Advances in Chemistry and Biochemistry Sciences
Journal homepage: www.ajournals.com/journals/acbs

Volume 3 | Issue 1 | Pages 01-09
ISSN: 2423-6748

Research Article Open Access

Preliminary phytochemical and *in-vitro* pharmacological evaluation of whole plant extracts of *Ziziphus nummularia* Burn. f.

Manjusha Choudhary [1]*, Ojashvi Sharma [1], Nitesh Choudhary [2], Vikaas Budhwaar [3], Vineet Mehta [1]

[1] *Institute of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra-136118, Haryana, India.*
[2] *R. P. Inderprastha Institute of Technology, Karnal-132001, Haryana, India.*
[3] *Department of Pharmaceutical Sciences, Maharishi Dyanand University, Rohtak-124001, Haryana, India.*

ARTICLE INFO

Article history:
Received: August 07, 2015
Revised: October 10, 2015
Accepted: October 21, 2015
Available online October 26, 2015

Keywords:
Ziziphus nummularia
Immunostimulating
Neutrophils
Chemotaxis
Anti-inflammatory

* Corresponding Author;
E. Mail:
manjushachoudhary@gmail.com

ABSTRACT

The aim of the present study is to evaluate the *in-vitro* pharmacological activities of various fractions of hydroalcoholic extracts of *Ziziphus nummularia* Burn. f. The anti-inflammatory activity was performed by heat induced haemolysis, membrane stabilization and protein denaturation inhibition assay. Diclofenac sodium was used as standard drug. The immunomodulatory activity was carried out on human neutrophils by assessing nitrobluetetrazolium test, neutrophil locomotion and chemotaxis. All the extracts were tested at concentrations viz. 100, 250, 500 and 1000 µg/ml. The various extracts showed significant activity on *in vitro* human neutrophils and protein denaturation which are comparable to standard and control at different concentration indicating the possible immune-stimulating, anti-inflammatory and antimicrobial effect.

1. Introduction

Immunomodulation is the regulation and modulation of immune system either by enhancing or by reducing the immune response. Modulation of immune response may involve induction, expression or amplification of immune responses. An immunomodulator is a biological or non-biological substance that directly influences a specific immune function or modifies one or more components of the immunomodulatory network to achieve an indirect effect on a specific immune function (Dashputre and Naikwade, 2010). This dysfunction is responsible for various diseases like arthritis, ulcerative colitis, asthma, allergy, parasitic diseases, cancer and infectious diseases (Nicoara and Crisan, 2003). The suppression of the immune system is characterized by reduction in the

Cite this article as: Choudhary, M., Sharma, O., Choudhary, N., Budhwaar, V., Mehta, V., 2016. Preliminary phytochemical and in-vitro pharmacological evaluation of whole plant extracts of *Ziziphus nummularia* Burn.f. Advances in Chemistry and Biochemistry Sciences 03 (01), 01-09.

number and phagocytic function of the neutrophils and macrophages, as well as an impairment of the intracellular bactericidal capacity of these cells (Patwardhan et al., 1990). This problem can be overcome by boosting the immune system by the use of immunomodulatory drugs (Rao et al., 1994). Inflammation is a complex process, which is normally associated with pain and involves occurrences such as the enhancement of vascular permeability, increase of protein denaturation and membrane alteration. When cells in the body are injured by microbes, physical agents or chemical agents it causes inflammation of the cells in response towards stress. It is a defensive response which is characterized by redness, pain, heat, and swelling and loss of function in the injured area (Tortora, 1993). Lysosomal enzymes released during inflammation produce a variety of disorders which leads to the tissue injury by damaging the macromolecules and lipid peroxidation of membranes which are assumed to be responsible for certain pathological conditions such as heart attacks, septic shocks and rheumatoid arthritis etc. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. Widespread use of antibiotics has promoted the emergence of resistant pathogens with multidrug resistant strains and unresponsive infectious disease against patients worldwide. Bacteria in general have the genetic susceptibility to heritage and acquire resistance to drugs, which have been used as therapeutic agents. One way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based upon existing synthetic drugs (Murphy, 1999).

Due to risk of adverse effects encountered with the use of synthetic drugs, medicinal plants may offer an alternative source for antimicrobial agent and immunomodulators with significant activity against pathogenic, infective microorganisms and decrease the immunosuppression. It is believed that current drugs available like opioids NSAIDS are not useful in all the cases of inflammatory treatments as it can cause side effects (Berahou et al., 2007). The literature survey indicates that the above mentioned activities of plant are not much explored due to its small size and wildly grown habitat. The aim of the study is to evaluate the *in-vitro* antimicrobial, anti-inflammatory and immunomodulatory activities.

2. Materials and Methods

2.1. Plant procurement and authentification

Ziziphusnummularia known as wild jujube in English was collected in the month of November, 2014 from the campus of Kurukshetra University, Kurukshetra, Haryana, India. Sample of leaves were authenticated by Dr.B.D.Vashistha, Professor, Department of Botany, Kurukshetra University, Kurukshetra, Haryana, India. Plant sample has been kept in the herbarium of Department of Botany, Kurukshetra University for future reference.

2.2. Extraction

Parts of the plant like roots, leaves, stem and fruits of *Ziziphus nummularia* were washed and dried under shade at room temperature for around two weeks. The dried parts of the plant were ground into coarse powder using pestle and mortar and stored in air tight container. 650 g of the coarse powder of plant was extracted with hydro-alcohol (95%) (30:70) by using Soxhlet extraction at temperature of 70°C for around 96 hours. The solvent was removed at 40-50°C under reduced pressure. The concentrated extract was weighed and percentage yield was calculated. The dried hydro-alcoholic extract so obtained was stored in an air tight container at 4-8°C for further investigations.

2.3. Fractionation of the plant extract with solvents

The dried hydroalcoholic extract was suspended in distilled water and successively partitioned in n-hexane, ethyl acetate and ethanol in separating

funnel. Each part was filtered and dried in rotary evaporator.

2.4. Preliminary phytochemical screening

The various extracts were subjected to different qualitative chemical tests to detect the presence of different phytoconstituents (Khandelwal, 2004; Kokate, 1994).

2.5. Anti-microbial activity

Antimicrobial activity of various extracts were tested using Agar well diffusion methods by using various gram positive and gram negative strains like *Echerichia coli* (MTCC 232)*, Bacillus subtilis* (MTCC 209), *Enterobacter hormaechei* (MTCC 236)*, Aeromonas hydrophila (MTCC* (1321). The bacterial strains obtained from Institute of Microbial Technology, Chandigarh, were used for evaluating antimicrobial activity. Stock cultures were maintained in nutrient agar. Active cultures for the experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller- Hinton broth (MHB) for bacteria by culturing overnight at 37◦ C. Standard antibiotic disks ampicilin 25 µg was included and tested for their antibacterial activity against the test micro organisms Melted and cooled sterile nutrient agar media was poured into sterilized petri dishes and was allowed to solidify. Broth culture of the test organisms was swabbed on the nutrient agar plate uniformly. The wells (5mm diameter) were made and filled with 0.01 ml of extract fractions. These plates were then incubated at 37°C ± 0.2°C for 24 h. The zone of inhibition was recorded as the presence of antimicrobial action. Different concentrations of the fractions were tested against each organism in triplicates (Perez et al., 1990).

2.6. Anti-inflammatory activity

2.6.1. Membrane stabilization test

a) The human red blood cell membrane stabilization (HRBC**) method**

Blood was collected from blood bank and mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) and centrifuged at 3000xg. The packed cells were washed with isosaline and a 10% suspension was made. Various concentrations of extracts were prepared (100,250,500 and 1000µg/ml) using distilled water. Each concentration consists of 1 ml of PBS, 2 ml hyposaline and 0.5 ml of HRBC suspension. It was incubated at 37°C for 30 min and centrifuged at 3,000 rpm for 20 minutes and the haemoglobin content of the supernatant solution was estimated on UV spectrophotometer at 560 nm. Diclofenac (100,250,500 and 1000 g/ml) was used as reference standard and a control was prepared by omitting the extracts (Gandhidasan et al., 1991; Samnelsson et al., 1995).

Percentage membrane protection= (Abs $_{control}$ -Abs $_{sample}$/ Abs $_{control}$) X 100

b) Heat induced haemolysis

Concentrations of test and standard drug were prepared as described in the above method. Only saline was added to the control test tube. To the test sample each concentration consists of 2 mL of test sample, 1 ml of PBS, 2 ml hyposaline and 0.5 ml of 10% HRBC suspension. All the centrifuge tubes containing reaction mixture were incubated in a water bath at 56°Cfor 30 min followed by cooling under running tap water. The reaction mixture was centrifuged for 3 min at 3000 rpm. The supernatant was decanted and absorbance was measured at 540 nm. The percentage inhibition of haemolysis in tests and control was calculated (Gandhidasan et al., 1991).

Percentage heat induced haemolysis = (Abs $_{control}$ - Abs $_{sample}$/ Abs $_{control}$) X 100

2.6.2. Inhibition of albumin denaturation

The reaction mixture (5 mL) consisted of 0.45 mL of egg albumin (from fresh hen's egg), and 2 mL of test sample (100,250, 500 and 1000 μgmL^{-1}) 2.6ml of PBS solution. With a small amount of 1 N HCl, pH was adjusted at 6.3. Diclofenac was used as the reference drug. The samples were incubated for 20 min at 37°C and then heated at 57°C for 3min. Aftercooling the samples, 2.5 mL PBS (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm. For control 0.05 mL distilled water was used instead of sample (Grant et al., 1970).

Percentage inhibition of protein denaturation= (Abs $_{control}$ -Abs $_{sample}$/ Abs $_{control}$) X 100

2.7. Immunomodulatory activity

2.7.1. Nitrobluetetrazoliumtest (NBT)

A suspension of leucocytes (5 x 106/ml) was prepared in 0.5 ml of PBS solution in 5 tubes. A volume of 0.1 ml PBS as control and 0.1 ml of endotoxin-activated plasma as standard was added to the 1st and 2nd tube respectively and to the rest of 4 tubes, 0.1ml of different concentrations (100, 250, 500, and 1000 µg/ml) of test samples. About 0.2 ml of freshly-prepared 0.15% NBT solution was added to each tube and incubated at 37°C for 20 min and then centrifuged at 3000rpm for 3-4 min to discard the supernatant. The cells were re-suspended in the small volume of PBS solution. A thin film was made with the drop on a slide, dried, fixed by heating, counterstained with methyline blue for 15 sec. The slide was washed under tap water, dried and focused under 100X. 100 neutrophils were counted for the percentage of NBT-positive cells containing blue granules/lumps (Wilkinson, 1981; Gooi, 1990).

2.7.2. Neutrophil adherence

Neutrophil adherence was analyzed by the initial count of total leucocyte (TLC) and dead leucocyte (DLC) from the blood sample. It was analysed by getting an initial count of total WBCs and differential or dead WBCs. After the initial count, blood samples were incubated in sterile nylon fiber column (80mg/ml) packed in a silicanized Pasteur pipette (column length 15 mm). After 30 minutes of incubation, blood sample was again analyzed for count of total WBCs and differential or dead WBCs. TLC and DLC. The product of initial and differential WBCs gave the neutrophil index (NI) of blood sample [16].

Percentage of neutrophil adherence =

(Neutrophil index of untreated blood samples) – (neutrophil index of treated blood sample) / (neutrophil index of untreated blood samples) x100

3. Results and discussion

The percentage w/w value of the extract obtained by the fractionation of n-hexane, ethyl acetate and ethanol are 5.71, 10.08, and 14 respectively.

The preliminary phytochemical analysis of n-hexane, ethyl acetate and ethanolic extract of *Zizyphus nummularia* showed the presence of flavonoids, carbohydrates, proteins, saponins, alkaloids, and tannins. Ethyl acetate and ethanol revealed the presence of carbohydrates, protein, alkaloids, phenol, flavonoids, tannins, saponins and glycosides, while only fixed oil, fats and volatile oil were present in n-hexane fraction as shown in the Table-1.

In the present study, the antimicrobial activity of *Z.nummularia* plant extract obtained from different solvents showed varying degree of response against the selected gram-positive and gram-negative pathogens. For all the tested organisms *Bacillus subtilis* showed maximum activity with zone of inhibition 20 mm,followed by *Enterobacter hormaechei* with zone of inhibition 13.5 mm of ethyl acetate fraction. Whereas *Aeromonas hydrophila*did not show any antimicrobial effect as illustrated in Table-2.

Table1. Preliminary phytochemical screening of various fractions of *Ziziphus nummularia* Burn.f. hydroalcoholic extract.

S.No	Constituent	Test	n-hexane	Ethyl acetate	Ethanol
1	Carbohydrates	Fehling's test	-	+	+
2	Proteins	Ninhydrine	-	+	+
3	Alkaloids	Hagers test	-	+	+
4	Phenol	Ferric chloride test	-	+	+
5	Flavanoids	Alkali test	-	+	+
6	Tannins	Alkali test	-	+	+
7	Saponins	Frothing test	-	+	+
8	Glycosides	Killer killini	-	-	-
9	Fixed oils	Spot test	+	-	-

Table 2. Antimicrobial activity of n-hexane, ethyl acetate and ethanolic fractions of *Ziziphus nummularia* extract.

S.No	Microorganisms	Zone of inhibition (mm)			
		n-hexane	Ethyl acetate	Ethanol	Amikacin
1	*Echerichia coli*	4.2	12	2.3	24
2	*Bacillussubtilis*	3.6	20	5.3	30
3	*Enterobacter hormaechei*	1	13.5	3	20
4	*Aeromonas hydrophila*	-	-	-	16

Table 3. Effect of various fractions of *Ziziphus nummularia* on membrane stabilization method.

S. No	Conc (µg/ml)	Absorbance				% inhibition			
		n-hexane	Ethyl acetate	Ethanol	Diclofenac	n-hexane	Ethyl acetate	Ethanol	Diclofenac
1	100	0.221	0.236	0.216	0.217	21.42	29.58	27.53	44.73
2	250	0.216	0.201	0.203	0.204	27.58	32.43	30.62	52.63
3	500	0.163	0.144	0.153	0.128	45.82	51.72	49.27	59.89
4	1000	0.145	0.123	0.143	0.09	50.63	59.62	51.72	71.42

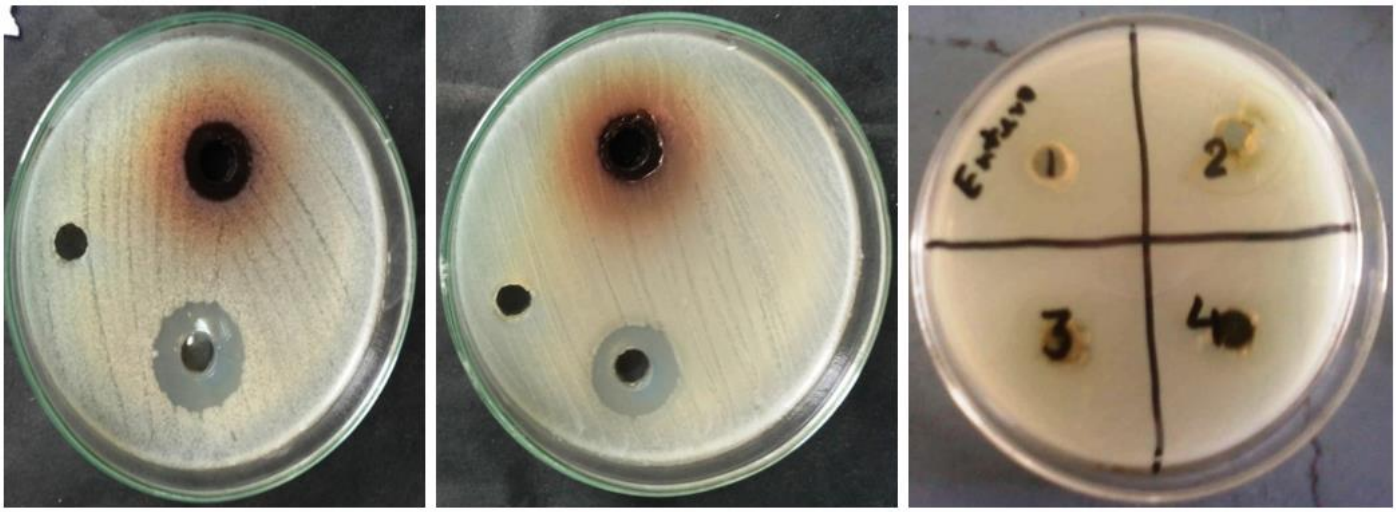
During inflammation, lysosomal hydrolytic enzymes are released which causes the damage of surrounding organelles and causes variety of disorders. The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may stabilize lysosomal membrane. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release bactericidal enzymes and proteases which cause further tissue inflammation and damage upon extracellular release (Perez et al., 1990).It is reported that ethyl acetate fraction of *Ziziphus nummularia* at a concentration of 1000µg/ml showed 59% protection with diclofenac (Table 3). The above results show that with the increase in concentration the percentage inhibition increases.

The extract was effective in inhibiting the heat induced haemolysis at different concentrations. The heat causes the denaturation of the proteins and destabilizes the lysosomal membrane. This inflammation is due to external stress on the lysosomal membrane (Chatterjee and Das, 1996). As the concentration of the extract increases then the haemolysis of the lysosomal membrane also decreases as the percentage inhibition increases with the increase in concentration. The extract prevents the haemolysis of membrane and thus decreases inflammation. The % protection of heat induced haemolysis was found to be maximum by ethyl acetate followed by ethanol which is71.79and 69.23 respectively when compared to diclofenac (71.42) as shown in Table 4. Both the fractions ethyl acetate and ethanol revealed the result to the greater extent when compared to standard. The ethyl acetate and ethanol fractions of *Ziziphus nummularia* showed the comparable result and prevent the inhibition of heat induced haemolysis.

Protein denaturation is a process in which proteins lose their metabolic secondary and tertiary structure by some external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when they get denatured. Denaturation of proteins is a well-recognized cause of inflammation and rheumatoid arthritis (Mizushima, 1966). Several synthetic anti-inflammatory drugs have shown dose dependent ability to decrease thermally induced protein denaturation (Grant et al., 1970).Maximum percentage of inhibition 71was observed from ethyl acetate extract followed ethanol 68 and lesser by hexane 56. Diclofenac was used as standard anti-inflammatory drug showed 71% inhibition that was comparable to inhibition induced by ethyl acetate at the concentration of 1000 µg/ml (Table 5). Both ethyl acetate and ethanol has shown comparable result as compared to diclofenac. The percentage inhibition shown by the fractions is lesser than standard but on the extent of inhibition of denaturation they show remarkable results.

During the process of phagocytosis by the polymorphonuclear leucocytes, there occur significant increases in oxygen consumption due to the reduction of NBT to formazon. Then-hexane, ethyl acetate, and ethanol extract of *Ziziphus nummularia* Burn.f has stimulated the neutrophills to the phagocytic activity to the extent of 44.81, 62.67 and 60.95 respectively at 1000µg/ml when compared to PBS 22.53 and to standard i.e endotoxin activated plasma as 57.97. The results revealed that ethyl acetate and ethanol fractions stimulated the neutrophils to phagocytic activity to wide extent when compared to PBS and endotoxin activated plasma. However, at low concentration 100, 250, 500 µg/ml the stimulation of neutrophils is comparatively lower. The results are shown in Table- 6.

All the three extracts showed significant increase in neutrophil adhesion at all the concentrations of fractions. The ethyl acetate extract was observed to be effective in increasing neutrophil adhesion due to the up regulation of β2 integrins. The main attractive substances for neutrophils are pro-



Bacillus subtilis *Echerichia coli* *Enterobacter hormaechei*

Figure 1. Antimicrobial activity of various fractions of *Ziziphus nummularia.*

Table 4. Effect of various fractions of *Ziziphus nummularia* on membrane stabilization by heat induced haemolysis.

S. No	Conc (µg/ml)	Absorbance				% inhibition			
		n-hexane	Ethyl acetate	Ethanol	Diclof enac	n-hexane	Ethyl acetate	Ethanol	Diclofe nac
1	100	0.311	0.288	0.462	0.217	18.42	26.31	21.05	44.73
2	250	0.264	0.261	0.366	0.186	31.57	31.57	23.61	52.63
3	500	0.227	0.168	0.197	0.168	42.10	57.89	50.01	57.89
4	1000	0.164	0.118	0.143	0.125	57.88	71.05	63.15	71.42

Table 5. Effect of various fractions of *Ziziphus nummularia* on protein denaturation.

S. No	Conc (µg/ml)	Absorbance				% inhibition			
		n-hexane	Ethyl acetate	Ethanol	Diclof enac	n-hexane	Ethyl acetate	Ethanol	Diclofe nac
1	100	0.311	0.303	0.314	0.217	20.51	23.07	20.51	44.73
2	250	0.276	0.299	0.291	0.204	25.64	28.20	25.64	52.63
3	500	0.132	0.163	0.160	0.168	58.97	60.12	57.41	57.89
4	1000	0.120	0.110	0.121	0.125	68.23	71.79	69.23	71.42

Table 6. *Immunomodulatory activity of various fractions of Ziziphus nummularia by nitroblueterazolium test.*

S.no	Concentration (µg/ml)	PBS	Endotoxin activated plasma	n-hexane	Ethyl acetate	Ethanol
1	100	19.20	50.37	27.81	36.21	37.49
2	250	20.12	52.21	31.65	49.65	47.83
3	500	22.17	52.21	36.40	56.83	53.95
4	1000	22.53	57.97	44.81	62.67	60.95

Table 7. Effect of various extracts of *Ziziphus nummularia* on immunomodulation by neutrophil locomotion method.

S.No	Concentration (µg/ml)	PBS	n-hexane	Ethyl acetate	Ethanol
1	100	12.50	110.82	134.36	123.60
2	250	13.75	129.54	139.83	132.61
3	500	14.25	132.01	148.54	146.41
4	1000	15.25	138.03	162.37	157.38

inflammatory cytokines, such as IL-Iβ, that regulate endothelial molecule expression on vascular endothelial cells and promote neutrophil adherence to these cells. The mean numbers of the neutrophills for n-hexane, ethyl acetate and ethanol fractions are 138.03, 162.37 and 157.38 respectively at 1000 µg/ml. whereas at low concentration as 100, 250 µg/ml the chemotactic activity shown by all the fractions are comparatively lower. The stimulation of the chemotactic activity is shown in Table-7.

4. Conclusion

From the present study it can be concluded that various fractions of *Ziziphus nummularia* showed significant *in-vitro* immunostimulating, anti-inflammatory and antimicrobial activities. *In-vitro* anti-inflammatory studies of plant demonstrated the suppression of both inflammation and arthritis. The present study indicates the ethyl acetate and ethanol fractions showed maximum activity. These activities may be due to the presence of phytochemicals such as alkaloids, flavonoids, tannins, steroids and phenols. As these results are encouraging enough to pursue bioactivity guided fractionation of this extract and structure elucidation of the active phytochemical constituents.

References

Berahou, A.A., Auhmani, A., Fdil, N., Benharref, A.,Jana, M., Gadhi, C.A. Antibacterial activity of *Quercus ilex* bark's extracts. *Journal of Ethnopharmacology*.

2007:112:426-429.

Chatterjee, S., Das,S.N. Antiarthritic and anti-inflammatory effects of a polyherbal drug (Ease): its mechanism of action. *Indian Journal of Pharmacology*. 1996;28: 116-119.

Dashputre, N.L., Naikwade, N.S. Immunomodulatory activity of *Abutilon Indicum* linn in albino mice. *International Journal of Pharma Sciences and Research*. 2010; 1(3): 178-184.

Gandhidasan, R., Thamaraichelvan, A., Baburaj, S. Anti-inflammatory action of *Lannea coromandelica* by HRBC membrane stabilization. *Fitoterapia*. 1991;62(1); 81- 83.

Gerard, J.Tortora., Sandra,Reynolds., eds. Principles of Anatomy and Physiology. Harper Collins College Publishers 7th edition, 1993; 695

Gooi, C. A practical approach. *Clinical Immunology*. 1990:51-78.

Grant, N.H., Alburn, H.E., Kryzanauskas, C. Stabilization of serum albumin by anti-inflammatory drugs. *Biochemistry and Pharmacology*. 1970;19: 715 -722.

Khandelwal, K.R. Practical Pharmacognosy, Techniques and experiments 12th Edn. NiraliPrakashan, Pune India.2004;157.

Kokate, C.K. Practical Pharmacognosy. 4th Edn. Vallabh Prakashan: New Delhi. 1994:120-124.

Mizushima, Y. Screening test for anti-rheumatic drugs. *Lancet*. 1966;2: 443.

Murphy, M.C. Plant products as antimicrobial agents. *Clinical Microbiology Review*. 1999; 12:564-572.

Nicoara, E., Crisan, A. Possibilities to modulate the immune response during infections. *Timisoara Medical Journal*. 2003; 53(4): 303-307.

Patwardhan, B., Kalbag, D., Patki, P.S., Nagsampagi, B.A. Search of immunomodulatory agents. *Indian Drugs*.1990; 28:56-63.

Perez, C., Paul, M., Bazerque, P. An antibiotic assay by the agar well diffusion method. *Acta Bio Med Exp,* 1990;15:113-115

Rao, C.S., Raju, C., Gopumadhavan, S., Chauhan, B.L, et al. Immunotherapeutic modification by an Ayurvedic formulation Septilin. *Indian Journal of Experimental Biology*.1994; 32:553-8.

Samnelsson, G., Kyeremater,G., Farah, M.H. *Journal of Ethnopharmacology*. 1985, 14: 193.

Thakur, M., Bhargava, S. Dixit, V.K. Immunomodulatory activity of *Chlorophytum borivilianum* Sant. *F. eCAM, doi*2006:1093.

Wilkinson PC. Neutrophil leucocyte function test, Tech in ClinImmunol, 2nd edition, Edited by Thomson RA, Blackwell Scientific Publication, Oxford, London, 1981 :273-93.