International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Assessment of *In Vitro* Immunomodulatory Activity of Ethanolic Extract of *Crataeva nurvula Buch. Ham*. Bark.

Aparna Lakshmi I[1], Prakashita Goud[2], CH. Sudha Bhargavi[3]

[1]Asst Prof, Department of Pharmacology, NET Pharmacy College, Raichur, Karnataka
[2]Asst Prof, Department of Pharmacy Pratice, NET Pharmacy College, Raichur, Karnataka
[3]Asst Prof, Department of Pharmacology, Vision college of Pharmaceutical Sciences and Research, Boduppal, Hyderabad, Telangana

ABSTRACT:
The current study was carried out to assess the immunomodulatory activity of Ethanolic extract *Crataeva nurvula* bark (EECNB) using in vitro methods.
In vitro immune modulatory activity was investigated by cellular lysosomal enzyme assay and phagocytosis evaluation.
The release of cellular lysosomal enzyme was found to be significant ($p<0.05$) at 200 µg/ml (Stimulation Index = 1.982) and 100 µg/ml (S.I = 1.564) of EECN with respect to control PHA (Stimulation Index =1.00) maximum phagocytic activity of EECNB was evident at 500µg/ml and with gradual increase in the dilutions of the plant extract there was a corresponding decrease in phagocytic activity.
The present has demonstrated the immunomodulatory potential of EECNB (as it has good stimulation index and maximum phagocytic activity) based on the results of lysosomal and phagocytosis assay. Further studies are needed to support its immonumodulatory activity.

KEY WORDS: Immunomodulatory, *Crataeva nurvula*, phagocytosis, lysosomal enzyme

INTRODUCTION

The immune system of vertebrate is a versatile defence system which is mainly involved in protecting the host from pathogenic micro-organisms. It acts as a surveillance system that continuously monitors the integrity of host [1]. The host immune system has a major influence on pathophysiology of many infectious diseases. Such diseases can be relieved by modulation of immune responses. The objectives of immunomodulation are to develop immunity by stimulation of the immune system and subdue of undesired immune responses. The traditional medicines play an important role in modulation of the host immune responses. Natural substances obtained from plants can serve as immonomodulators to control immune responses [2].

Innate immunity is the first line of defence against invading pathogen. In this, the phagocyte accumulates immediately at the site of infection [3]. Polymorphonuclear neutrophil (PMN) cells are the most abundant and the first cells to migrate towards the site of infection and play an important role to destroy the pathogens [4]. The migration of phagocytes to the site of infection occurs in multiple step

processes like rolling, adhesion of cells to vascular endothelium, diapedesis, followed by migration to the site of infection, phagocytosis and finally degradation of pathogens [5].

During innate immunity adhesion of phagocyte cells to the endothelium involves specific membrane receptors called beta-2-integrin (CD18/11 a,b,c) of proteins [6]. The beta-2-integrin receptors facilitates adhesion of phagocyte to endothelium cell receptors known as intercellular adhesion molecule 1 and 2. This phagocyte-endothelial interaction will promote the movement of phagocyte into infected tissue which later results in phagocytosis of pathogen [7,8].

Phagocytosis is a process in which phagocyte cells engulf, ingest solid particles and microbial pathogens. Through this process the micro-organism is not only destroyed but also processed for antigen presentation for later immune responses towards this pathogen [9]. Phagocyte cells contain surface receptors such as Fc gamma receptor and complement fragment C3b that are involved in phagocytosis process [10,11]. Pathogen that enters our body will be firstly opsonised by C3b which is the result of complement activation. Then, the phagocytosis process will be initiated after C3b binds into a complement fragment C3b receptor [12].

75% of the overall population uses plants or their extracts for their curative abilities [13]. Around 80% of the developing countries in the world rely on plants and their products for basic healthcare needs [14]. World Health Organization (WHO) states that "traditional medicine" implies the knowledge and practices of herbal healing for the prevention, diagnosis and elimination of physical, mental or social imbalance [15].

The genus Crataeva was named in honor of the Greek botanist "Crataevas" [16]. In Ayurveda, *C.nurvala* is known as Tikta saka (due to bitter taste of leaves), Setu briksh (because its leaves remain young for many days) and sweta puspa (due to its whitish flower) [17,18,19,20]. This plant softens and expels hardened faeces, cures the diseases of kapha and suppresses difficulty in micturation [21]. It is also used as a blood purifier and in treating cardiac and lung diseases, fever, arthritis, dementia, metabolic disorders, wound healing and also has diuretic effect.

The stem bark of *createva nurvula* contains (-)- epiafzelechin 5-o-□- D-glucoside, betulinic acid, cetyl alcohol, (-)- catechin, diosgenin, friedelin, gluco-capparin, lupeol, and □-sitosterol. The alkaloids like cadabicine, cadabicine diacetate have also been reported [22]. Immunomodulatory activity in plants was attributed for the presence of alkaloids, Polysaccharides, terpenoids, flavonoids, alkaloids, glycosides, and lactones.

The current study was aimed to investigate the immunomodulatory effect of the ethanolic extract of stem bark of *Crataeva nurvala*

MATERIALS AND METHODS

COLLECTION AND AUTHENTIFICATION OF PLANT MATERIAL:

Createva nurvula stem bark was collected from the local areas of talakona forest, chittor district, Andhra Pradesh. The plant material was authentified by Dr.K. Madhava chetty, Asst. Prof, Department of Botany, S.V. University, Tirupathi, Andhrapradesh, India.

PREPARATION OF PLANT EXTRACT:

Freshly collected stem bark of *C. nurvula* was air dried and powdered at a quantity of 3 kg. The powdered crude extract of *C. nurvula* was extracted with 95% ethanol by using simple maceration technique for 7 days at room temperature. The extract was concentrated to dryness using rotary vacuum evaporator under

reduced pressure. The yield of the extract was 3.78% w/w and the extract were stored at 4-5^0 C until used to avoid. Double- distilled water was used to redissolve the extract prior to experimentation to evaluate immunomodulatory activity [23].

PRELIMINARY PHYTOCHEMICAL SCREENING:

Standard screening test of the extract was carried out for various plant constituents. The crude extract was screened for the presence or absence of secondary metabolites using standard procedures [24].

In vitro Cellular lysosomal enzyme assay

Cellular lysosomal enzyme activity was measured by determining acid phosphatase in macrophages as described by Suzuki et al [25]. Macrophage suspension was seeded in each well of a 96- well plate and incubated in 5% CO_2 humidified incubator for 2 h; then non-adherent cells were removed by washing in RPMI-1640 medium. The remaining adherent cells (1 x 10^6 cells/well) were cultured in 100 ml RPMI-1640 medium with ethanolic extracts of *Crataeva nurvula* in different concentartions and were dissolved in 0.1% dimethyl sulfoxide (DMSO) in PBS so that their final concentrations were 10 mg/ml, 25 mg/ml, 50 mg/ml, and 100 mg/ml and incubated for 24 h at 37^{0}C in 5% CO_2 humidified atmosphere.

The 0.1% DMSO in PBS (without plant extract) was used as control. After incubation, the medium was removed by aspiration and 20 ml of 0.1% Triton X-100 (Himedia, India), 50 ml of 0.1 M citrate buffer (pH 5.0) and 100 ml of 10 mM p-nitrophenyl phosphate (p- NPP) (Himedia, India) solution were added into each well. The plate was further incubated for 30 min, 150 ml of 0.2M borate buffer was then added. The optical density (OD) was measured at 405 nm by using a microplate reader. Stimulation index (SI) for lysosomal enzyme activity was calculated as the ratio of OD of test extract/ drug treated cells to vehicle treated control cells (RPMI-1640 containing 10% DMSO).

$$\text{Lysosomal Enzyme activity (\%)} = \frac{\text{OD sample} - \text{OD Control}}{\text{OD Control}} \times 100$$

In vitro phagocytosis evaluation:

Capillary blood (0.2 ml) was obtained by finger prick method and was placed on a clean grease free glass slide and spread to 1.5×1.5 cm. Blood was allowed to clot at 37ºC for 25 min. The clot was removed using sterile normal saline. The polymorphonuclear leukocytes (PMN's) were found adhered to the glass surface while the rest of the blood components are washed away. Slides in duplicates were prepared and used for each dilution of the plant extract and for control. *Candida albicans* was confirmed using germ tube test and was inoculated in Saboraud Dextrose Broth. Overnight culture was centrifuged at 2000 rpm for15 min. The cell pellet was washed four times with sterile Hank's balanced salt solution (HBSS). The final cell button was suspended in sterile HBSS and human serum in a proportion of 4:1 and the cell density was adjusted to 2×108 cfu/ml with the help of MacFarlands standard. Different dilutions of the ethanol extracts of *crataeva nurvula* (0.1 ml) and control were flooded over the PMN layer on the slides, after which the slides were incubated at 37º for 15 min followed by the addition of 100 µl of *C. albicans* cell suspension. The slides were further incubated at37º for 60 min. After incubation, the film was washed twice with sterile normal saline. The film was fixed with methanol for 5 min. Diluted Giemsa stain was flooded over the film and was left undisturbed for 25 min. The excess stain was removed using HBSS and air dried. The slides were observed under the oil immersion (×100) objective. The mean number of *Candida* cells phagocytosed by PMNs on the slide were determined microscopically for 100 granulocytes

using morphological criteria (26). This number was taken as the phagocyte index (PI) and was compared with the PI of the control. Immunostimulation (%) was calculated by using the following Eqn., Stimulation

$$Immunostimulation(\%) = \frac{Phagocytic\ Index\ (test) - Phagocytic\ Index(control)}{Phagocytic\ Index\ (control)} \times 100$$

RESULTS

PRELIMINARY PHYTOCHEMICAL SCREENING:

The revealed results of the preliminary phytochemical screening of the ethanolic extract of stem bark of *Crataeva nurvala* were shown below. The ethanolic extract gave positive results for alkaloids, terpenoids, phytosterols, flavonoids, flavonones, glycosides, saponins, triterpenoids and tannins. Preliminary Phytochemical test for Ethanolic extract of *Crataeva nurvala*

LYSOSOMAL ENZYME ACTIVITY:

The release of cellular lysosomal enzyme was found to be significant ($p<0.05$) at 200 µg/ml (Stimulation Index = 1.782) and 100 µg/ml (S.I = 1.564) of EECN with respect to controls (Stimulation Index =1.00) (Table 1). Positive control PHA (Phytohaemagglutinin- M showed significant stimulation ($p<0.05$) of lysosomal enzyme release (S.I = 1.982)

Table-1 Effect on Lysosomal Activity

Sl. No	Conc. (µg/ml) CNE	Lysosomal activity
1	Control	1.000±0.05
2	800	1.450±0.08
3	400	1.552±0.03
4	200	1.782±0.05
5	100	1.564±0.08
6	50	1.495±0.03
7	25	1.275±0.06
8	12.5	1.250±0.09
9	6.25	1.305±0.06
10	PHA (Phytohaemagglutinin-M – 10□g/ml)	1.982±0.07

IN VITRO PHAGOCYTOSIS EVALUATION

While performing the *in vitro* evaluation of phagocytosis, ethanol extract of *Crataeva nurvula* was found to be cytotoxic at a concentration of 1000 µg/ml. Maximum phagocytic activity was evident at 500 µg/ml. With gradual increase in the dilutions of the plant extract there was a corresponding decrease in phagocytic activity.

Table-2 showing the % immunostimulation with reference to the concentration of extract

Sl.No	Concentration of ethanol extract of *Crataeva nurvula* (ug/ml)	Phagocytosis index	% Immunostimulation
1	500	83	87.65
2	250	80	84.42
3	125	78	82.32
4	62.5	66	69.75
5	31.25	65	68.61
6	15.60	61	64.34
7	7.80	53	55.91
8	3.90	47	49.63
9	1.95	44	46.42
Control	PHA (10□g/ml)	95	99.70

DISCUSSION

Immunomodulators are the substances which alter the immune response by augmenting or reducing the ability of the Immune System to produce antibodies or sensitized cells [26]. Due to increased stress conditions, mind and body are significantly showing impact on Immune response in general. There has been a tremendous increase for the drugs which are effective on immune system. Now, there is a need for the identification and development of natural and herbal Immunomodulators [27]. Specific antibodies bind to specific antigen leading to its activation or even phagocytosis [28]. Macrophages play an important role in the defence mechanism against host infection by innate and acquired immunity. Macrophages are the cells that present antigen to lymphocytes during the development of specific immunity and also increase the phagocytic activity. In the process of phagocytosis, they produce cytokines, reactive oxygen species (ROS) and nitrogen species (RNS) which are involved in the destruction of pathogen [29].

The main objective of the study was to investigate the preliminary immunomodulatory effects of stems of *Crataeva nurvula* using its ethanol extracts (CNE). This study demonstrated that the extract stimulated phagocytic activity on the tested *In vitro* assays,

*Candida albicans*enters inside the secondary lysosomes of the intact poly morpho nuclear leucocytes (PMN's), they are killed probably by the activity of myelopeorixdase. The viability of intra cellular *Candida albicans* could be determined by staining. The dead phagocytosed *C. albicans* appeared blue while the viable cells remained unstained.

For lysosomal enzyme activity, the transformation of *p*- NPP to coloured compound by acid phosphatase of the stimulated macrophage correlates to the extent of degranulation in phagocytosis [30].

CONCLUSION

The present study has demonstrated the *in vitro* immunomodulatory property of ethanol extracts of *Crataeva nurvula*. The results suggest that the ethanolic extract of CNE influence the non- specific immune system by the process of phagocytosis. Further elucidation of effective immunomodulation constituents of *Createva nurvula* is needed to supports its immonomodulatory activity.

REFERENCES

1. Delves PJ, Martin SJ, Burton DR, Roitt IM. Roitt's essential immunology. 12th ed. 2011.
2. Saravanan S, Prakash Babu N, Pandikumar P, Karunai Raj M, Gabriel Paulraj M, Ignacimuthu S. Immunomodulatory potential of Enicostema axillare (Lam.) A. Raynal, a traditional medicinal plant. J Ethnopharmacol 2012,140,239-46.
3. Underhill DM, Goodridge HS. Information processing during phagocytosis. Nat Rev Immunol 2012,12, 492-502.
4. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 2013,13,159-175.
5. Muller WA. Getting leukocytes to the site of inflammation. Vet Pathol 2013, 50(1), 7-22.
6. Schmidt S, Moser M, Sperandio M. The molecular basis of leukocyte recruitment and its deficiencies. Mol Immunol 2013, 55(1), 49-58.
7. Zhang Y, Wang H. Integrin signalling and function in immune cells. Immunology 2012, 135, 268-275.
8. Li N, Mao D, Lü S, Tong C, Zhang Y, Long M. Distinct binding affinities of Mac-1 and LFA-1 in neutrophil activation. J Immunol 2013, 190, 4371-4381.
9. Haugland GT, Jakobsen RA, Vestvik N, Ulven K, Stokka L, Wergeland HI. Phagocytosis and respiratory burst activity in lumpsucker (Cyclopterus lumpus L.) leucocytes analysed by flow cytometry. PloS One 2012; doi: 10.1371/journal.pone.0047909.
10. Goodridge HS, Underhill DM, Touret N. Mechanisms of Fc receptor and dectin-1 activation for phagocytosis. Traffic 2012, 13(8), 1062- 1071.
11. Ballanti E, Perricone C, Greco E, Ballanti M, Di Muzio G, Chimenti MS, et al. Complement and autoimmunity. Immunol Res 2013, 56, 477-491.
12. Flannagan RS, Jaumouillé V, Grinstein S. The cell biology of phagocytosis. Annu Rev Pathol 2012, 7, 61-98.
13. Abelson PH. Medicine from plants. Science 1990, 247,513–514
14. Farnsworth NR, Akerele O, Bingel AS. Medicinal plants in therapy. Bull World Health Organ 1985,63,965–981.
15. Akerele O. Summary of WHO guidelines for the assessment of herbal medicine. Herbalgram 1993,28,13–16
16. Bhattacharjee A, Sashidhara SC, Aswathanarayana. Phytochemical and ethno-pharmacological profile of C. nurvala Buch-Hum (Varuna): a review. Asian Pac J Trop Biomed 2012, S1162–68.
17. Bal N, Chaubey S, Tiwari RC, Kour GD, Dhyani S Varun (Crataeva nurvala Buch-Ham): a critical review with respect to urinary tract disorder. Int J Ayurveda Pharma Res2016,4,49–51.
18. Sastry JLN Ayurvedokta Oushadha Nirukta Mala, Ist edn. 2001: Jaikrishn Ayurveda Granthmala, Delhi.
19. Sastry JLN Dravyaguna Vijnana. Part-II, 2005; 2nd edn. Chaukhamba Orientalia, Varanasi.
20. Nripa Madanpala. Madanpala Nighaṇṭu. Hariharprasad Tripathi. Editior. Abhyadi varga, Chaukhamba Krishnadas Academy; Varanasi; 2009.
21. Chunekar KC, Pandey GS Bhavaprakash Nighantu. Chaukambha Bharati Academy, Varanasi 2009
22. Lakshmi V, hauhan JS Triterpenoids and related compounds from ratava nurvula. Planta M 1975,27,254-256.
23. Kokate CK, Purohit AP, Gokhale SB, Pharmacognosy, Nirali Prakashan, Pune, India, 2004,29, 54.

24. Khandelwal K, Practical pharmacognosy, Nirali prakashan, Pune, India. 2005, 149.
25. Suzuki I, Tanaka H, Yoshiyuki A, Yadomae T. Rapid measurement of phagocytosis by macrophages. Chem Pharm Bull 1988,36,4871-5.
26. Brune K, Schmid L, Glatt M, Minder B. Correlation between antimicrobial activity and peroxidase content of leukocytes. Nature 1973,245,209-10.
27. Nawale Rand PoojariS : Review on Chemicals and parts of plants as immunomodulators. Research Journal of Pharmaceutical, Biological and Chemical Sciences 2013,4(1),76 -84.
28. Agarwal SS and Singh VK: Immunomodulators: A review of studies on medicinal plants and synthetic peptides Part II synthetic peptides. PINSA 2013, 6, 377-392.
29. Garci D, Delgado R, Ubeira FM and Leiro J: Modulation of rat macrophage function by MangiferaindicaL extracts and mangiferin., Int. Immunopharmacol 2002, 2, 797–806.
30. Ding AH, Nathan CF and StuehrDJ : Release of reactive nitrogen intermediates and reactive oxygen Intermediates from mouse peritoneal macrophages. J. Immunol 1988, 141, 2407-2412.