ACUTE TOXICITY AND ANALGESIC ACTIVITY OF CRUDE FLAVONOIDS OF ACHILLEA WILHELMSII AND TEUCRIUM STOCKSIANUM

Niaz Ali[1✉], Ulfat Sultana[2], Syed Wadood Ali Shah[3], Muhammad Nabi[2], Shakirullah[2], Ismail Shah[4], Ghayour Ahmed[4]

[1✉] Associate Professor of Pharmacology, Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Khyber Pakhtunkhwa, Pakistan
Email: niazpharmacist@yahoo.com
Phone: 0092-91-9217703
[2] Department of Pharmacology, Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Khyber Pakhtunkhwa, Pakistan
[3] Department of Pharmacy, University of Malakand, Chakdara, Dir, Khyber Pakhtunkhwa, Pakistan
[4] Department of Pharmacy, Kohat University of Science and Technology, Khyber Pakhtunkhwa, Pakistan.
Date Submitted: October 16, 2015
Date Revised: February 22, 2016
Date Accepted: February 28, 2016

ABSTRACT

OBJECTIVES: To isolate total flavonoids from *Achillea Wilhelmsii* and *Teucrium Stocksianum* and screen them for possible analgesic activity and to determine the safe dose range of total flavonoids for preclinical studies in mice.

METHODS: Acute toxicity studies were performed in mice to determine its safe dose range for total flavonoids of Achillea wilhelmsii and Teucrium stocksianum. Writhing tests were performed in mice for possible analgesic activity. Tail immersion method was used in rats for possible analgesic activity.

RESULTS: Respective LD50 for crude flavonoids of *Achillea wilhelmsii* (CFA) and crude flavonoids of *Teucrium stocksianum* (CFT) are 1312±12.76 mg/kg and 1248 ±29.6 mg/kg. In acetic acid induced writhing, analgesic effect of CFA and CFT in dose 10 mg/kg is almost comparable with standard acetylsalicylic acid ($p \leq 0.05$). Similarly, in tail immersion method, reaction time was significantly prolonged in test doses of 5 and 10 mg/kg (both for CFA and CFT) suggesting supraspinal analgesic effect.

CONCLUSION: The results confirm that total crude flavonoids of both the species, which are safe up to dose of 100 mg/kg, have analgesic activity. It further warrants for isolation and purification of analgesic flavonoids for new drug development.

KEY WORDS: Achillea (MeSH), Achillea wilhelmsii (Non-MeSH); Teucrium stocksianum (Non-MeSH); flavonoids (MeSH), crude flavonoids (Non-MeSH); Analgesics (MeSH).

THIS ARTICLE MAY BE CITED AS: Ali N, Sultana U, Shah SWA, Nabi M, Shakirullah, Shah I, et al. Acute toxicity and analgesic activity of crude flavonoids of Achillea Wilhelmsii and Teucrium Stocksianum. Khyber Med Univ J 2016; 8(1): 7-11.

INTRODUCTION

Flavonoids are low molecular weight bioactive polyphenols, which play a vital role in photosynthesizing cells.[1] Naturally occurring flavonoids are generally classified into six classes according to their chemical structures of carbon ring and functional groups attached with the carbon ring. They are flavanones, flavones, isoflavonoids, flavans (flavanols), flavonols and anthocyanins.[2] They have been described to possess numerous biological activities such as antioxidant, anti-inflammatory, estrogenic, cytotoxic, antitumor, antiviral. Thus their actions in humans have been the subject of extensive research.[3] In animals and humans, flavonoids have been known for diverse biological effects including antibacterial, antioxidant, antitumor, antiviral, anti-inflammatory, anti-allergic and vasodilatory actions.[4,5] Flavonoids exhibit several other biological effects such as anti-inflammatory, anti-hepatotoxic and anti-ulcer actions.[6,7]

So far flavonoids have shown strong antioxidant and free radical scavenging activity.[8] Thus flavonoids have been directly associated with reduction of risks for certain chronic diseases, particularly for the prevention of some cardiovascular disorders.[8-10] Flavonoids have anticancer activity as well.[5] Study of literature proves that flavonoids have antiviral,[11] antimicrobial,[12] anti-inflammatory activities,[13] beneficial effects on capillary fragility,[14] inhibit human platelet aggregation,[15] antiulcer activity,[16] anti-allergenic activity,[18] and analgesic properties.[13,18] Thus the pharmacological properties of flavonoids are very diverse and beneficial. Hence, we set our objectives to screen the total flavonoids of some of medicinal plants that are traditionally used for analgesic purposes that may help in future for new drug development.[19]

Achillea wilhelmsii C. Koch (Local name: Zawal) belongs to family Asteraceae, While Teucrium stocksianum Bioss belongs to family Lamiaceae.[20] Traditionally, the species of Achillea and Teucrium genus are used as analgesic.[20,21] It has been reported that Achillea wilhelmsii and Teucrium stocksianum contain flavonoids.[22-24] Based on the literature for diverse activities of flavonoids and our reports for the presence of flavonoids in *Achillea wilhelmsii* and T*eucrium stocksianum,* we carried out current work to isolate total flavonoids from Achillea Wilhelmsii and Teucrium Stocksianum and screen them for possible analgesic

activity and to determine the safe dose range of total flavonoids for preclinical studies in mice.

METHODS

Plant Materials

Achillea wilhelmsii was purchased from the local market of Board Bazaar of Peshawar, Khyber Pakhtunkhwa (KP). Teucrium stocksianum was collected from the nearby hills of University of Malakand in the month of May-June 2011. The plants were identified by Professor Dr. Jehandar Shah, plant taxonomist, the then vice chancellor Shaheed Benazir Bhuto University, Sheringal Dir Upper, Khyber Pakhtunkhwa, Pakistan. Voucher specimens, respectively, AW-2011 for Achillea wilhelmsii and T-01-2011 for Teucrium stocksianum were deposited in the herbarium of Department of Botany, University of Malakand.

Preparation of extract and the crude flavonoids

140 grams of each powdered plant materials were extracted with ethanol (95%) in the ratio of 1:8 (w/v) for two hours. The menstruum was filtered through a porcelain cloth. The process was repeated three times. The filtrates were combined and concentrated on 45°C using a rotary evaporator. The extract was dissolved using sufficient distilled water. To remove the fatty contents, the materials were exhaustively extracted with distilled petroleum ether. Finally, crude flavonoids were obtained when the defatted fractions were extracted with distilled ethyl acetate to get crude flavonoids of Achillea wilhelmsii (CFA) and crude flavonoids of Teucrium stocksianum (CFT) respectively yielding 14.35 g and 11.80 g.[25] The qualitative analysis of CFA and CFT was carried out with $NaBH_4$, 1% $FeCl_3$-ethanol and 1% NaOH reagent to confirm the presence of polyphenols i.e. flavonoids.[26]

Ethical approval: The study protocols were approved by the Advanced Studies and Research Board of the Khyber Medical University, Peshawar. Ethical approval (No. Dir/KMU-EB/AA/000081) was also accorded by the Ethical Board of the Khyber Medical University, Peshawar.

Acute toxicity studies

The acute toxicity LD_{50} of CFA and CFT was carried out on Swiss albino mice. The animals (either sex) were divided in 7 groups (A-G), 5 mice in each group for each of the crude flavonoids. The animals were fasted overnight before the experimentations. The study was performed in two phases. In first phase, three groups (A-C) of mice were treated intra-peritoneal (i.p) with CFA at doses of 10, 100 and 1000 mg/kg body weight, respectively and observed for next 24 hours for any signs of toxicity and death. In 2nd phase, three groups (D-F) of mice were treated with CFA (crude flavonoids of Achillea wilhelmsii) (i.p) at doses of 1250, 1500 and 1750 mg/kg body weight, respectively. Group G (negative control) received 3 ml/kg of normal saline. Symptoms for toxicity and death were observed with in twenty four hours in each group. The numbers of death in each group in 24 hours were noted. Mean lethal dose that killed fifty percent of test animals (LD_{50}) were calculated. The same protocols were repeated for the CFT (27).

Analgesic activity (Writhes Test)

In this experiment Swiss albino mice were selected. Mice were divided in six groups (1-6) having 5 mice in each group. Group 1 received normal saline in a dose of 10 ml/kg and considered as negative control. Group 2 received standard analgesic drug acetylsalicylic acid (ASA) at a dose of 150 mg/kg. Group 3 and group 4 received intra-peritoneal doses in 5 and 10 mg/kg of CFA respectively. While group 5 and group 6 received intra-peritoneal doses in 5 and 10 mg/kg of CFT respectively. After 30 minutes of dosing of test samples and standards (both negative and positive control), 0.7% solution of acetic acid (prepared in sterilized normal saline) was injected (i.p) in dose 0.1 ml/10g. Mice were then kept in separate cages. The number of abdominal constrictions were observed and counted five minutes after stimulation during period of ten minutes.[18,28] The percent inhibitions of writhes were calculated.

Tail immersion method

The tail immersion method was used to determine analgesic activity of crude flavonoids of both species with slight modifications. Briefly, young Wistar rats (150-170 mg) were randomly divided into 6 groups (1-6) each group had 6 rats. Rats were fasted overnight before the starts of experiments. Rats had free access to water during experiments. The Group 1st & 2nd received oral dose of 5 and 10 mg per kg body weight of CFA, respectively. Group 3rd & 4th received oral dose of 5 and 10 mg per kg body weight of CFT, respectively. Group 5th received tramadol® (a narcotic analgesic) in dose of 30 mg/kg as standard narcotic analgesic. Group 6th served negative control group which was treated with vehicle (0.8ml of 0.5% CMC suspension in saline). All the vehicle/drugs and extract were administered orally.

With the help of thermostat the temperature of the organ bath was set at 55 ± 1°C. The lower portion of tail (5 cm) of the rats (hold in rat restrainers) were marked and immersed in the hot water bath and the time taken by the rats to withdraw its tail from the water already maintained at 55 ± 1°C was recorded by mean of stopwatch. After each reading, the tail was dried with soft tissue or clean cloth. The reaction time was recorded before and after oral dose at an interval of 30 minutes for 120 hours. The cut off time of the immersion is 15 seconds.

Statistical Analysis: Graph Pad Prism version 5 was used to test the significances at $P \leq 0.05$, CI=95% for key variables like number of writhes and time for tail withdrawing on respective test doses of crude flavonoids and standard drugs. ANOVA followed by Dunnet's test was used to test the hypothesis.

RESULTS

Results of acute toxicity studies are expressed in Table 1. It is important to mention that crude flavonoids of Achillea wilhelmsii and Teucrium stocksianum are safe up to dose of 100 mg/kg. Lethality was prominent in test dose of 1000 mg/kg body weight for Crude flavonoids

of Achillea wilhelmsii and Teucrium stocksianum. Respective LD_{50} for crude flavonoids of Achillea wilhelmsii (CFA) and crude flavonoids of Teucrium stocksianum (CFT) are 1312 ± 12.76 and 1248 ±29.6 mg/kg (Figure 1). Results of analgesic activity using acetic acid induced writhing are expressed in Table II.

Crude flavonoids of *Achillea wilhelmsii* produced statistically significant analgesic effect in dose 5 mg/kg and 10 mg/kg ($p \leq 0.05$). Analgesic effect of CFA at dose of 10 mg/kg is almost comparable with analgesic effect of acetyl salicylic acid (positive control).

Similarly, crude flavonoids of Teucrium stocksianum significantly inhibited the number of writhings in experimental animals in dose of 10 mg/kg. Like crude flavonoid of Achillea, analgesic effect of crude flavonoid of *Teucrium stocksianum* is comparable in dose of 10 mg/kg versus the analgesic effect of positive standard, acetic acid induced writhing method is well recommended for pharmacological screenings of test samples for possible analgesic effects. Percent inhibition of writhing is expressed in Figure 2. Results of thermal nociception model (tail immersion test) are expressed in Table III.

TABLE I: RESULTS OF ACUTE TOXICITY STUDY IN MICE (N=3)

Phases	Groups		% lethality (Mean ±SEM)		
		Dose (mg/kg)	Negative control (normal saline only)	CFA	CFT
1st phase	1	10		0	0
	2	100	0	0.000±0	0.000±0
	3	1000	0	17.500±2.5	26.500±1.5
2nd phase	4	1250	0	40.500±4.5	50.000±2
	5	1500	0	81.000±4	89.500±0.5
	6	1750	0	100.000±0	100.000±0

TABLE II: EFFECTS OF TOTAL FLAVONOIDS AND STANDARDS ON NUMBER OF WRITHING IN TEST ANIMALS

Group	Dose (mg/kg)	CFA	CFT
		No. of writhing	No. of writhing
Negative control	—	39±2.2	43±2.5
Acetyl salicylic acid	150	8.16±0.44**	8.16±0.72**
1	5	23.33±0.88**	21.00±0.86**
2	10	14.00±0.76**	11.66±0.44**

Values are mean ± SEM, n=6; **p≤0.01 (ANOVA) vs. control

DISCUSSION

Pain is produced due to release of some autacoids like prostaglandins (PGE2, PGF2 α) involving the cyclooxygenase pathway.[29] In this method, local peritoneal receptors may be the possible cause of writhing in test animals.[30] The writhing is widely used to assess the possible analgesic activity that follows the peripheral pathway. Thus a substance which reduces the number of writhings may have analgesic activity. Hence, crude flavonoids of Achillea and Teucrium stocksianum have analgesic activity that not only confirms the traditional use the plant for analgesic activity but also confirms that flavonoids, in general, have analgesic activity as well.[13,18]

Thus it is stated that analgesic activity of the flavonoids of Achillea wilhelmsii and Teucrium stocksianum follow peripheral analgesic activity by inhibition of possible mediator(s) of inflammation like prostaglandins. It may also be attributed to inhibitory effect of local peritoneal receptors that translate the pain indication effect and passes it onward to central nervous system. On the other hand, thermal nociception model (tail immersion test) is used to evaluate analgesic activity following effect on central nervous system. The CFA and CFT showed significant analgesic activity. The analgesic effects started after 30 minutes and reached to maximum as time increases (60-120 minutes) significant changes and reaction time is observed in 30, 60, 90

TABLE III: ANALGESIC EFFECTS OF TOTAL FLAVONOIDS ON TIME FOR TAIL WITHDRAWING EFFECTS ON DIFFERENT INTERVALS

Samples	Dose mg/kg	Time for tail withdrawing (Seconds) on different intervals				
		0 min	30 min	60 min	90 min	120 min
Negative Control	0.5% CMC in saline	3.31±0.032	3.42±0.020	3.35±0.028	3.47±0.015	3.53±0.021
Tramadol	30	3.37±0.009	5.10±0.029***	5.89±0.014***	6.12±0.014***	5.73±0.017***
CFA	5	3.38±0.060	3.61±0.015*	3.89±0.032*	4.12±0.343*	4.14±0.265*
	10	3.44±0.029	3.95±0.025	4.16±0.140*	4.27±0.049*	4.33±0.026*
CFT	5	3.36±0.017	3.89±0.030	4.19±0.132*	4.47±0.015**	4.56±0.037**
	10	3.41±0.020	4.33±0.026*	4.54±0.037**	4.93±0.111**	4.76±0.055**

Values are mean ± SEM, n=6; **p≤0.05; **p≤0.01; ***p≤0.01 (ANOVA followed by Dunnet's test) vs. control

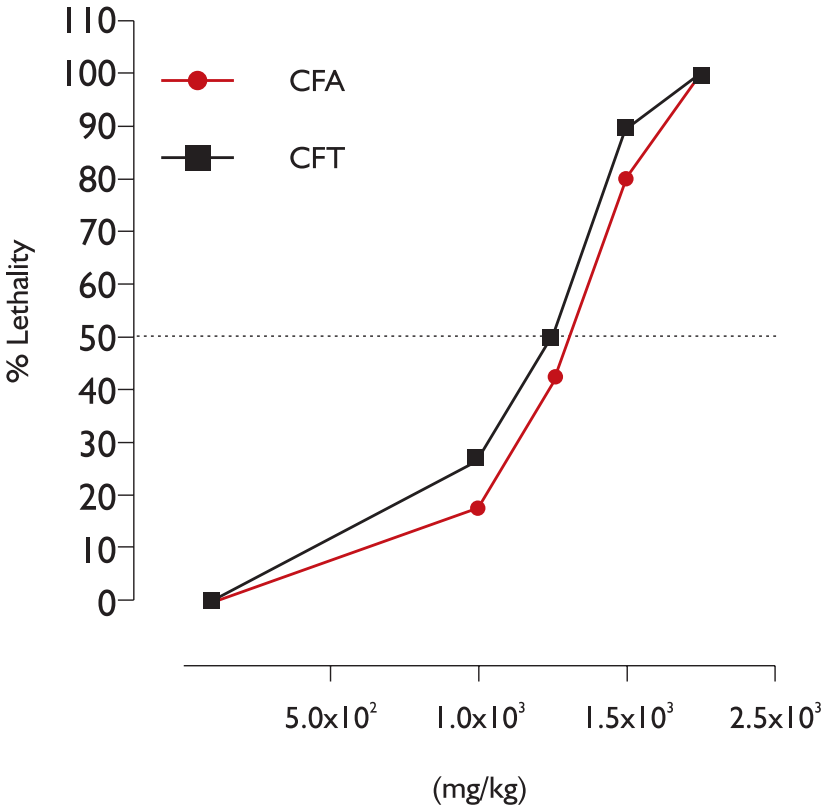

Figure 1: Acute toxicity study results of total flavonoids of Achillea wilhelmsii and Teucrium stocksianum.

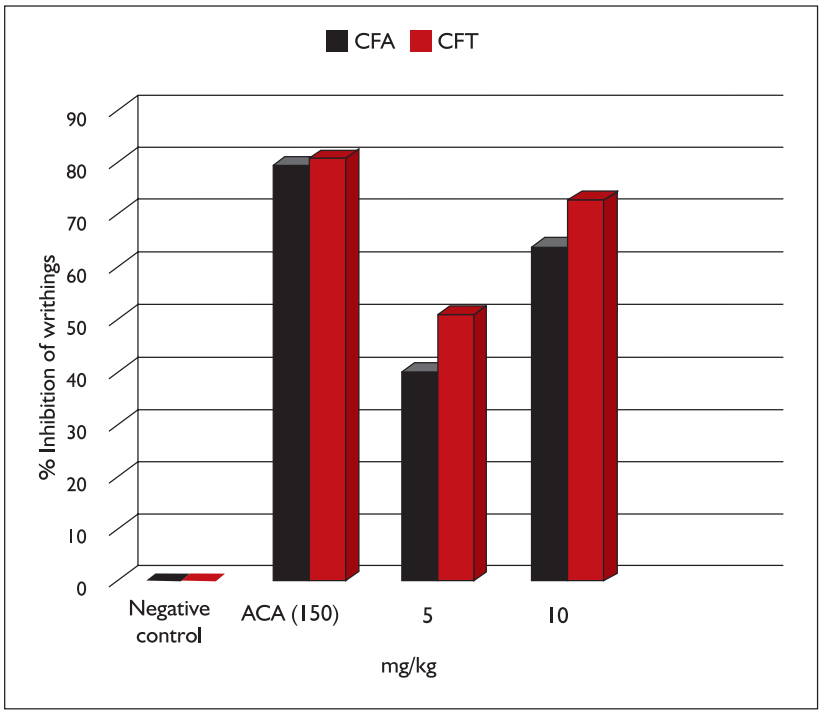

Figure 2: Percent inhibition of writhing by standards and total flavonoids of Achillea wilhelmsii and Teucrium stocksianum.

and 120 minutes in test doses of 5 and 10 mg/kg of the both the flavonoid of *Achillea wilhelmsii* and *Teucrium stocksianum*. After an hour (60 minute) administration, CFT in test dose of 10 mg/kg, produced significant analgesic effects ($P<0.05$) which was comparable to analgesic effect of tramadol®, an opioid analgesic which raises the pain threshold and thus increases the reaction time. Hence, likely in manner, both CFA and CFT increased the reaction time suggesting its possible mode of action through involvement of opioid receptors. Hence, the flavonoids of *Achillea wilhelmsii* and Teucrium stocksianum may have spinal and supraspinal analgesic effects.[31] This concludes that flavonoids of *Achillea wilhelmsii* and *Teucrium stocksianum* have analgesic effect that requires further work for its purification and isolation. Subsequent pharmacological screenings of the isolated and purified contents of total flavonoids may help in developing new analgesics from the flavonoids of Achillea wilhelmsii and Teucrium stocksianum.

CONCLUSION

Crude flavonoids of Achillea wilhelmsii and Teucrium stocksianum have analgesic effect that may either act peripherally or through central nervous system. Flavonoids of Achillea wilhelmsii and Teucrium stocksianum may help in developing new analgesics from natural products.

ACKNOWLEDGMENTS

The authors want to thank Higher Education Commission, Islamabad for providing grant (Grant No:20-1723/R&D/10) for establishment of Pharmacology Laboratory at Institute of Basic Medical Sciences, Khyber Medical University Peshawar, Pakistan.

REFERENCES

1. Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 2005;26:343-356.
2. Peterson J, Dwyer J. Flavonoids: dietary occurrence and biochemical activity. Nutrition Research 1998;18(12):1995-2018.
3. Fernández SP, Wasowski C, Loscalzo LM, Granger RE, Johnston GA, Paladini AC, et al. Central nervous system depressant action of flavonoid glycosides. Eur J Pharmacol 2006;539(3):168-76.
4. Bors W, Heller W, Michel C, Saran M. Flavonoids as antioxidants: determination

of radical-scavenging efficiencies. Methods Enzymol 1990;186:343.
5. Sahreen S, Khan MR, Khan RA, Shah NA. Estimation of flavoniods, antimicrobial, antitumor and anticancer activity of Carissa opaca fruits. BMC complementary and alternative medicine. 2013;13:372.
6. Narayana KR, Reddy MS, Chaluvadi M, Krishna D. Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. Indian J Pharmacol 2001;33(1):2-16.
7. Zhang HY, Yang DP, Tang GY. Multipotent antioxidants: from screening to design. Drug Discov Today 2006;11(15):749-54.
8. Gross M. Flavonoids and cardiovascular disease. Pharm Biol 2004;42(s1):21-35.
9. Moon YJ, Wang X, Morris ME. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicol In Vitro 2006;20(2):187-210.
10. Asres K, Seyoum A, Veeresham C, Bucar F, Gibbons S. Naturally derived anti-HIV agents. Phytother Res 2005;19(7):557-81.
11. Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. J Pharmacol Sci 2004;96(3):229-45.
12. Benavente-García O, Castillo J, Marin FR, Ortuño A, Del Río JA. Uses and properties of citrus flavonoids. J Agric Food Chem 1997;45(12):4505-15.
13. Parveen Z, Deng Y, Saeed MK, Dai R, Ahamad W, Yu YH. Antiinflammatory and analgesic activities of Thesium chinense Turcz extracts and its major flavonoids, kaempferol and kaempferol-3-O-glucoside. Yakugaku Zasshi 2007;127(8):1275-9.
14. Wightman JD, Red berries and their health benefits in Nutraceutical Beverages: Chemistry, Nutrition, and Health Effects (ACS Symp. Ser. 871) 2004;123-132.
15. Middleton Jr E, Kandaswami C. Effects of flavonoids on immune and inflammatory cell functions. Biochem Pharmacol 1992;43(6):1167-79.
16. Yamaguchi F, Saito M, Ariga T, Yoshimura Y, Nakazawa H. Free radical scavenging activity and antiulcer activity of garcinol from Garcinia indica fruit rind. J Agric Food Chem 2000;48(6):2320-5.
17. Kawasaki M, Toyoda M, Teshima R, Sawada J-i, Hayashi T, Arisawa M, et al. In vitro antiallergic activity of flavonoids in histamine release assay using rat basophilic leukemia (RBL-2H3) cells. Shokuhin eiseigaku zasshi. Journal of the Food Hygienic Society of Japan 1994;35(5):497-503.
18. Sannigrahi S, Mazumder UK, Pal D, Mishra ML, Maity S. Flavonoids of Enhydra Fluctuans exhibits analgesic and anti-inflammatory activity in different animal models. Pak J Pharmaceut Sci 2011;24(3):369-75.
19. Botta B, Vitali A, Menendez P, Misiti D, Delle Monache G. Prenylated flavonoids: pharmacology and biotechnology. Cur Medicinal Chem 2005;12(6):717-39.
20. Ali N, Shah SW, Ahmed G, Shah I, Shoaib M, Junaid M, et al. Acute toxicity and antispasmodic activities of Achillea wilhelmsii C. Koch. Pak J Pharmaceut Sci 2014;27(2):309-15.
21. Shah SM, Sadiq A, Ullah F. Antioxidant, total phenolic contents and antinociceptive potential of Teucrium stocksianum methanolic extract in different animal models. BMC Complement Altern Med 2014;14:181.
22. Ali N, Ali Shah SW. Antispasmodic activity of Teucrium stocksianum Boiss. Pak J Pharmaceut Sci 2011;24(2):171-4.
23. Rasheed RA, Ali BH, Bashir AK. Effect of Teucrium stocksianum on paracetamol-induced hepatotoxicity in mice. Gen Pharmacol 1995;26(2):297-301.
24. Ali N, Shah SW, Shah I, Ahmed G, Ghias M, Khan I. Cytotoxic and anthelmintic potential of crude saponins isolated from Achillea Wilhelmsii C. Koch and Teucrium Stocksianum boiss. BMC Complement Altern Med 2011;11:106.
25. Andersen OM, Markham KR. Flavonoids: chemistry, biochemistry and applications: CRC Press; 2010.
26. Lu W, Yang Y, Li Q, Liu F. Crude flavonoids from Carya cathayensis Sargent inhibited HeLa cells proliferation through induction of apoptosis and cell cycle arrest. Lat Am J Pharm 2009;28(4):568-73.
27. Lorke D. A new approach to practical acute toxicity testing. Arch Toxicol 1983;54(4):275-87.
28. Hunskaar S, Hole K. The formalin test in mice-dissociation between inflammatory and non- inflammatory pain. Pain 1997;30(7):103-14.
29. Duarte ID, Nakamura M, Ferreira SH. Participation of the sympathetic system in acetic acid-induced writhing in mice. Braz J Med Biol Res. 1988;21(2):341-3.
30. Mbiantcha M, Kamanyi A, Teponno RB, Tapondjou AL, Watcho P, Nguelefack TB. Analgesic and Anti-Inflammatory Properties of Extracts from the Bulbils of Dioscorea bulbifera L. var sativa (Dioscoreaceae) in Mice and Rats. Evid Based Complement Alternat Med: eCAM.2011;2011.
31. Muhammad N, Saeed M, Khan H. Antipyretic, analgesic and anti-inflammatory activity of Viola betonicifolia whole plant. BMC Complement Alternat Med 2012;12:59.

CONFLICT OF INTEREST

Authors declared no conflict of interest

GRANT SUPPORT AND FINANCIAL DISCLOSURE

Funded by Higher education commission Islamabad (Grant No:20-1723/R&D/10) for establishment of Pharmacology laboratory at Institute of Basic Medical Sciences, Khyber Medical University Peshawar

AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

NA: Concept & study design, supervision, critical revision, drafting the manuscript, final approval of the version to be published

US: Acquisition analysis and interpretation of data, drafting the manuscript, final approval of the version to be published

SWAS: drafting the manuscript, final approval of the version to be published

MN, Sh, IS: Acquisition, analysis and interpretation of data, final approval of the version to be published

GA: Acquisition of data, critical revision, final approval of the version to be published

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.