ISSN 0975-6299

Vol 2/Issue 2/Apr-Jun 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

PHARMACOLOGY

PHARMACOLOGICAL SCREENING OF COMBINED EXTRACT OF *ANNONA* SQUAMOSA AND NIGELLA SATIVA

F V MANVI,B K NANJAWADE AND SANJIV SHING*

K. L. E. S's College of Pharmacy, Belgaum-590 010, India

SANJIV SINGH
K. L. E. S's College of Pharmacy, Belgaum-590 010, India

ABSTRACT

Anti-arthritic, anti-inflammatory and analgesic activity of combined extract of *Annona squamosa* and *Nigella sativa* was evaluated and validated in various animal models. Arthritis was induced by Complete Freund`s Adjuvant (CFA) injection in metatarsal footpad of Sprague-Dawley rats. Degree of inflammation was evaluated by hind paw swelling and body weight, estimation of AST, ALT and TP supported by histopathology of knee joint. Combined extract reduced hind paw swelling, body weight, AST, ALT and TP Histopathology revealed significant reduction in mononuclear infiltration, pannus formation and bone erosion. Combined extract decreased the paw volume in carageenan treated rats. Combined extract (PHF) shows moderate central and peripheral analgesic activities in hot plate method and acetic acid induced writhing method in mice.

ISSN 0975-6299 Vol 2/Issue 2/Apr-Jun 2011

KEYWORDS

Anti-Arthritic, Anti-Inflammatory, Analgesic Activity, Combined extract.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic, destructive inflammatory polyarticular joint and systemic autoimmune disease of unknown cause[1]. The prevalence of RA is consistent worldwide affecting, about 0.5-1.0% of the population. It usually occurs in people between 25 and 55 year of age. Women are affected more often than men at ratio of 3 to 1[2]. It is characterized by synovial hyperplasia, angiogenesis and mononuclear infiltration. RA progresses in three stages. The first stage is the swelling of the synovial lining, causing pain, warmth, stiffness, redness and swelling around the joint. Second is the rapid division and growth of cells, or pannus, which causes the synovium to thicken. In the third stage, the inflamed cells release enzymes that may digest bone and cartilage, often causing the involved joint to lose its shape and alignment, more pain and loss of movement[3]. As the result of the inherent problems associated with current non-steroidal as well as steroidal anti-inflammatory agents, there is a continuous search especially from natural source. Recently there is a greater global interest in non synthetic, natural drugs derived from plant/herbal sources due to better tolerance and minimum adverse drug reactios[4]. Herbal drugs used in Indian system of medicines are however claimed to be effective and safe for treatment of inflammations. Plant medicines are more often used in combination rather than in a single in order to get maximum benefit from their combiner strength[5]. Annona squamosa[6] (Annonaceae) and Nigella sativa[7] (Ranunculaceae) are the medicinal plants used for centuries in the Ayurvedic system of medicine. The anti-inflammatory activity of both of constituents of polyherbal extract, Nigella sativa[8, 9] and Annona squamosa[10, 11] has been reported in scientific literature; hence the present study was undertaken to evaluate and to validate the anti-arthritic, anti-inflammatory and analgesic activity of combination of both herbs *Annona squamosa* and *Nigella sativa* in form of Combined extract (PHF).

MATERIAL AND METHODS

Animals: SD rats weighing 140 ± 10 g of either sex and Swiss albino mice of either sex weighing 22 ± 3 g, procured from Venkateshwara Enterprises, Bangalore were used in this study. The animals were procured at least 2 weeks prior to the study and maintained in institutional animal house (registration no. 29/CPCSEA), so, that animals could adapt to the new environment. The Institutional Animal Ethics Committee`s permission was obtained before starting the experiments on animals. The studies were conducted from 2007 to 2008.

***Preparation of drug*:** The seeds of *Nigella sativa* (Kalonji) obtained from Prgati Ayurvedic Drug store Belgaum and matured fruit of *Annona squamosa* (Sharifa) from local market of Belgaum and they were authenticated from Botanical Survey of India, Pune (Maharastra). The extracts of the both antidiabetic plants in 1:1 ratio were mixed and polyherbal extract was prepared. Five hundred grams of each plant (chopped into small pieces) was extracted individually and were soaked overnight in 1 l of water. This extract was filtered and the filtrates were pooled and the solvents were evaporated in a rotavapor at 40–50 ^{0}C under reduced pressure[12] then the extract was mixed with 0.5% w/v Carboxy Methyl cellulose (SCMC) to get 1 mg mL^{-1} of

Polyherbal suspension (PHF). The suspension was freshly prepared before use.

***Chemicals*:** ALT, AST and TP kit from RMS Ltd., Baddi, Freund`s adjuvant emulsion from Difco Lab, USA, Pethidine Sulphate from AstraZenica, Bangalore, Carageenan from Sigma Labs and all other chemicals, reagents used were of analytical grade.

Grouping and treatment of experimental animals: For Adjuvant induced arthritis model, Female Sprague-Dawley rats weighing 130-150 g were divided into five groups of six animals each. Control (Group 1) animals were administered the vehicle, Group 2 (Arthritic control) animals were administered the vehicle and CFA 0.1 mL to sub planter region of hind paw, Group 3 and Group 4 animals were administered the combined extract 270 mg kg^{-1}, 540 mg kg^{-1} and reference standard Indomethacin, 5 mg kg^{-1} p.o., respectively[13].

For Carageenan induced hind paw edema Albino Wistar rats weighing between 150-200 g were divided into four groups of six animals each; Control (group 1) animals were administered saline, Group 2 animals were administered Indomethacin (10 mg kg^{-1}), Group 3 animals were administered the combined extract lower dose, 270 mg kg^{-1}, Group 4 animals were administered the combined extract higher dose, 540 mg kg^{-1}.

For Eddy`s hot plate Swiss albino mice of either sex were divided into four groups of six animals each. Control (Group 1) animals were administered the vehicle, Group 2-4 animals were administered the combined extract 400 mg kg^{-1}, 800 mg kg^{-1} and Pethidine sulfate 5 mg kg^{-1} i.p., respectively.

For acetic acid induced writhing, Swiss albino mice of either sex were divided into four groups of six animals each. Control (Group 1) animals were administered the vehicle, Group 2-4 animals were administered the PHF 400 mg kg^{-1}, 800 mg kg^{-1} and indomethacin 10 mg kg^{-1} p.o., respectively.

EXPERIMENTAL

Adjuvant induced arthritis model: Arthritis was induced by a 0.1 mL injection of complete Freund`s adjuvant emulsion (CFA) into the sub-planter surface of right hind paw[14]. Drugs was administered orally once a day, from the day of injection of CFA and continued up to 14th post CFA challenge day. The change in the inflammatory reaction was measured using mercury plethysmograph on 0, 4, 7, 14 and 21 day from the day of CFA injection. The animals were weighed, using digital weighing balance, on 0, 4, 7, 14 and 21day from the day of CFA injection[15] at the end of 21st day rats were anaesthetized with diethyl ether. Blood was withdrawn by puncture of retro orbital plexus, centrifuged (Remi) and serum ALT, AST and TP estimated[16].

Histopathological assessment: After euthanasia on day 21st, the hind paws amputated above the knee joint and were fixed in 7.4% formalin solution. The paws were then decalcified using 10% Nitric acid embedded in paraffin and sectioned in a mid-sagittal plane. The sections of articulation of the tarsal joints were stained with heamtoxylin and eosin and were examined microscopically for mononuclear infiltration, pannus formation and bone destruction[17, 18].

Carageenan induced hind paw edema in rats: Rats of all the groups were injected 0.1 mL of carageenan (1%) in normal saline into sub planter area of right hind paw. The drugs were given orally 1 h prior to carageenan injection. Paw volume was measured by mercury plethysmograph at 0, 3, 6, 12 and 24 h after the carageenan injection[19].

***Eddy`s hot plate method*:** The time for licking paws or jumping in hot plate was recorded as response, prior and 30, 60, 120 and 150 min after administration of combined extracr /reference standard drug[20].

***Acetic acid induced writhing test in mice*:** Writhing was induced 30 min after the last dose by intraperitoneal injection of 10 mL kg^{-1} of 0.6% acetic acid in distilled water. The number of writhes was counted for 30 min immediately after the acetic acid injection[21]. The percentage inhibition of abdominal constrictions between control animals and poly herbal formulation treated animals using the ratio was calculated using formula:

$$\text{Inhibition (\%)} = \frac{\text{Control mean-Treated mean}}{\text{Control mean}} \times 100$$

Statistical analysis: All values were reported as Mean ± SEM. Results were analyzed using One way ANOVA, followed by Dunnet`s/Tukey`s test, $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

As shown in Table 1, CFA treatment caused increase in the paw volume and decrease in the body weight. CFA elevates the levels of ALT, AST and TP. After treatment with combined extract there is a significant decrease in paw volume, increase body weight and reduction in elevated levels of ALT, AST and TP. Table 2. Histopathology of knee joint of CFA treated rat, reveals enhanced neutrophil infiltration, pannus formation and bone erosion, whereas in combined extract treated rats, there is significant reduction in neutrophils infiltration, pannus formation and bone.

Table 1

Effect of combined extract on paw volume and body weight in CFA induced arthritis in rats

Treatments	Paw volume(mL)					Body weight (g)				
	0 day	4th	7th	14th	21st	0 day	4th	7th	14th	21st
Control	0.124±0.002	0.106±0.002	0.106±0.002	0.106±0.002	0.106±0.002	173.3±2.47	178.5±2.32	185.0±2.295	189.6±1.909	194.6±2.028
Arthritic control	0.123±0.003	0.181±0.003*	0.183±0.002*	0.210±0.003*	0.226±0.003*	150.0±4.47	154.3±4.63	158.5±4.610	161.3±4.210	164.5±4.250
PHF 540 mg kg^{-1} p.o	0.120±0.007	0.156±0.007**	0.180±0.006	0.178±0.006**,*	0.173±0.006**,*	141.3±9.09	149.1±9.25	153.0±9.160	158.3±9.180	162.5±9.630
PHF 270 mg kg^{-1} p.o.	0.116±0.004	0.178±0.003	0.176±0.004	0.180±0.003**,*	0.195±0.004**	175.0±2.88	179.5±2.97	184.0±3.120	185.3±2.950	186.0±3.180
Indom.5 mg kg^{-1} p.o.	0.121±0.004	0.180±0.002	0.183±0.004	0.168±0.003**,*	0.165±0.002**,*	175.0±2.88	181.8±2.75	188.6±2.670	191.8±2.760	195.5±2.930

*Values are expressed as (Mean ± SEM) N = 6; One way ANOVA followed by Tukey`s multiple comparison test, *p<0.001 vs control, **p<0.01 vs. arthritic control ***p<0.001 vs arthritic control*

Table 2
Effect of combined extract on lysosomal enzyme and total protein

Treatments	ALT(IU)	AST(IU)	TP(g dL^{-1})
Control	34.910±0.7720	104.81±3.031	12.81±0.083
Arthritic control	49.850±6.0620*	145.33±11.45*	13.70±0.186
Standard	21.016±0.738***	65.41±2.460***	9.78±0.546***
PHF 270 mg kg^{-1}	29.750±0.4039**	93.30±1.018***	11.90±0.143**
PH 540 mg kg^{-1}	26.210±0.5256***	76.13±1.860***	10.22±0.336***

*Values are expressed as (Mean ± SEM) N = 6; One way ANOVA followed by Dunnet`s test, p<0.001vs control, **p<0.01, ***p<0.001 vs. arthritic control*

Combined extract significantly inhibited the paw edema in a dose dependent manner as shown in Fig. 1. Combined extract shows analgesic effect in dose dependent manner and the results are comparable with the reference standard drugs, pethidine sulfate and indomethacin, respectively (Fig. 2,3).

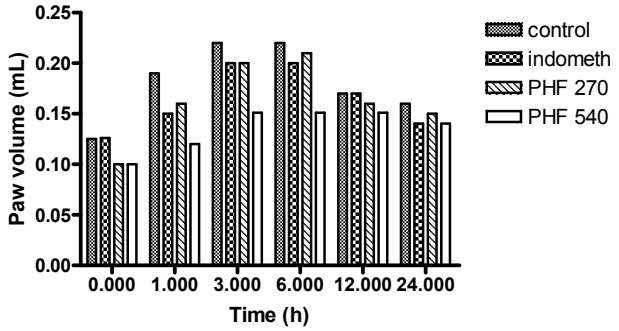

Fig. 1
Effect of combined extract on paw volume in carrageen induced paw edema in rats

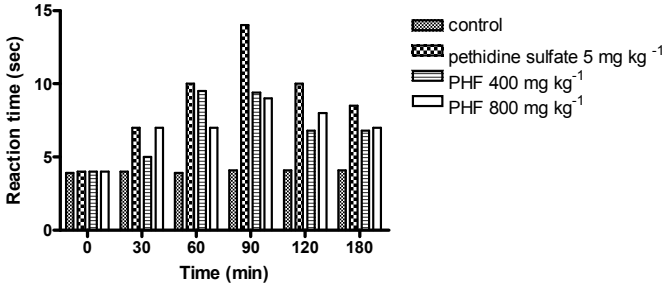

Fig. 2
Effect of combined extract on reaction time (sec) in Eddy`s hot plate

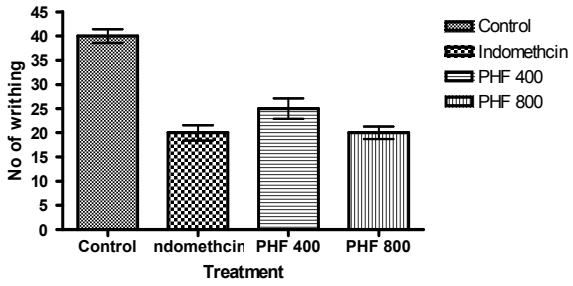

Fig. 3

Peripheral analgesic effect of combined extract in acetic acid induced writhing in mice

RA is a chronic, cytokine-mediated destructive inflammatory polyarticular joint disease, characterized by massive synovial proliferation, systemic and local inflammation resulting in cartilage and bone destruction. Adjuvant Arthritis (AA) in rat mimics many of the clinical and pathological features of human RA, such as paw swelling, joint erosions and ankylosis and it is the most commonly used animal models for RA[22].

In the present study, we used AA rats to demonstrate the inhibiting effects of a combined extract on RA. The *in vivo* experiments confirmed that combined extract (270 and 540 mg kg^{-1}, orally) significantly reduced paw volume and increased the body weight in AA rats. The inhibition of the increase in hind paw volume may be associated with inhibition of neutrophil infiltration, Pannus formation and bone erosion[23]. It is supported by histological studies of knee joints. Fig. 4 is TS of knee joint of control rat. Severe neutrophil infiltration, Pannus formation and bone erosion is seen in knee joint of Arthritic control rat as shown in Fig. 5. On treatment with combined extract 270 mg kg^{-1} there is slight reduction in the neutrophil infiltration, Pannus formation and bone erosion but combined extract 540 mg kg^{-1} showed significant reduction neutrophil infiltration, Pannus formation and bone erosion, which is comparable with reference standard drug as shown in Fig. 6-8, respectively[24]. combined extract in AA model, combined extract decreased the elevated level of lysosomal enzymes which may be due to inhibition of either release or by stabilizing lysosomal enzymes and Cytokines which play key role in the development of inflammation[25].

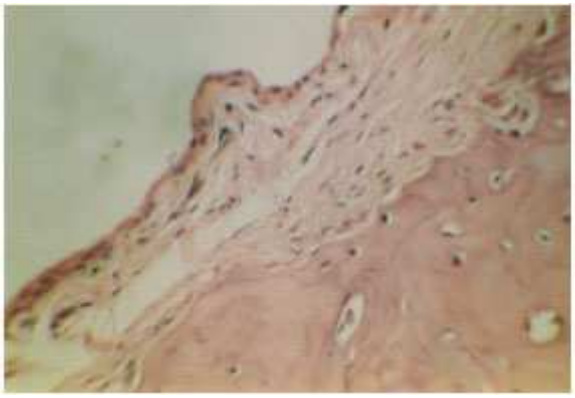

Fig. 4
TS of knee joint of control rat

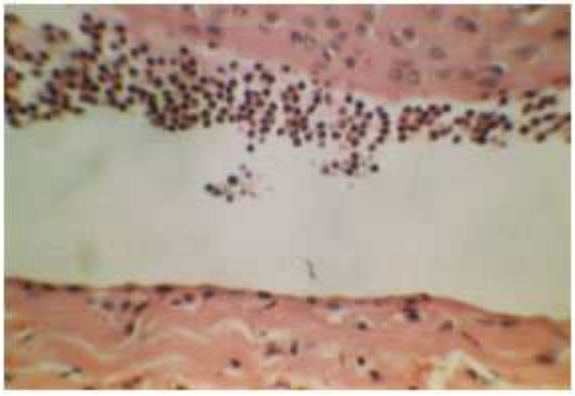

Fig. 5
TS of knee joint of Arthritic rat showing Mononuclear infiltration, bone erosion

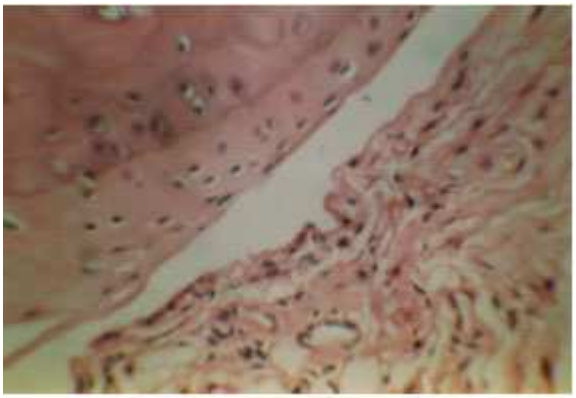

Fig. 6
TS of knee joint of indomethacin treated rat

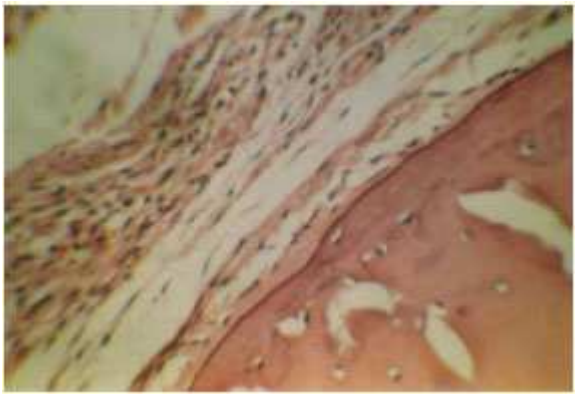

Fig. 7
TS of Knee joint of combined extract 270 mg kg^{-1} treated rat

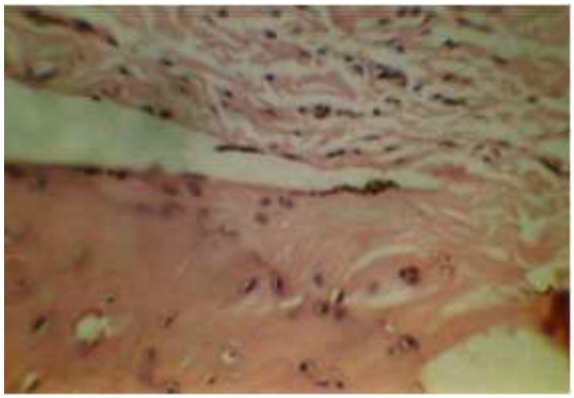

Fig. 8
TS of Knee joint of combined extract 540 mg kg^{-1} treated rat showing reduced mononuclear infiltration and bone erosion

The development of edema in the paw of the rat after injection of carageenan is a biphasic event. The initial phase of the edema has been attributed to the release of histamine and serotonin, the edema maintained during the plateau phase to kinin like substances and the second accelerating phase of swelling to the release of prostaglandin like substances. Inhibition of edema observed in various inflammatory models induced experimentally in the present study may, therefore be attributed to the ability of the combined extract to inhibit various chemical mediators of inflammation like histamine and 5-HT during the initial phase[26].

In the present study, combined extract significantly increased the reaction time in hot-plate est suggesting its central analgesic activity; the probable mechanism could be by inhibition of prostaglandin synthesis. Prostaglandins play significant role in different phases of inflammatory reactions and elicit pain by direct stimulation of sensory nerve endings and also sensitize sensory nerve endings to other pain provoking stimuli. Further studies are required on both ingredients of Combined extract to explore the mechanism of anti-inflammatory activity. The peripheral analgesic activity of combined extract against acute inflammatory pain was good as compared to potent inhibitory activity of indomethacin. Aspirin and Indomethacin often give relief from inflammatory pain by suppressing the formation of pain substances in the peripheral tissues, where prostaglandins and bradykinin were suggested to play an important role in the pain process[27]. Therefore, it is likely that

combined extract might suppress the formation of these substances and they exert its analgesic activity in acetic acid-induced writhing test. Further studies are required on each ingredients of combined extract to explore the mechanism of analgesic activity.

CONCLUSION

Combined extract of *Annona squamosa* and *Nigella sativa*, possesses anti-arthritic, anti-inflammatory and analgesic property.

ACKNOWLEDGEMENTS

Authors are thankful to KLESs University College of Pharmacy, Belgaum for providing necessary facilities to conduct the experimental work.

REFERENCES

1- Hong-MeiXu, W. Wei, J. Xiao-Yi, Y. Chang and L. Zhang, Effect and mechanisms of total glucosides of paeony on adjuvant arthritis in rats. J. Ethnopharmacol., 109: 442-448, (2007).
2- Maya, B., M.R.C.P. and P.Emery, The aetiology and pathogenesis of rheumatoid arthritis. Hospital Pharmacist., 9: 5-10, (2002).
3- Atsushi, O., Y. Kawahito, I. Prudovsky, Y. Tubouchi and M. Kimuara et al., Copper chelation with tetrathiomolybdate suppresses adjuvant-induced arthritis and inflammation-associated cachexia in rats. Arthritis Res. THer., 7: 1174-1182 (2005).
4- Kimmatkar N, Thawani V, Hingorani L, Khiyani R. Efficiency and tolerability of *Boswellia serrata* extract in treatment of oesteo arthritis of knee: A randomized double blind plaCcebo controlled trial. Phytomedicine; 10:3-7, (2003).
5- Nadeem M, Dandiya PC, Pasha KV, Imran M, Balani DK, Vohora SB., Hepatoprotective activity of some herbal formulations available in India. Indian Drugs; 33:390-6, (1996).
6- C. Ramankutty, R. Vasudevan Nair, An Indian Medicinal Plant: a compendium of 500 species: Orient Black Swan, 1: 160-163, (1996).
7- Nadkarni AK., ***Indian materia medica***. 3rd ed. Mumbai: Popular Prakashan Pvt. Ltd; pp. 301–40, (1976).
8- Al Chandi, MS, The anti-inflammatory, analgesic and antipyretic activity of Nigella sativa. J. of Ethnopharmacol., V. -76, Issue 1, June, 45-48, (2001).
9- Chehl N, Chipitsyna G, Gong Q, Yeo C J, Arafat H A, Anti-inflammatory effects of the *Nigella sativa* seed extract, thymoquinone, in pancreatic cancer cells. International Hepato-Pancreato-Biliary Association, 11(5): 373–381, (2009).
10- Y Shang-Hsin, C. Fang-Rong, WU Yang-Chang, Y. Yu-Liang, Z. Shi-Kai, H. Tsong-Long, An anti-inflammatory ent-kaurane from the stems of Annona squamosa that inhibits various human neutrophil functions. Planta medica. 71: 10, 904-909, (2005).
11- Saluja, Ak., Santani DD., Pharmacological screening of ethanol extract of defatted seeds of Annona squamosa.

Pharmaceutical Bio, 32, 2: 154-162, (1994).
12- Pandey, V.N., Rajagopalan, S.S., Chowdhorry, D.P., An effective ayurvedic hypoglycemic formulation. J. Res. Ayur. Siddha. 16, 1–14, (1995).
13- Chamundeeswari, D., J. Vasantha, S. Gopalakrishanan and E. Sukumar, Effect of the alcoholic extract of *Trewia polycarpa* roots on Cathepsin D in arthritic rats. Ind. J. Pharm. Edu. Res., 89:51-53, (2005).
14- Mi-Jung, Y., L. Han-Chang, K. Gun-Ho, L. Hye-Jung and S. Insop et al., Anti-arrthritic effect of *ephedra sinica* stapf herb acupuncture: Inhibition of lipopolysaccharide-induced inflammation and adjuvant-induced polyarrthritis. J. Pharmacol. Sci., 100: 41-50, (2006).
15- Abdul-shakoor, B., S.K. Tandan, D. Kumar, V. Krishna and V.R. Prakash, Interaction between inhibitors of inducible nitric oxide synthase and cyclooxygenase in adjuvant-induced arthritis in female albino rat: An isobolographic study. Eur. J. Pharmacol., 556: 190-199, (2007).
16- Colin, G.E., J.C. Lockhart and W.R. Ferrell, Pathophysioogy of vascular dysfunction in a rat model of chronic joint inflammation. J. Physiol., 557: 635-643, (2004).
17- Choi, J., B.J. Yoon, Y.N. Han, K.T. Lee and J. Ha *et al.,* Antirheumatoid arthritis effect of Rhus verniciflua and of the active component, sulfuretin. Planta-Med., 69:899-904, (2003).
18- Michele, M.B., E. Roffe, C.M. Yokoro, W.L. Tafuri and D.G. Souza et al., Anti-inflammatory and analgesic effects of atorvastin in a rat model of adjuvant-induced arthritis. Eur. J. Pharmacol., 516: 282-289, (2005).
19- Olumayokun, A., J. Olajide, M. Makinde and O. Awe, Effects of the aqueous extract of *Bridelia ferrugined* stem bark on carageenan-induced oedema and granuloma tissue formation in rats and mice. Olumaykun, 66:113-117, (1999).
20- Somchit, M.N.., M.R. Sulaiman, A. Zuraini, L. Samsuddin and N. Somchit et al., Antinociceptive and anti-inflammatory effects of *centella asiatica*. Indian. J. Pharmacol., 36: 377-380, (2004).
21- Nivsarkar, M.M. Mukhaerjee, M. Patel, H. Padh and C. Babu, *Launaea nudicaulis* leaf juice exhibits anti-inflammatory action in acute and chronic inflammation models in rats. Indian Drugs, 39: 290-292, (2002).
22- Hong-MeiXu, W. Wei, J. Xiao-Yi, Y. Chang and L. Zhang, Effect and mechanisms of total glucosides of paeony on adjuvant arthritis in rats. J. Ethnopharmacol., 109: 442-448, (2007).
23- Abdul-shakoor, B., S.K. Tandan, D. Kumar, V. Krishna and V.R. Prakash, Interaction between inhibitors of inducible nitric oxide synthase and cyclooxygenase in adjuvant-induced arthritis in female albino rat: An isobolographic study. Eur. J. Pharmacol., 556: 190-199, (2007).
24- Michele, M.B., E. Roffe, C.M. Yokoro, W.L. Tafuri and D.G. Souza et al., Anti-inflammatory and analgesic effects of atorvastin in a rat model of adjuvant-induced arthritis. Eur. J. Pharmacol., 516: 282-289, (2005).
25- Vijayalakshmi, T., V. Muthulakshmi and P. Sachdanandam, Effect of milk extract of *Semecarpus anacardium* nuts on glycohydrolase and lysosomal stability in adjuvant stability in adjuvant arthritis in rat. J. Ethnopharmacol., 58: 1-8, (1997).
26- Harsh, M., A Text Book of pathology. 4th edn., Jaypee Brother Publishers, New Delhi, ISBN: 81-8061-368-2, (2000).
27- Hajare, S.W., C. Suresh, S.K. Tandan, J. Sarma, J.L.La and A.G. Telang, Analgesic and antihyperetic activities of *Dalbergia sissoo* leaves. Indian J. Pharmacol., 32:357-360, (2000).