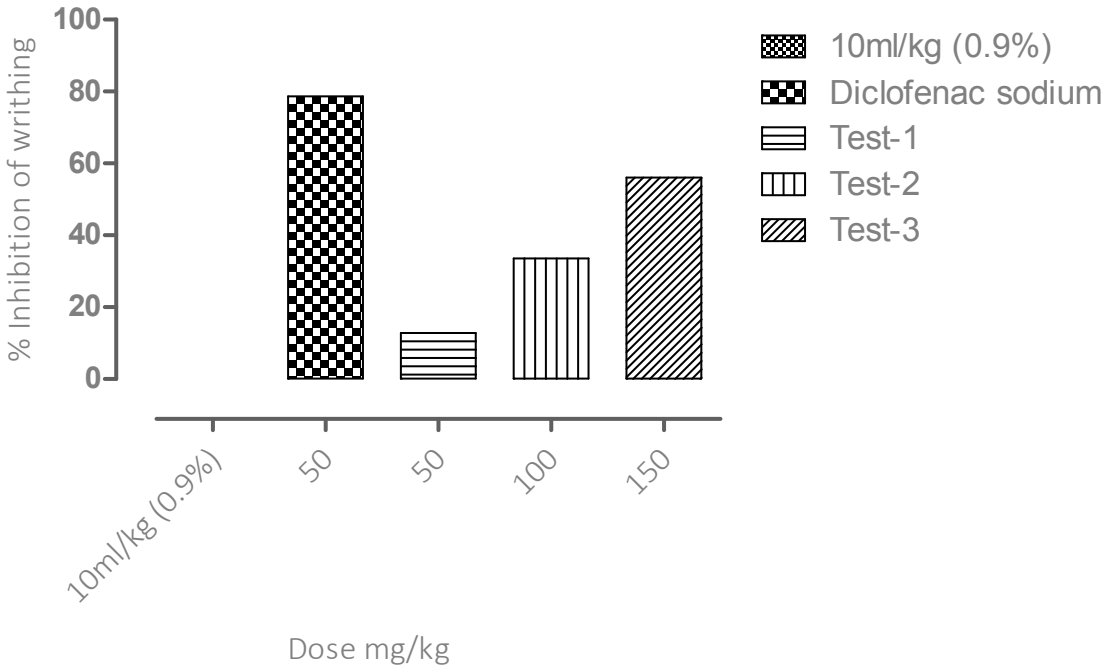

Figure 1 Percent inhibition of writhing by TFSW (50, 100, 150 mg/kg) and standard Diclofenac sodium (50 mg/kg).

Percent analgesic activity of TFSW (50, 100 and 150 mg/kg) in acetic acid induce pain model. Graph represents percent analgesic effect as mean±SEM of six animals. Statistic was done through ANOVA and then Dunnett's test was applied.

Hot plate

A significant increase ($P<0.05$) in latency time was observed from 13.16% to 22.22% at a dose of 50mg to 150 mg/kg. A dose-dependent effect was observed, the maximum effect of 22.22% was observed after 90 mins with 150mg/kg dose while standard opioid analgesic (Tramadol) showed a maximum effect of 88.14% shown in **Fig-2**. It was observed that the analgesic effect of standard opioid agonist Tramadol (30 mg/kg) and different test doses (50, 100, 150 mg/kg)of TFSW was reversed profoundly by central opioid antagonist naloxone (0.5mg/kg) as shown in **Fig-3**.

Table 5 Latency time for nociceptive response for different test doses of TFSW (50,100, 150 mg/kg), Diclofenac sodium (50 mg/kg) and Saline group.

Treatment	Dose (mg/kg)	The latency of nociceptive response in seconds (mean ± SEM)				
		0 min	30 min	60 min	90 min	120 min
Saline	10 ml/kg	9.015 ±0.562	9.035 ± 0.3939	9.027 ± 0.2609	9.028 ± 0.7016	8.748 ± 0.8071
Standard	50	9.960 ±0.821	23.37 ± 0.8287	23.38 ± 0.8356	26.27 ± 0.9331	27.48 ± 0.5328
TFSW	50	9.230 ±0.911	10.40 ± 1.056	10.12 ± 0.4214	11.79 ± 0.9969	11.44 ± 0.9820
	100	9.419 ±0.262	11.07 ± 0.9329	11.78 ± 0.5496	12.60 ± 0.9899	12.31 ± 1.033
	150	9.335 ±0.656	13.62 ± 0.9088	14.52 ± 0.3931	13.27 ± 1.147	12.94 ± 1.356
Antagonizing effect of naloxone						
TFSW	50	9.150 ±0.6682	9.432 ± 0.3184	9.733 ± 0.5855	9.99 ± 0.5415	9.74 ± 0.5408
	100	9.265 ± 0.3605	9.717 ± 0.350	10.08 ± 0.7114	10.36 ± 0.6821	10.17 ± 0.4725
	150	9.115 ± 0.5611	9.913 ± 0.4931	10.45 ± 0.6764	10.88 ± 0.7718	10.65 ± 0.7398

Mean ±S.E.M for a group of six animals. Statistic was done through ANOVA and then Dunnet,s test was applied.

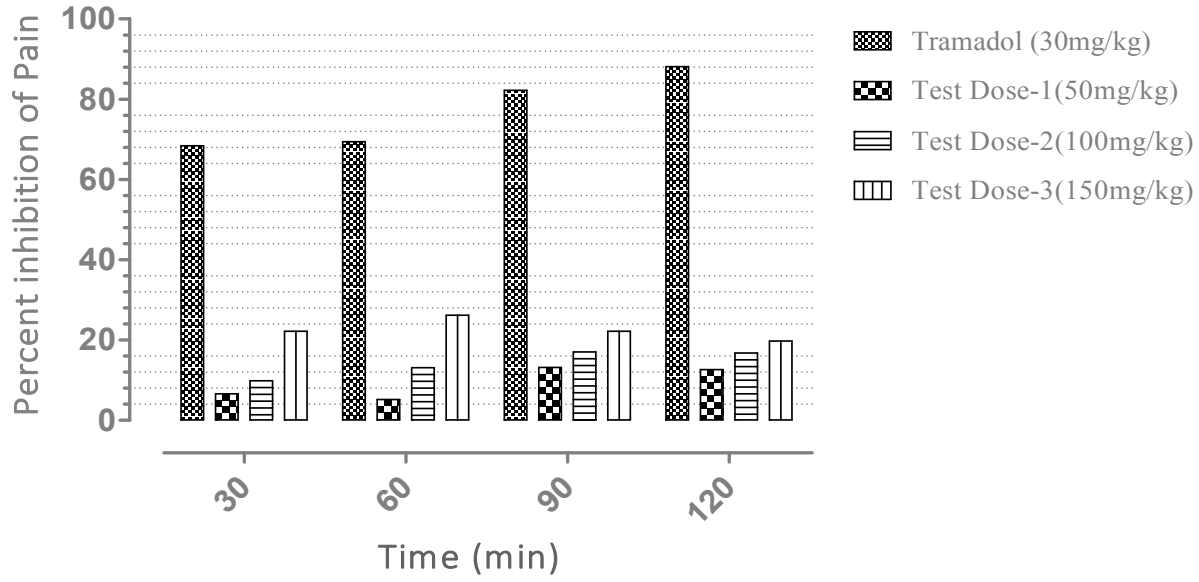

Figure 2 Percent analgesic effect of TFSW (50, 100, 150mg/kg) and Tramadol (30mg/kg) in hot plate pain induced mice.

Graph showing percent analgesic effect of TFSW(50,100and150mg/kg)and Tramadol (30 mg/kg). Graph shows mean±SEM of six animals .Statistic was done through ANOVA and then Dunnett's test was applied.

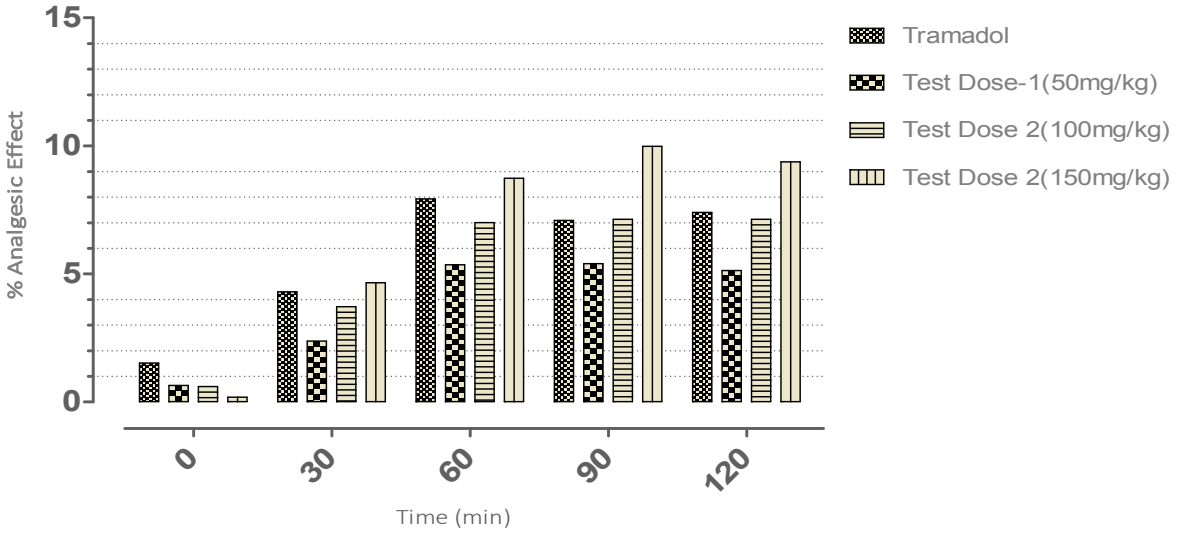

Figure 3 Percent inhibit of pain effect of TFSW (50, 100, 150mg/kg) and Tramadol (30mg/kg) antagonized with naloxone (0.5mg/kg) in hot plate pain induced mice

Graph showing antagonizing effect of Naloxone (0.5mg/kg)on hot plate pain model in mice. Graph shows mean±SEM of six animals. Statistic was done through ANOVA and then Dunnett's test was applied.

DISCUSSION

Lorke technique was used to find out acute toxic dose. It was observed that TFSW was safe upto500mg/kg while at 750 mg/kg there occurred 20% deaths. In some animal's certain behavior changes such as shivering, stimulation, tremors, and an increase in motor activities were observed at a dose of 500 and 700 mg/kg. Acetic acid induced writhing that involves peripheral receptors and Hot plate induced model that involves the central receptors of pain(17).Cyclo-oxygenase and lipoxygenase pathways play the main role in producing inflammatory and pain mediators like prostaglandins, cytokines and other endogenous substances. Most of the NSAIDs act through inhibition of these pain producing mediators' pathways [18]. Acetic acid causes the activation of these pathways thus produces

prostaglandins and cytokines. These mediators disturb the vascular permeability hence results in an increase in the permeability from the vascular lumen and activates the immune system [19]. Peritoneal receptors causes the stimulation of peripheral nervous system that causes writhing. Agents that decreased number of writhing inhibit the Cyclo-oxygenase, lipoxygenase pathways and are said to act on peripheral on the nervous system [20]. Diclofenac sodium (50 mg/kg) a prominent NSAID was used as a positive control that showed 78.65 % inhibition of writhing as compared to the negative controlled group following peripheral inhibition of prostaglandin synthesis pathway. Similarly, TFSW in different test doses decreased the number of writhing in test animals. Maximum percent inhibition of writhing of TFSW 50, 100 and 150 mg/kg was calculated to be 12.804%, 33.54%, and 56.10% respectively. It is concluded from the acetic acid-induced pain model that TFSW causes the peripheral inhibition of pain similar to the positive control Diclofenac sodium. The central action of total flavonoids of *Sambucus wighitana*was confirmed through the hot plate pain induced model. Tramadol (30 mg/kg) has a central mode of action on opioid receptors similar to morphine (standard group). The latency time of test animals was significantly increased ($P<0.05$) from 13.16% to 22.22% at a dose of 50mg to 150 mg/kg. A dose-dependent effect was observed, the maximum effect of 22.22% increase in latency time was observed after 90 mins with 150mg/kg dose while a positive controlled standard Tramadol (30 mg/kg) showed 88.14% increase in latency time. TFSW is less potent than Tramadol (30 mg/kg). The difference Tramadol (30 mg/kg) and TFSW in their latency time were probably due to a difference in pharmacokinetic or pharmacodynamics profile or may due to the low potency of TFSW. When nonselective opioid receptor antagonist (naloxone) was administered to the groups receiving Tramadol (30 mg/kg) and TFSW (50, 100 and 150 mg/kg) the analgesic potential of both were reversed in a significant manner. In the presence of naloxone, a weak analgesic activity was produced which shows that TFSW mainly acts through opioid receptors but it also acts through other mechanisms but in a less significant manner. All test animals were injected subcutaneous Brewer's yeast which increases the synthesis of prostaglandins [21]. This test is an important tool to test the antipyretic profile of natural as well as synthetic compounds. This type of inducted pyrexia is termed as pathogenic pyrexia [22]. Fever is induced by several chemical mediators like prostaglandins. Agents that cause a relief from fever (Paracetamol) mainly acts through inhibition of the cyclooxygenase enzyme pathway that further reduce down prostaglandins synthesis [23]. Yasilada *et al* [6] conducted the study which is quite similar in a sense that isolation was made but at the same hand this study is inconsistent with the current study that current study is aimed on the analgesic activity while the former conducted on the ulcerative activity. Zulfikar [18] Conducted the study which I similar to the present study that he made research on ethanol extract and found out the analgesic activity ,

CONCLUSION

S.wightiana has been used for various mankind disease in the Northern hilly areas of Pakistan and India without any proper scientific bases. Here in our study, we confirmed the presence of different phytochemicals through preliminary phytochemical tests (qualitative analysis) on the methanolic extract and its consequent fractions and also performed a quantitative analysis of total flavonoids. Furthermore, total flavonoids were isolated from the ethyl acetate fraction of *S.wightiana*and different in vivo pharmacological activities like acute toxicity, analgesic and antipyretic was performed. We observed that the TFSW possess pain relieving properties which are central as well as peripheral in action and it also has the capability of fever-reducing properties.

CONTRIBUTION OF AUTHORS

Niaz Muhammad designed the study and performed in the Lab ,Shafiq Ahmed Tariq Analysed the results and discussion ,Altaf Ali Mangi Helped in Lab and wrote the manuscript ,Fazle Khuda and maria Qubtia participated in conceiving the study ,Muhammad Asif Shahzad helped in Literature review , Ali Said helped in literature search ,Abid Ali and Tahseen Ahmed Chano helped in result interpretation , All authors read and approved th final manuscript .

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