European Journal of Medicinal Plants
11(3): 1-7, 2016, Article no.EJMP.21894
ISSN: 2231-0894, NLM ID: 101583475

SCIENCEDOMAIN *international*
www.sciencedomain.org

Suppression of Inflammatory Mediators by the Ethanol Extract of *Crotalaria verrucosa* L. leaf – *in vivo* and *in vitro* Analysis

Kashfia Nawrin[1], Mohammad Mustakim Billah[1], Mohammed Sahek Ullah Jabed[1], Mohammad Ibrahim Khalil[1], Md. Main Uddin[2] and Md. Nurul Islam[1*]

[1]Department of Pharmaceutical Sciences, North South University, Dhaka, Bangladesh.
[2]Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Bangladesh.

Authors' contributions

This work was carried out in collaboration between all authors. Authors KN and MMB performed in vivo experiments, participated in all the tests and prepared the manuscript. Authors MSUJ and MIK collected the plant and performed the in vitro experiments. Author MMU prepared the extract and participated in all the tests. Author MNI designed the methodologies and coordinated the whole project. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2016/21894
Editor(s):
(1) Marcello Iriti, Professor of Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Bhaskar Sharma, Suresh Gyan Vihar University, India.
(2) Wouyo Atakpama, University of Lome, Togo.
Complete Peer review History: http://sciencedomain.org/review-history/12214

Original Research Article

Received 8th September 2015
Accepted 14th October 2015
Published 9th November 2015

ABSTRACT

Objective: The present study demonstrated the potential of *Crotalaria verrucosa* L., a widely used medicinal plant of Bangladesh, ethanolic leaf extract in inhibiting the cascades of inflammation.
Methods: Carrageenan induced rat paw edema and xylene induced mice ear edema tests were performed as *in vivo* assessment of anti-inflammatory activity. Moreover, these methods were supported by the *in vitro* heat induced protein denaturation and haemolysis tests.
Results: In all experiments CVE extract shows moderate to significant efficacy. CVE 600 mg/kg significantly suppressed the biological response (edema) in comparison to both steroidal and non-steroidal anti-inflammatory drugs. Moreover *in vitro* study suggested that the leaf extract at doses

**Corresponding author: E-mail: itsnurulislam@outlook.com;*

of 300, 400 and 500 µg/ml possess moderate to high inhibition capacity for auto antigen production which exerts extract's potential against Type III hypersensitivity.
Conclusion: All these data support the safe use of this plant traditionally. However, the extract is yet to be explored for responsible compounds and specific mechanisms.

Keywords: C. verrucosa; paw edema; ear edema; protein denaturation; haemolysis.

1. INTRODUCTION

The plant *Crotalaria verrucosa* L., well-known as blue rattlesnake and also known as Jhanjhania/ Bansan in Bengali belongs to the family of Fabaceae. It is a woody erect plant with multiple branches growing up to 80-100 cm high in the fallow lands of Chittagong, Khulna, Mymensingh, Rajshahi and Sylhet district of Bangladesh [1]. Its 5-15 cm ovate-rhomboid or ovate-deltoid to obtuse leaves has been reported to be used as juice for scabies and impetigo both internally and externally and also considered effective in diminishing salivation [2]. Literature review suggests the leaf's potential in many pharmacological activities including expectorant, emetic; in biliousness, dyspepsia, fever, cardiac abnormalities and oral diseases. Leaves are also found in use by Chakma and Marma tribes in case of skin allergies and applied in affected areas [3,4]. Leaves contain tropane alkaloids [5]. In Nigeria the leaves are also used for colic, flatulence and skin disease [2]. However, though wound healing activity of the plant leaf has been previously reported, no experimental evaluation of anti-inflammatory activity was undertaken. Therefore, the present study was performed to investigate its anti-inflammatory potential to support the scientific basis of its traditional use.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of the Plant Leaf

Samples of *C. verrucosa* leaves were collected from Mymensingh district of Bangladesh in April, 2014 and authenticated from Bangladesh National Herbarium (DACB 42010). After collection it was thoroughly washed, dried, crushed into powder and finally soaked in ethanol (approx. 500 g in 1 L) for 3 days with occasional shaking. It was filtered and the filtrate was condensed using rotary evaporator. A viscous mass (5 gm approx.) was obtained as ethanol extract of *C. verrucosa* (CVE).

2.2 Reagents and Chemicals

Carrageenan, Xylene, prednisolone, indomethacin and acetyl salicylic acid were obtained from Merck, Germany and were of analytical grade.

2.3 *In vivo* Anti-inflammatory Tests

2.3.1 Institutional ethical committee approval

Institutional ethical committee adopted standard guideline for treating and handling of animal was followed for the *in vivo* tests [6].

2.3.2 Carrageenan induced rat paw edema

The method was performed as described by Winter *et al.* with slight modifications [7,8]. Wister rats weighing 130-150 gm of both sex were taken and divided into six group (N=5); control (water), test drugs (CVE 100 mg/kg, CVE 200 mg/kg and CVE 400 mg/kg body weight) and standards (prednisolone 5 mg/kg and indomethacin 10 mg/kg body weight) were orally administered to the animals 1 hour prior to injection of carrageenan (inflammation inducer) to the right paw whereas left paw served as own control. A base value was recorded before the injection by plethysmometer by dipping the paw in the tube containing 0.7% normal saline. The paw volumes were measured in 1, 2, 3, 4 and 24 hour after injection.

2.3.3 Xylene induced ear edema

Swiss albino mice of both sex weighing 22-27 gm, similarly grouped as above experiment (N=5) were orally treated with test and standard drugs with same doses 1 hour prior the bilateral application of xylene (inflammatory substance) to the right ear of each animal. 15 minutes later animals were sacrificed by dislocating their spinal cord from the neck and a 16 diameter circular area was cut from each ear of the animal. The weight of the inflamed ear was then compared to that of control (left) ear [9,10].

2.4 *In vitro* Anti-inflammatory Tests

2.4.1 Heat induced protein denaturation

Fresh hen's egg albumin was extracted using the method described by S.J. Carter [11]. A reaction mixture containing 0.2 ml of egg albumin, 32.8 ml

of phosphate buffer saline (pH 6.4) and 2 ml of CVE extract at different concentration (100, 200, 300, 400, 500 µg/ml) was used to investigate the inhibition capacity of albumin denaturation. Aspirin at the same doses served as the standard whereas distilled water served as control. The tubes containing the mixture were incubated at 37ºC for 10 min and heated at 70ºC for 5 min. After cooling, the absorbance was measured using UV spectrophotometer at 660 nm [12].

2.4.2 Heat induced haemolysis

Fresh human blood was collected and washed with 0.9% saline and centrifuged at 3000 rpm for 10 minutes. The packed cells were again washed three times with 0.9% normal saline and a 10% v/v HRBC suspension was obtained using isotonic phosphate buffer (154 mM NaCl in 10 mM Sodium Phosphate Buffer at pH 7.4). The final reaction mixture (4 ml) contained 1 ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hyposaline [0.36 %], 0.5 ml HRBC suspension with 0.5 ml of extracts of various concentrations (50, 100, 250, 500, 1000, 2000 µg/ml) or same concentrations of ASA for standard or distilled water for control. It was then incubated at 37ºC for 30 min and centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was taken at 560 nm [13].

2.5 Statistical Analysis

All data were statistically examined for significance of the result in 95%, 99%, and 99.99% confidence level using SPSS.

3. RESULTS

3.1 Carrageenan Induced Rat Paw Edema

Fig. 1 shows the gradual inhibition of the degree of paw inflammation exerted by the crude extract of *C. verrucosa* leaf against the standards where at 600 mg/kg dose for body weight the extract exhibits maximum activity among all its doses. From 3rd to final observation CVE 600, Prednisolone and Indomethacin decreased the edema. Prednisolone though a non-selective steroidal drug, successfully resulted in maximum suppression among all groups. Indomethacin also showed its potential to decrease the accumulations of mediators.

The figure shows the edema status of the inflamed right paw (N=5) of the rat in different time intervals which was induced by carrageenan. Hour 0 represents the base value and all other values were compared to this. All groups were compared to the control groups for the inhibition capacity. The minimum value of $P < 0.05$ was considered significant. *$P < 0.05$, **$P < 0.01$ as compared with control group.

3.2 Xylene Induced Ear Edema

Prednisolone found to be most potent drug in inflammatory pathway of xylene (Fig. 2). However, Indomethacin proves its strong involvement to decrease this inflammation. CVE 600 as like the previous experiment showed its activity to suppress the inflammatory cascades.

The figure shows percentage inhibition of ear edema of different experiment groups where N=5 in each group and all groups were compared with the control group. These data found not to be significant at 95% confidence interval however showed efficacy compared to standards.

3.3 Heat Induced Protein Denaturation

ASA in a dose dependent manner showed to be potent in inhibiting the egg albumin protein denaturation with moderate to significant extent. However, CVE at the dose of 300-500 µg/ml exhibit the same pattern of inhibition (Fig. 3).

The figure shows percentage of inhibition capacity for protein denaturation induced by heat in different concentrations where three samples were assessed for each concentration groups. All groups were compared to the control groups for the inhibition capacity. The minimum value of $P < 0.05$ was considered significant. *$P < 0.05$, ***$P < 0.001$ as compared with control group.

3.4 Heat Induced Haemolysis

Fig. 4 represents the percentage inhibition of haemolysis induced by heat where Aspirin showed a linear relationship with gradient doses. However the extract at last three doses exerts almost similar but moderate efficacy.

The figure shows percentage of inhibition capacity for haemolysis induced by heat in different concentrations where three samples were assessed for each concentration groups. All groups were compared to the control groups for the inhibition capacity. The minimum value of $P < 0.05$ was considered significant. *$P < 0.05$, ***$P < 0.001$ as compared with control group.

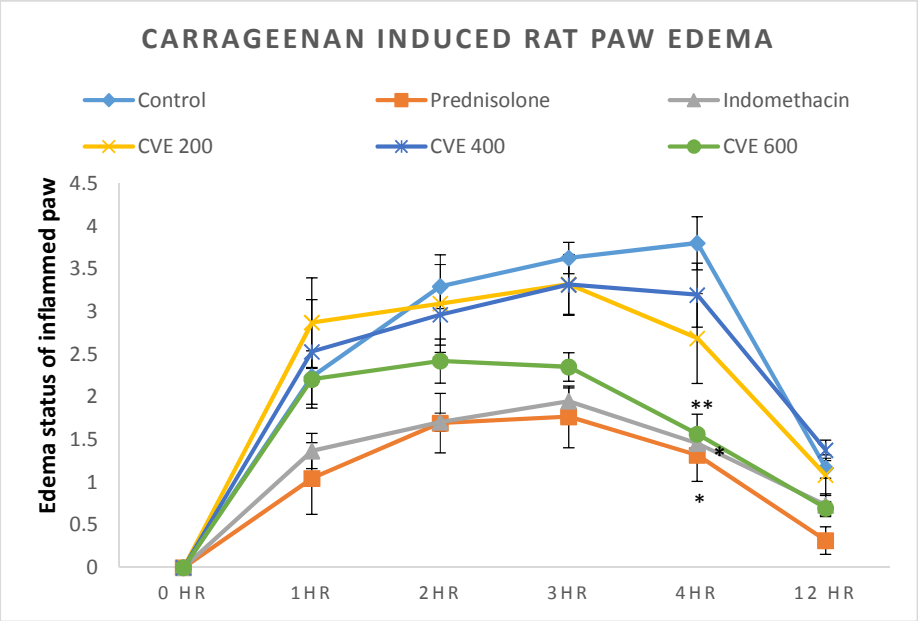

Fig. 1. Edematous condition of carrageenan induced inflammation
*Data are presented as the mean ± SEM (n=5). **P < 0.01, *P < 0.05*

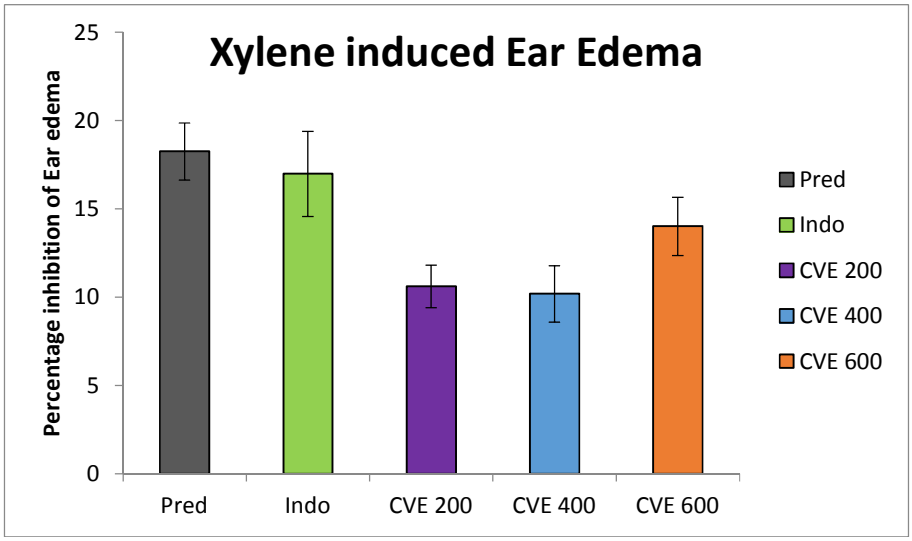

Fig. 2. Percentage inhibition of ear edema induced by xylene
Data are presented as the mean ± SEM (n=5)

4. DISCUSSION

Anti-inflammatory activities belongs to receptor bindings and mediator's involvement in cascades of inflammation. Carrageenan and xylene induced inflammatory methods are well established and widely used for the assessment of any drugs or samples for its anti-inflammatory potentials [14]. The involvement of mediators in inflammatory pathways induced by these two agents are still under investigation. Carrageenan treatment lead two phases of biological response- primarily by releasing inflammatory mediators like histamine, serotonin and kinin (0-2 hr) and later by producing excessive prostaglandins, oxygen-derived free radicals and cyclooxygenase [15]. Xylene-induced inflammation is partially associated with substance P which is widely distributed in the central and peripheral nervous system. The release of this neuromodulator causes vasodilatation and plasma extravasations which

involves in neurogenic inflammation, thus causing the swelling of ear in mice [16]. In both of this processes indomethacin has been reported to have greater involvement in suppression of mediators [17,18]. However, prednisolone was also used to set the maximum efficacy range in case of comparison. Prednisolone found to be highly effective being a steroidal agent [19]. On the other hand Indomethacin is a common non-steroidal anti-inflammatory (NSAID) agent with high efficacy. Now in comparison, *C. verrucosa* crude extract also found to possess potential activity against the mediators involved.

In case of in vitro assessment, heat induced denaturation and haemolysis are also popular methods [20]. Diseases like serum sickness and glomerulo-nephritis are attributed to the expression of antigens formed from the denaturation of proteins and are associated to Type III hypersensitivity reactions [21]. Like native proteins these heat-denatured proteins (egg albumin and haem) provokes delayed hypersensitivity response [22]. Moreover, conventional NSAID like indomethacin and acetyl salicylic acid (aspirin) has been proved to act not only by blocking COX enzyme and synthesis of endogenous PGE2 but also by suppressing the

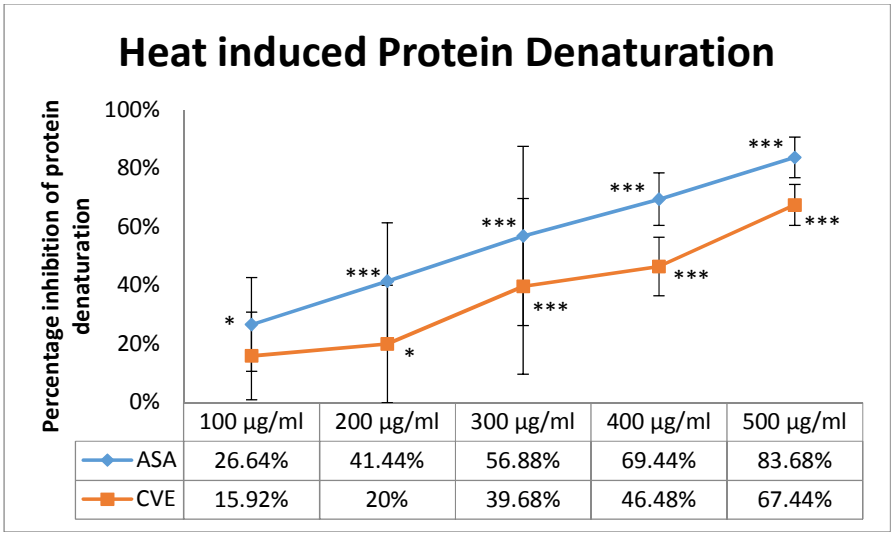

Fig. 3. Percentage inhibition of heat induced protein denaturation
*Data are presented as the percentage ± SEM (n=3 samples). **P < 0.001, *P < 0.05*

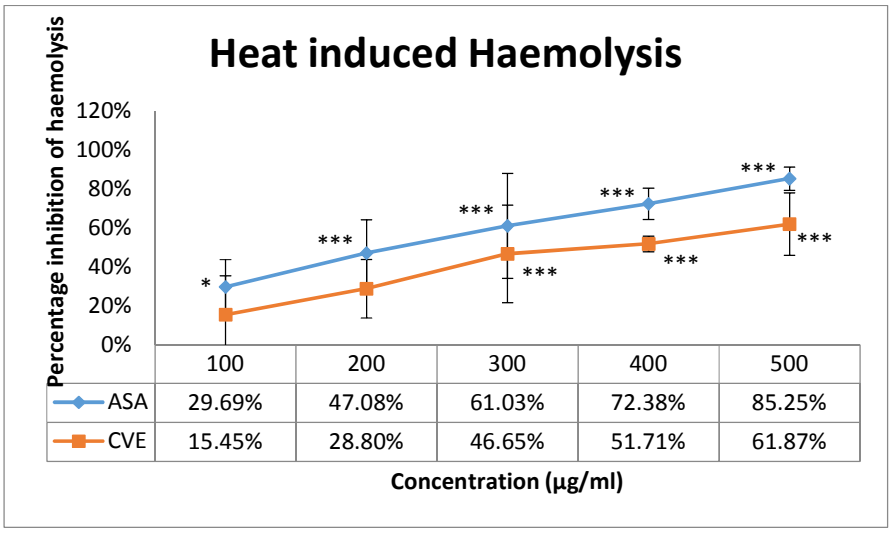

Fig. 4. Percentage inhibition of heat induced haemolysis
*Data are presented as the percentage ± SEM (n=3 samples). **P < 0.001, *P < 0.05*

process of protein denaturation [23]. Therefore these two in vitro experiments were performed to support the results of in vivo experiments. The result of the study demonstrated that the leaf extract of *C. verrucosa* possess considerable anti-inflammatory activity as capable of inhibiting the auto antigen production to significant extent compared with the standard. The leaves has been reported to contain tannins and phenolic content which can be the attributor for these activities.

5. CONCLUSION

The present study of anti-inflammatory activity concludes that the use of the plant leaf of *C. verrucosa* in ailments of inflammation has scientific basis. However, responsible compounds for this activity is yet to be investigated. Chromatographic isolation of the compound and cell line study is to be performed to pinpoint the involved compounds, receptor bindings and further elucidation of the process. This study also concludes the saftey of this plant for use and declares it to be non-toxic in acute use at the doses executed.

CONSENT

The authors themselves served as the volunteers for the donor of fresh samples of HRBC for heat induced haemolysis test.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Md. Salah Uddin. *Crotalaria verrucosa* L.- plant profile: Taxonomic information, phyto-chemical constituents &traditional uses of plants. Ethnobotanical Database of Bangladesh (EDB); 2010. Available:http://www.ethnobotanybd.com/index.php?action=Taxonomy2&key=ill(Accessed 29 March 2015).
2. Kumari M, Eesha BR, Amberkar M, Babu S, Rajshekar, Kumar N. Wound healing activity of aqueous extract of *Crotalaria verrucosa* in Wistar albino rats. Asian Pac J Trop Med. 2010;783-787. DOI: 10.1016/S1995-7645(10)60187-3
3. Rahman MA, Uddin SB, Wilcock CC. Medicinal plants used by Chakma tribe in hill tracts districts of Bangladesh. Indian J Tradit Know. 2007;6(3):508-517.
4. Yusuf M, Begum J, Hoque MN, Chowdhury JU. Medicinal Plants of Bangladesh. Bangladesh Council of Scientific and Industrial Research; 2009.
5. Rastogi Ram P, Mehrotra BN. Compendium of Indian medicinal plants. Central Drug Research Institute, Lucknow and Publicatios & Information Directorate, New Delhi, India. 1993;2.
6. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain. 1983;16:109–10. PMID: 6877845.
7. Winter CA, Risley EA, Nuss GW. Carrageenan-induced edema in the hind paw of rat as an assay for anti-inflammatory activity. Proc Soc Exp Biol Med. 1962;111:544–547.
8. Adeyemi OO, Okpo SO, Ogunti OO. Analgesic and anti-inflammatory effects of the aqueous extract of leaves of *Persea americana* Mill (Lauraceae). Fitoterapia. 2002;73(5):375–380.
9. Kou J, Ni Y, Li N, Wang J, Liu L, Jiang ZH. Analgesic and anti-inflammatory activities of total extract and individual fractions of Chinese medicinal ants *Polyrhachis lamellidens*. Biol Pharm Bull. 2005;28(1): 176–180.
10. Li Y-C, Xian Y-F, Ip S-P, Su ZR, Su JY, He JJ, et al. Anti-inflammatory activity of patchouli alcohol isolated from *Pogostemonis herba* in animal models. Fitoterapia. 2011;82(8):1295-1301. DOI: 10.1016/j.fitote.2011.09.003
11. Carter SJ. Blood and related products. Part 3, Chapter 34, 6th ed. Pitman, London: Cooper and Gunn's Tutorial Pharmacy; 2000.
12. Ullah HMA, Zaman S, Juhara F, Akter L, Tareq SM, Masum EH, et al. Evaluation of antinociceptive, *in-vivo* & *in-vitro* anti-inflammatory activity of ethanolic extract of *Curcuma zedoaria* rhizome. BMC Complement Altern Med. 2014,14:346. DOI: 10.1186/1472-6882-14-346
13. Anosike CA, Obidoa O, Ezeanyika LUS. Membrane stabilization as a mechanism of the anti-inflammatory activity of methanol extract of garden egg (*Solanum aethiopicum*). DARU J Pharm Sci. 2012; 20:76. DOI: 10.1186/2008-2231-20-76

14. Hajhashemi V, Sadeghi H, Minaiyan M, Movahedian A, Talebi A. Effect of fluvoxamine on carrageenan-induced paw edema in rats evaluation of the action sites. Iran J Pharm Res. 2011;10(3):611–8.
15. Ashok P, Koti BC, Thippeswamy AHM, Tikare VP, Dabadi P, Viswanathaswamy AHM. Evaluation of antiinflammatory activity of *Centratherum anthelminticum* (L) Kuntze Seed. Indian J Pharm Sci. 2010; 72(6):697–703.
DOI: 10.4103/0250-474X.84577
16. Agbaje EO, Fageyinbo MS. Evaluating Anti-Inflammatory activity of aqueous root extract of *Strophanthus hispidus* DC. (Apocynaceae). Int. J. Appl. Res. Nat. Prod. 2012;4(4):7-14.
17. Amann R, Schuligoi R. Inhibition of carrageenan-induced edema by indomethacin or sodium salicylate does not prevent the increase of nerve growth factor in the rat hind paw. Neurosci Lett. 2000;278(3):173–176.
DOI: 10.1016/S0304-3940(99)00931-3
18. Gamache DA, Ellis EF. Effect of dexamethasone, indomethacin, ibuprofen, and probenecid on carrageenan-induced brain inflammation. J Neurosurg. 1986; 65(5):686-92.
19. Okoye FB, Osadebe PO, Proksch P, Edrada-Ebel RA, Nworu CS, Esimone CO. Anti-inflammatory and membrane-stabilizing stigmastane steroids from *Alchornea floribunda* leaves. Planta Med. 2010;76(2):172-7.
DOI: 10.1055/s-0029-1186032
20. Das B, Choudhury MD, Dey A, Talukdar AD, Nongalleima K, Deb L. Antioxidant and anti-Inflammatory activity of aqueous and methanolic extracts of rhizome part of *Drynaria quercifolia* (l.) J. Smith. Int J Pharm Pharm Sci. 2014;6(6):43-49.
21. Ahmad F, Khan RA, Rasheed S. Study of analgesic and anti-inflammatory activity from plant extracts of *Lactuca scariola* and *Artemisia absinthium*. J Islamia Acad Sci. 1992;5:111–114.
22. Gell PGH, Benacerraf B. Studies on hypersensitivity-II delayed hypersensitivity to denatured proteins in guinea pig. Immunology. 1959;2:64.
23. Insel PA: Analgesic-antipyretics and antiinflammatory agents: Drugs employed in the treatment of rheumatoid arthritis and gout. In goodman and Gilman's the pharmacological basis of therapeutics. Edited by Gilman AG, Rall T, Nies A, Taylor P. Pergamon, NY. 1990:638–681.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/12214