Khandelwal & Choudhary, *J. Pure Appl. Microbiol.,* **14**(**1**), 641-646 | March 2020
Article 6103 | https://doi.org/10.22207/JPAM.14.1.66

Print ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Immunomodulating potential of *Neolamarckia cadamba* (Roxb.) Bark extract

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Abstract

***Neolamarckia cadamba* (Roxb.) Bosser Miq., holistic tree, well narrated in Charak Samhita and Sushrut Samhita. Traditionally different parts of *N. cadamba* has been used by many communities and tribes to treat sour throat, cough, fever, infections and inflammation. Present work concern with study of immunomodulatory activities of hydro-methanolic extract (HME) of *N. cadamba* bark with reference to humoral and cell mediated immune responses of experimental animals. On the basis of mean body weight (g) of Wistar albino rats, their healthy status, change in their behavior, skin and fur texture, four groups- Group-I (control), Group-II (125 mg/kg HME), Group-III (250 mg/kg HME) and Group-IV (500 mg/kg HME) containing six animals each were made to determine mean serum antibody titer of treated and control groups against *Salmonella typhimurium* 'O' antigen using indirect ELISA. Determination of *in vitro* cell mediated immune response was done by MTT assay using optimum concentration (5 µg/mL) Con A with various concentration of HME (20-500 µg/mL). Result suggested various doses of HME (125/250/500 mg/kg b.wt) causes significant increase ($p < 0.01$) in antibody titer when compared to the control group, which concludes enhanced humoral immune response. Result suggested that different concentrations of HME bring about significant increase ($p < 0.01$) in proliferation of splenocytes, depicting enhanced cell mediated immune response. Study concludes immunostimulatory potential of HME of *N. cadamba* bark and can be possibly used in drug and food preparation.**

Keywords: Humoral immune response, cell mediated immune response, MTT, ConA

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(Received: January 18, 2020; accepted: March 23, 2020)

Citation: Vishal Khandelwal and Pradeep Kumar Choudhary, Immunomodulating potential of *Neolamarckia cadamba* (Roxb.) Bark extract, *J. Pure Appl. Microbiol.*, 2020; **14**(**1**):641-646. https://doi.org/10.22207/JPAM.14.1.66

INTRODUCTION

Plant derived products and their derivatives have been the most successful source of drug leads[1]. In spite of popularity of the synthetic products due to its production cost, time effectiveness, easy quality control, stringent regulation and quick effects in recent decades, their safety and efficacy has always remained questionable. Natural products still make a significant contribution to health care because of its time tested safety and efficacy[2,3]. Recent advancement in natural product drug discovery has resulted in compounds that are being developed to treat cancer, viruses and resistant bacteria and immunosuppressive disorders[4]. Further, clinical trials are ongoing on more than 100 natural product derived drugs and at least 100 molecules/ compounds are in preclinical development stage highlighted the existing viability and significance of the use of natural products as sources of new drug candidates[5,6].

The immunomodulatory properties of plants are being studied broadly with ever-increasing interest due to the benefits by immune system modulation for disease prevention and treatment in recent years[7]. These findings have now given many empirical therapies a rationale, scientific basis and thereby a means for 'intelligent' development[8].Immunomodulatory potential of 100 plants have been investigated in India by studying the mechanism of immune system and involvement of cytokines at molecular level[9]. Immunomodulatory plants in our Classics are *Tinospora cordifolia* (Guduchi), *Emblica officinalis* (Amalaki), *Terminalia chebula* (Haritaki), *Glycyrrhiza glabra* (Yashtimadhu), *Commiphora mukul* (Guggul), *Allium sativum* (Lahsuna), *Withania somnifera* (Ashwagandha), *Azardicta indica* (Neem), *Asparagus racemosus* (Shatavari), *Samecarpus anacardium* (Bhallatak), *Piper longum*(Pippali), *Aloe vera*(Ghritkumari), *Boerhaevia diffusa* (Punarnava), *Ocimum sanctum* (Tulasi), and Shilajit (Asphalt)[10].

Neolamarckia cadamba is one of such important medicinal plants belonging to the Rubiaceae family. In traditional Indian system of medicine, it has been used to treat fever, uterine complaints, blood diseases, skin diseases, eye inflammation, anemia, diarrhea, leprosy, dysentery and stomatitis[11]. It has the largest number of phytochemicals and secondary metabolites (viz., cadambagenic acid, cadamine, quinovic acid, β-sitosterol, cadambine, etc.) having various biological and pharmacological properties[12]. *Neolamarckia cadamba* has a wide spectrum of biological activities including anti-inflammatory[13], anti-helminthic[14], antioxidant[15], and anti-hepatotoxic activities[16]. However, no study to date has investigated the immunomodulatory role *Neolamarckia cadamba* bark extract.Therefore, the present study has been designed to study immunomodulatory potential of hydromethanolic extract of *N. cadamba* bark.

MATERIALS AND METHODS

Experimental animals

Wistar albino rats weighing 80-100 g were procured from animal house, IPR, GLA University, Mathura. Four groups containing six animals were made. Animals of Group-II, III & IV were fed orally with 125, 250, 500 mg/kg body weight of HME of *N. cadmba* bark respectively while control group (Group-I) animals were orally fed with rat pellet and water *ad libitum* for 21 days.

Chemicals

Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), Concanavalin A (Con A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue, were procured from Sigma chemicals Co, USA and Anti rat IgG-HRP conjugate antibody was obtained from Thermo Scientific, USA.

Plant material

Neolamarckia cadamba bark was gathered from Mathura (27°31'2" N – 77°39'26'' E). Identification and authorization of plant material was done by Dr. A. S.Updadhye, Agharkar Research Institute, Pune with voucher deposition No. L-084. Dried bark powder was used in preparation of hydo-methanolic extract (HME) of *N. cadamba*.

Preparation of extract

Coarsely powdered bark (300-350 g) was taken in soxhlet apparatus in presence of hydro-methanolic (80:20, v/v) solvent and subjected to boil at 65°C for 8-10 hrs. Evaporation was done at controlled temperature and reduced pressure using rotary evaporator. Finally, dark brown crystals (% yield: 14) were obtained.

Experimental animal

Wistar albino rats (80-100 g) were taken from central animal house, GLA University, Mathura and used for immunomodulatory studies. Ethical clearance was obtained with IAEC approval vide GLAIPR/CPCSEA/IAEC/2014/Biotech/02. Four groups (Groups-I, II, III & IV) containing six animals each were made for assessment of *in vivo* humoral immune response.

Determination of safe and non-toxic dose of HME of *N. cadamba*

To determine the safe and non-toxic dose of hydro-methanolic extract (HME) of *N. cadamba*, four groups of Wistar albino rats, comprising six rats in each group were made. Safe dose level was determined according to Organization for Economic Co-operation and Development guidelines No. 423[17]. Animals of Group-II, III & IV were fed orally with 125, 250, 500 mg/kg b.wt respectively for twenty one days whereas animals of Group-I (control) were fed with rat pellet and water *ad libitum*. All the groups of albino rats were monitored for the development of clinical signs, changes in physical health conditions and body weight.

Apparent heath condition and toxic signs

Rats of test groups (HME fed rats and unfed rats) were kept separately and observed every day for any toxic sign / symptoms / abnormal changes throughout the experiment. The dose causing no adverse effect was taken as safe and nontoxic dose (NTD) for further studies.

Body weight gain/loss

Animals fed with different doses of HME of bark including control group were weighed at weekly interval and the mean body weight was recorded at 0 and 21st day. The mean body weight of rats of test groups was recorded and compared with the control group.

Measurement of antibody titre for determination of humoral immune response

Salmonella typhimurium 'O' antigen was prepared as per method suggested[18]. Smooth colonies of *S. typhimurium* grown on Tryptose agar medium were selected and inoculated in nutrient broth. Inoculated broth was incubated for 6-8 hours at 37°C and then boiled at 100°C for two hours thirty minutes. Heated *S. typhimurium* culture was used as 'O' antigen for determination of humoral immune response in rats. Antibodies were raised against animals of all groups subcutaneously immunized with *Salmonella typhimurium* 'O' antigen. On 1st day, all animals of Group-I (control), Group-II (125 mg/kg b.wt), Group-III (250 mg/kg b.wt) and Group-IV (500 mg/kg b.wt) were subcutaneously immunized with equal volume (0.5 mL) of *S. typhimurium* "O" antigen (2 mg/ml) and FCA. Then after at 7th and 14th day all groups including control were immunized with equal volume (0.5 mL) of *S. typhimurium* "O" antigen and FIA. At 21st day serum from all experimental animals was used for determination of humoral immune response. Antibody titer of experimental and control group animals was determined in accordance to protocol[19]. Assessment of humoral (antibody mediated) immune response was done by conventional indirect ELISA.

Determination of cell mediated immune response

Splenocyte preparation

Rat splenocytes preparation was carried out in accordance to method suggested [19]. Spleen was macerated and filterated through nytex membrane to remove large particles. Cells were then resuspended in RPMI-1640 medium containing 10 % fetal calf serum and centrifuged at 2500 rpm for 10 minutes. Centrifuged cell were then treated with 0.15 M NH_4Cl (lysis buffer) for erythrocytes lysis and then washed with RPMI-1640 medium. Cell viability was determined by trypan blue dye (0.1% solution) and viable cell concentration was adjusted to 2 x 10^6 cells/mL by haemocytometer and was used for splenocytes proliferation assay.

***In vitro* effect of HME of *N. cadamba* bark on splenocytes proliferation/inhibition**

Efficacy of HME of *N. cadamba* bark over cell mediated immune response of Wistar albino rats was determined by studying the effect of HME over rat's splenocytes. 200µL of 2x10^6 spleen cells/ml in triplicate seeded with 10% FBS with 5µg/ml Con-A (optimum conc.) was cultured in RPMI-1640 medium. Different concentrations 20, 50, 100, 250, 500 µg/mL of HME of *N. cadamba* bark were added in triplicate to respective wells of plate. Incubation of culture plate was carried at 37°C for 72 hours using CO_2 incubator (5% CO_2). After that 20 µL of MTT solution (5 mg/mL) was added to each well. Formation of formazone crystals occurs due to reduction of MTT. Plate was further incubated at 37°C for 4 hours in CO_2 incubator (5% CO_2 and

80% relative humidity), followed by removal of supernatant. The plate was allowed to dry and formazone crystals were dissolved by adding 100µL DMSO. Mean OD values were determined with respect to control by ELISA reader.

Result and Discussion

Determination of safe and nontoxic dose of HME of *N. cadamba*

Various doses of HME (125, 250, 500 mg/kg body weight) of *N. cadamba* bark was given orally to the respective groups of Wistar albino rats comprising of six rats in each group for 21 days. All the orally fed (Group-II, Group-III and Group-IV) and control (Group-I) groups were kept under regular observations. 125, 250 and 500 mg/Kg b.wt of HME of *N. cadamba* were safe and non-toxic and were used in further studies.

Effect of HME of *N. cadamba* bark on apparent health status and toxic sign(s)

All the extract-fed rats were found healthy, showing no significant change in their behavior, skin, and fur texture, impairment in food intake and water consumption and no toxic symptom was observed.

Effect of HME of *N. cadamba* bark over body weight of experimental rats

Body weight of albino rats fed with different concentrations of HME was found to have increased significantly ($p < 0.01$) when compared to those in the control group, this is shown in Table 1.

Effect of HME of *N. cadamba* on humoral immune responses in Wistar albino rat model against *S. typhimurium*"O" antigen

Mean values of serum antibody titer in Group-I, II, III and IV were $1710^{a} \pm 271.97$, $3840^{ab} \pm 572.43$, $4266.67^{bc} \pm 539.69$ and $6400^{c} \pm 1280$ respectively as shown in Table 2. Dose dependent significant increase ($p < 0.01$) in antibody titer was found when compared with the control group.

***In vitro* effect of HME of *N. cadamba* bark on splenocytes proliferation/inhibition**

20.87, 48.87, 81.71, 159.70 & 209.06 percentage proliferation in spleen cell culture was noticed at 20 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL and 500 µg/mL HME respectively (Table 3). Maximum % stimulation index (209.06) was noticed at 500 µg/mL HME treated spleen cell culture.

Various doses of HME viz. 125/250/500 mg/kg b.wt were found to be safe and nontoxic and were used for determination of humoral immune response in Wistar albino rats. Dose dependent significant increase ($p < 0.01$) in serum antibody titer was found in rats orally fed with 125, 250 and 500 mg/kg b.wt. HME of *N. cadamba* bark with respect to control which is in agreement with previous findings[19]. Presently, enhanced antibody titer suggests that various doses of HME brings about stimulation and proliferation of B lymphocytes, which play a key role in antibody production[20,21]. Study signifies that oral intake of different doses of HME of *N. cadamba* bark causes augmentation in adaptive immune response of experimental groups, since they are based mainly on the antigen-specific receptors found on the

Table 1. Mean body weight (g) of Wistar albino rats of different Groups

S. No	Groups	0 day	21st day
1	Group-I (Control)	61.50 ± 1.67	80.83^{a}± 3.19
2	Group-II (125 mg/kgb.wt)	66.33 ± 0.99	95.33^{b} ± 3.6
3	Group-III (250 mg/kgb.wt)	57.67± 2.3	97.50^{b} ± 2.24
4	Group-IV (500 mg/kgb.wt)	62.83 ± 5.35	98.83^{b} ± 3.19

All values are Mean ±S.E. of 6 rats. Results are significant at $p < 0.01$ as per one way ANOVA followed by DMRT.

Table 2. Determination of Humoral response of Wistar albino rats fed with HME against *S .typhimurium* 'O' antigen

Control		Antibody Titer HAE fed rats	
Group-1 (Control)	Group-2 (125 mg/kgb.wt)	Group-3 (250 mg/kgb.wt)	Group-4 (500 mg/kgb.wt)
1710^{a} ± 271.97	3840^{ab}± 572.43	4266.67^{bc} ± 539.69	6400^{c} ± 1280

The values represent the mean ± SEM of six rats. Results are significant at $p < .01$ as per one-way ANOVA followed by DMRT.

Table 3. *In vitro* effect of HME of *N. cadamba* bark on Con-A stimulated Wistar albino rat splenocytes proliferation/inhibition

Spleen cell culture	Mean Absorbance	% stimulation index
Control spleen cells without extract	$0.0618^{a} \pm 0.00151$	-
with 20 µg/mL HME	$0.0747^{b} \pm 0.00133$	20.87
with 50 µg/mL HME	$0.092^{c} \pm 0.00103$	48.87
with 100 µg/mL HME	$0.1123^{d} \pm 0.00552$	81.71
with 250 µg/mL HME	$0.1605^{e} \pm 0.00134$	159.70
with 500 µg/mL HME	$0.1910^{f} \pm 0.00393$	209.06

The values represent the mean ± SEM of six rats. Results are significant at p<.01 as per one-way ANOVA followed by DMRT.

surfaces of T- and B-lymphocytes[22]. Current study depicts immunomodulatory activity of HME of *N. cadamba* bark.

Current study deal with *in vitro* effect of different doses of HME (20, 50, 100, 250, 500 µg/mL) over spleen cell culture. Maximum stimulation index (%) was noticed with 500 µg/mL HME of bark of *N. cadamba* (Table 3). Dose dependent significant ($p < 0.01$) increase in proliferation of splenocytes was found at 20, 50, 100, 250 and 500 µg/mL conc. of HME of *N. cadamba*. Present findings on splenocytes proliferation are in concurrence with previous study[23].

Current study revealed escalation in humoral and cell mediated immune in experimental animals suggesting immunostimulatory potential of hydro-methanolic extract of *N. cadamba* bark and can therefore serve as herbal remedy to enhance immune responses in immunodeficient individuals.

CONCLUSION

Present study concludes synergistic effect of HAE of *N. cadamba* bark, responsible for enhancement of humoral and cell mediated immunity and can serve as remedy in treatment of immunodeficiency disorders. Moreover, there is a need to isolate and characterize the novel compound(s) present in *N. cadamba* bark extract responsible for enhanced immune response.

ACKNOWLEDGEMENTS

Authors express their thanks to Director, Institute of Applied Sciences and Humanities, GLA University, Mathura for providing necessary facility.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

VK and PK contributed significantly and equally.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Ethical clearance was obtained with IAEC approval vide GLAIPR/CPCSEA/IAEC/2014/Biotech/02.

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