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Immunomodulatory activity of *Neolamarckia cadamba* (Roxb.) Bosser with reference to IL-2 induction

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Neolamarckia cadamba (Roxb.) Bosser syn. *Anthocephalus cadamba* (Roxb.) Miq., an evergreen tropical tree, known in Hindu Mythology, for its various medical properties. In the present study, immunomodulatory potential of *N. cadamba* has been investigated in Wistar albino rat model. The effect of hot aqueous extract (HAE) of *N. cadamba* leaves over differential leukocyte count and humoral immune response was assessed using four groups of six animals each (Gp-I as control, Gp-II orally fed with 125 mg/kg body weight, Gp-III orally fed with 250 mg/kg body weight and Gp-IV orally fed with 500 mg/kg body weight of HAE of *N. cadamba* leaves). Differential leukocyte count (DLC) was measured in blood, collected from retro-orbital plexus of rats of control and experimental groups. *In vivo* humoral immune response was determined by estimating the serum antibody titer against *Salmonella typhimurium* 'O' antigen using indirect ELISA. Interleukin (IL)-2 was assayed by sandwich ELISA in presence of different concentrations of HAE (20-500 µg/mL) in the culture supernatant of splenocytes and its expression was determined by quantitative reverse transcription real-time PCR (qRT-PCR). Results suggested significant increase ($p < 0.01$) in lymphocytes (%) of animals orally fed with different concentrations of HAE of *N. cadamba.* Serum antibody titer was also significantly increased ($p < 0.05$) in *N. cadamba* fed animals. IL-2 level was augmented significantly ($p < 0.01$) in Concanavalin A (Con A) stimulated *in vitro* splenocytes culture of 250 µg/mL and 500 µg/mL HAE treated animals in comparison to controls. IL-2 expression was confirmed at molecular level by qRT-PCR analysis of mRNA transcripts of IL-2 gene. Fold expression of IL-2 gene was 28.84 and 330.84 at 250 µg/mL, 500 µg/mL concentrations of HAE respectively in comparison to control. It is concluded that HAE of *N. cadamba* leaves is a promising drug with immuno-stimulant properties.

Keywords: RT-PCR, Interleukin-2, Humoral immune response.

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Over the past century the modern and western medicine has revolutionized healthcare of humans and animals in the world, but still large percentage of populations in developing nations depends on phytomedicines[1]. Recently, Golden Triangle Partnership (GTP) program jointly by ICMR, AYUSH and CSIR has been initiated for validation of traditional *Ayurvedic* drugs/formulations and new drug development from Indian plant species[2].

Neolamarckia cadamba is one of evergreen tropical tree belongs to the Rubiaceae family, closely associated with the life of Lord Krishna (Hindu Deity) and has been used in folklore medicine to treat fever, uterine complaints, anaemia, blood diseases, skin diseases, eye inflammation, diarrhoea, leprosy, dysentery and stomatitis[3-8]. It contains the number of phytochemicals and secondary metabolites (viz., cadamine & isocadamine[9], 3β-dihydrocadambine & 3β-isodihydrocadambine[10], aminocadambine A & B[11], neolamarckines A & B[12], chlorogenic acid & β-sitosterol[13]) responsible for its various biological and pharmacological activities such as antiplasmodial[14], analgesic[15], antidiabetic[16], antioxidant[13], antipyretic[17], anticancer[18] and antimicrobial activities[19,20].

Modulation of host immune responses, to increase clearance of contagious agents and to reduce tissue damage due to inflammation, is the fundamentally new strategy for the cure of infectious diseases[21]. Phytotherapy might be useful to treat the diseases occurring due to dysfunction of immune system

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through dynamic regulation of informational molecules, viz. hormones, cytokines, chemokines, neurotransmitters, and other peptides[22]. Presently IL-2, IL-7, IL-10 IL-12, TNF-α and many other cytokines are comprehensively used in clinical and preclinical studies[23]. Many plants have immunomodulating properties which are being studied broadly with ever increasing interest due to benefits by immune system modulation for the prevention and treatment of disease in recent years[24].

Although *N. cadamba* plant has high therapeutic value but perhaps little information is available regarding its role in immunomodulation and in regulating cytokine biomolecules. Therefore, current study has been designed to find out the medicinal potential of *N. cadamba* plant for its immunomodulatory activity using various techniques including sandwich ELISA and qRT-PCR for assessing the differential expression (IL-2 metabolite/mRNA) between treated and control rats.

Methodology

Chemicals

Fetal Bovine Serum (FBS), Concanavalin A (Con A), TRI reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and RPMI-1640 medium were purchased from Sigma chemicals Co, USA. Rat IL-2 ELISA kit was obtained from R&D Systems, USA. Anti rat IgG-HRP conjugate antibody, revertaid reverse transcriptase and 2X SYBR green master mix were procured from Thermo Scientific, USA. Primers were designed from Xcelris, Ahmedabad, India.

Plant material

Anthocephalus cadamba leaves were collected from Mathura (27°31'2" N - 77°39'26'' E) and Vrindavan areas (27°33'39" N - 77°41'14'' E). The leaves were identified and authenticated by Dr AS Updadhye, Agharkar Research Institute, Pune with voucher deposition No. L-084. Coarsely powdered shade dried leaves were used in the preparation of hot aqueous extract (HAE) of *N. cadamba* leaves.

Extract preparation

HAE of dried leaves of *N. cadamba* was prepared by soxhlet apparatus by hot extraction method. HAE was concentrated to dryness using rotary evaporator under reduced pressure and low temperature (< 40 °C). The extract was then stored at 4 °C for further studies.

Experimental animal

Wistar albino rats of either sex weighing 60-100 g were procured from animal house facility of ICAR-IVRI, Izzatnagar, Bareilly with IAEC approval vide GLAIPR/CPCSEA/IAEC/2014/Biotech/02 and were used for immunomodulatory studies.

Effect of HAE over differential leukocyte count (DLC) of Wistar albino rats

Previously 125 mg, 250 mg and 500 mg/kg body weight (b.wt.) were found to be safe and non-toxic when given orally, depending upon the haematological and biochemical indices of Wistar albino rats[25]. Henceforth respective doses of HAE of *N. cadamba* were given orally up to 21 days to study its effect on DLC of experimental animals.

Blood samples were collected from innercanthus through retro-orbital plexus in EDTA coated sterile vials on 21st day, from the control and test groups of animals fed with different doses of HAE of *N. cadamba* leaves and immediately processed to determine differential leukocyte count (DLC) as described by Schalm & coworkers[26].

Determination of *in vivo* humoral immune response against *S. typhimurium* 'O' antigen

Study of humoral immune response was carried out using *Salmonella typhimurium* 'O' antigen (MTCC-3231) for raising antibodies in all the four groups. Test groups were fed with 125 mg/250 mg/500 mg/kg b.wt. of HAE of *N. cadamba* leaves for 21 days, while the control were given placebo during the period of experiment.

Preparation of S. typhimurium 'O' antigen for raising antisera[27]

Smooth colonies of *S. typhimurium* grown on tryptose agar medium were selected and inoculated in nutrient broth. Inoculated broth was incubated for 6-8 h at 37 °C, centrifuged at 3000 rpm for 20 min and supernatant was discarded. Pellet was washed three times with normal saline and then boiled at 100 °C for 2.5 h. Heat killed *S. typhimurium* culture was then used as 'O' antigen for determination of humoral immune response in treated and control albino rats.

Immunization of rats

All the four groups were inoculated subcutaneously with 0.5 mL *S. typhimurium* 'O' antigen & Freund's complete adjuvant (FCA) in 1:1 ratio at 1st day and subsequently immunized by 'O' antigen at 7th & 14th day along with Freund's incomplete adjuvant (FIA). One week after last dose of 'O' antigen, blood from all the groups of animals was collected for determining antibody titre in serum against 'O' antigen by indirect ELISA using anti rat IgG-HRP conjugated antibody.

Determination of antibody titre by indirect ELISA

ELISA test was carried out by following standard protocol[28]. The *Salmonella* antigen (100 µL) was used to coat the wells of polystyrene micro titer plate (Nunc) for overnight at 4 °C. Following three washings with PBST (*p*H-7.2, 0.01M) containing 0.05 % Tween-20, blocking was done by 3 % bovine serum albumin (BSA) dissolved in PBS and incubated at room temperature for 2 h. Diluted serum samples (1:10 to 1:10, 240) were added from 1st to 11th well of micro titer plate, while 12th well was used as blank. Rabbit anti-rat immunoglobulin-G (IgG) conjugated with horse radish peroxidase (HRP) and substrate solution tetramethyl benzidine (TMB) were added serially to each well of ELISA plate with the intermittent washing steps. Fifty µL of 1M sulphuric acid was added in each well to stop the colour development further. OD was measured at 450-570 nm. In between the each step, 3-5 washings were done with PBST to remove non specific binding.

In vitro effect of HAE of _N. cadamba_ leaves on induction of IL-2

Splenocyte preparation

Rat splenocytes were prepared as per the method suggested[29]. Normal rat reared under hygienic condition in the approved Lab animal house facility of Department of Biotechnology, was sacrificed and spleen was collected aseptically using sterilized petri dishes containing RPMI-1640 medium. Then spleen was teased with the help of sterilized toothed forceps to release spelnocytes and filtered through nytex membrane to remove the large clumps/debris. Cells were further suspended in RPMI-1640 medium containing 10 % fetal bovine serum (FBS) and centrifuged at 2500 rpm for 10 min at 25 °C. Centrifuged cells were then treated with 0.15M NH_4Cl (RBC lysis buffer) to lyse the erythrocytes and washed thrice with RPMI-1640 medium. Cells viability was determined by trypan blue (0.1 %) dye exclusion technique and the viable (unstained) cells were counted using Neubauers hemocytometer. The viable cells concentration was adjusted to $2x10^6$ cells/mL for IL-2 cytokine analysis. In addition, $1x10^6$ cells/mL in 10 cm^2 cultures dishes were used for RNA extraction to quantify mRNA transcripts for IL-2 gene using quantitative real time polymerase chain reaction (qRT-PCR).

IL-2 induction in presence of HAE of N. cadamba leaves

In vitro induction was done by adding 5µg/mL Con A mitogen per well in 200 µL of $2x10^6$ spleen cells/mL in triplicate, cultured in RPMI-1640 medium with 10 % FBS. 20 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL and 500 µg/mL HAE of *N. cadamba* leaves were added to respective wells of plate. The culture plate was incubated at 37 °C for 48 h with 5 % CO_2. Following 48 h of incubation, culture supernatant was collected for quantitation of IL-2 using sandwich ELISA.

Quantitation of IL-2 using sandwich ELISA

The collected supernatant from Con A stimulated splenocyte cultures were used for quantitation of IL-2 cytokine by sandwich ELISA as per protocol of R&D Systems. Different concentrations of IL-2 standard (Std1-4000 pg/mL, Std 2-2000 pg/mL, Std 3-1000 pg/mL, Std 4-500 pg/mL, Std 5-250 pg/mL, Std 6-125 pg/mL and Std 7-62.5 pg/mL) were used to quantitate the IL-2 in culture supernatant. Optical density was measured at dual wavelength (450-570 nm). Each experiment was run in triplicate.

Expression of IL-2 cytokine at molecular level by real time PCR

The expression of IL-2 cytokine gene in HAE treated and control rats were estimated by qRT-PCR.

RNA isolation from spleen cells

Con A stimulated splenocytes ($1x10^7$ cells/mL) were cultured in 10 cm^2 cell-culture petri dishes for 24 h in the absence and presence of 250 and 500 µg/mL of HAE. RNA extraction was done from using TRI reagent. One ml TRI reagent was added into each petri dish to lyse spleen cells and incubated for 5 min at room temperature (RT). The mixture was homogenized by pipetting several times. The

homogenate was incubated at RT after adding 250 µL of molecular grade chilled chloroform. Mixture was then centrifuged at 12,000 x g for 15 min at 4 °C. Aqueous phase containing RNA was collected into another microfuge tubes and further precipitated by addition of 500 µL of chilled isopropanol followed by centrifugation at 12,000 x g for 10 min at 4 °C. Without disturbing the RNA pellet, the supernatant was decanted and the pellet was washed with 75 % ethanol (in DEPC water), by centrifuging at 7500 x g at 4 °C for 5 min. The RNA pellet was dried and reconstituted with 30 µL DEPC water for checking its quality.

RNA Quantitation

RNA pellets obtained from control and 250 µg/mL and 500 µg/mL HAE treated spleen cells were re-suspended in nuclease free distilled water. Absorbance (A) was measured at 260 and 280 nm using the microphotometer (NanoDrop™ 2000 Spectrophotometers, Thermo Scientific, USA). The concentration was measured at A_{260} and the purity was checked using the ratio of absorbance (A_{260}/A_{280}).

For calculating the RNA concentration: [A_{260} x dilution factor x 40 µg/mL] was used.

Synthesis of cDNA

RNAase H Revertaid cDNA synthesis kit was used for reverse transcription following the manufacturer's instructions. 1µg of total RNA was used per reaction volume of 20 µL and reverse transcribed using oligo (dT) primers. The final concentration of cDNA is estimated using the formula:

[A_{260} x dilution factor x 33 µg/mL].

Prepared cDNA was stored at -80 °C for further use.

Real Time polymerase chain reaction of cDNA (qPCR)

The differential expression of IL-2 cytokine and β-actin (endogenous control) in spleen cells were carried out by Quantitative SYBR Green Real Time PCR (qRT-PCR) in CFX96 Touch Real-Time PCR detection system (Biorad, USA) by using specific primers. The sequence of primers advocated by Asa Melhus[30] were as following:

IL-2: 5'-AGCTGTTGCTGGACTTACAGG-3' (forward primer) & 5'-AATTCCACCACAGTTGCTGG-3' (reverse primer)

β-actin: 5'-TGGAGAAGAGCTATGAGCTGC-3'(forward primer) & 5'-TCCACACAGAGTACTTGCGC-3' (reverse primer) All the reactions were set in 20 µL of reaction volume in 8 strip tubes in triplicate. In short, 1µL of cDNA template (50 ng total RNA equivalent) for each sample was assayed in triplicates along with no template controls (NTC) and non-reverse transcription controls (NRT). The reaction mixture contained 10µL of 2XHot start Veri Quest™ Fast SYBR Greenq PCR Master Mix with Fluorescein (USB, Affymetrix, USA), 1 µL (10 pmol) of forward and reverse primer. PCR conditions were: hold at 50 °C for 2 min, initial activation at 94 °C for 5 min followed by 39 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec and extension at 72 °C for 30 sec. After 39 cycles of amplification, final extension was done at 72 °C for 7 min followed by melt curve analysis starting from 65 °C to 90 °C for 20 min with continuous imaging by CCD camera.

Real time qRT-PCR calculations

Relative fold expression of IL-2 gene were determined by $2^{-\Delta\Delta CT}$ method[31]. The ΔC_T values were calculated by subtracting β-actin C_T values from the C_T values of a specific target IL-2 cytokine for each sample. The average ΔC_T for samples collected from normal spleen cells was taken as the calibrator group (ΔC_T calibrator group) for each sample. Then $\Delta\Delta C_T$ values were calculated by subtracting the average ΔC_T for normal spleen cells (ΔC_T calibrator group) from ΔC_T of normalized target values (ΔC_T target group).

Statistical analysis

Statistical analysis of data was done with one way analysis of variance (ANOVA) using SPSS version 20.0 software and DMRT at $p < .05$ and .01 to determine the significant differences among treatment means. Values are expressed as mean ± SEM.

Results

Effect of HAE of *N. cadamba* leaves on differential leukocyte count (DLC) %

Lymphocytes (%) in Gp-I, Gp-II, Gp-III & Gp-IV was 62.65 ± 0.25, 64.47 ± 0.31, 65.95 ± 0.19 and 70.11 ± 0.44, respectively. HAE fed animals exhibited increase in the level of lymphocytes (%) as compared to control group. Dose dependent significant increase ($p < 0.01$) in lymphocytes (%) was found as shown in Table 1.

Determination of humoral immune response against *S. typhimurium* "O" antigen

Mean serum antibody titer in animals of Gp-II (125 mg/kg), Gp-III (250 mg/kg) & Gp-IV (500

mg/kg) was $3413.33^{a} \pm 539.70$, $3840.00^{ab} \pm 572.43$ and $5973.33^{b} \pm 1427.90$, respectively and found significant higher ($p < 0.05$) as compared to Gp-I (control) animals ($1493.33^{a} \pm 213.33$) (Fig. 1).

In vitro effect of HAE of *N. cadamba* on induction of IL-2 cytokine

Induction of IL-2 cytokine was evaluated by using Con A stimulated splenocytes in the presence of different concentrations of HAE. Quantitation of IL-2 was done by ELISA using supernatant of splenocytes culture 48 h post treatment. This experiment revealed that there was direct correlation between HAE concentration and IL-2 production, i.e., IL-2 production enhanced with increase in HAE conc. High concentrations (500 µg/mL and 250 µg/mL) of HAE induced 189 % and 152 % increase in IL-2 respectively as shown in Table 2.

Expression of IL-2 cytokine gene at molecular level by real time PCR analysis

Threshold cycle (C_T) or Quantification cycle (Cq) is the PCR cycle at which the fluorescent signal of reporter dye, i.e., SYBR green crosses an arbitrarily placed threshold or the value at which amplification of target gene gets started after crossing a cut-off value. Value of C_T or Cq will be inversely proportional to amount of amplicon (target gene). Cq value for IL-2 gene from normal spleen cells, spleen cells treated with 250 µg/mL HAE and 500 µg/mL HAE was found to be 28.12, 22.25 and 17.23, respectively (Table 3). Results of qRT-PCR were analyzed by comparative C_T values as suggested[32]. Standardization and optimization of these primers were initially checked using conventional PCR using the same reaction conditions and the products were verified by agarose gel electrophoresis in 1.5 % TAE-Ethidium Bromide stained gel in the presence of 50 bp DNA ladder (Hi-Media, MBT084-200LN) size run at 60V for 1-2 h (Fig. 2). The Amplicons were analyzed by Quantity One® (Biorad) software. Standard curve, melt curve, relative amplification curve of β-actin and IL-2 gene generated by machine is given in Fig. 3.

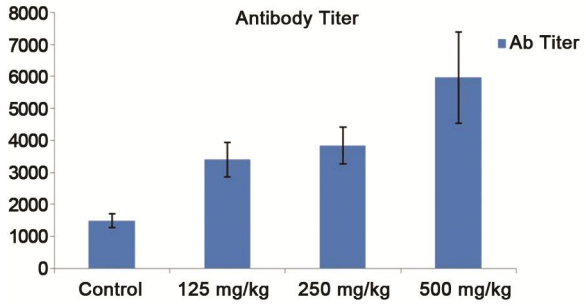

Fig. 1 — Humoral response using *S. typhimurium* "O" antigen in unfed and HAE fed groups

Table 1 — Differential Leukocyte count (%) of Wistar albino rats of control group and experimental groups orally fed with HAE of *N. cadamba* leaves

Parameters (Differential leukocyte count) DLC %	Gp-I (Control) (Average)	Orally treated with HAE Gp-II (125mg/kg) (Average)	Gp-III (250mg/kg) (Average)	Gp-IV (500mg/kg) (Average)
Lymphocytes**	$62.65^{a} \pm 0.25$	$64.47^{b} \pm 0.31$	$65.95^{c} \pm 0.19$	$70.11^{d} \pm 0.44$
Neutrophils^{NS}	31.38 ± 0.39	29.47 ± 0.31	27.90 ± 0.24	23.98 ± 0.51
Eosinophils^{NS}	2.47 ± 0.16	2.53 ± 0.16	2.53 ± 0.12	2.42 ± 0.13
Monocytes^{NS}	3.35 ± 0.13	3.40 ± 0.11	3.47 ± 0.17	3.33 ± 0.15
Basophils^{NS}	0.15 ± 0.02	0.13 ± 0.02	0.15 ± 0.02	0.15 ± 0.02

The values represent the mean ± SEM of six rats. Results are significant at**$p < 0.01$ as per one way ANOVA followed by Duncan's multiple range test (DMRT), NS = non significant.

Table 2 — *In vitro* effect of HAE of *N. cadamba* leaves on IL-2 production

Concentration of extract	Concentration of IL-2 (pg/mL)	% of stimulation
Con-A control	$881.83^{a} \pm 87.39$	-
Con-A control + 20µg/mL HAE	$1153.33^{ab} \pm 92.99$	30.79
Con-A control + 50µg/mL HAE	$1526.33^{b} \pm 102.76$	73
Con-A control + 100µg/mL HAE	$1960.83^{c} \pm 142.97$	122
Con-A control + 250µg/mL HAE	$2222.50^{cd} \pm 151.60$	152
Con-A control + 500µg/mL HAE	$2552^{d} \pm 196.99$	189

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

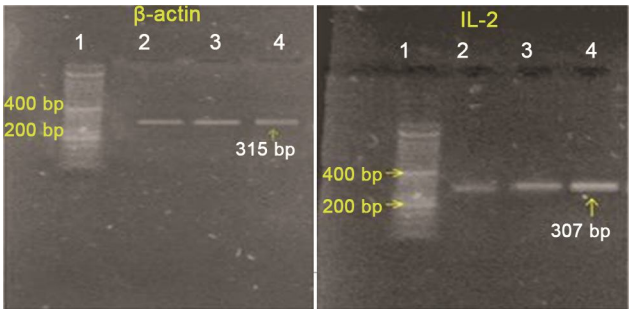

Fig. 2 — Agarose gel electrophoresis of RT-PCR products: Electropherogram of β-actin (house keeping gene) and IL-2 with lane 1 containing Molecular Marker (50 bp), lane 2 containing control (untreated) target gene, lane 3 containing target gene in presence of 250 µg/mL HAE of *N. cadamba* and lane 4 containing target gene in presence of 500 µg/mL HAE of *N. cadamba*

Discussion

In recent years the Scientific concept of immunomodulation has been put forward, and it now seems that few of the benefits of Indian medicinal plants, proposed in *Ayurveda* by '*Charaka*' and '*Sushruta Samhita*', may be due to these "immunomodulation and its effects"[33]. In *Ayurveda* termed '*Rasayanas*' is devoted to enhancement of body's resistance. The main therapeutic strategy involved in the treatment by *Ayurvedic* medicine is to increase body's natural resistance to the agent causing the disease rather than directly neutralizing the agent causing the disease[34]. No study to date has been done on immunomodulatory role of *Neolamarckia*

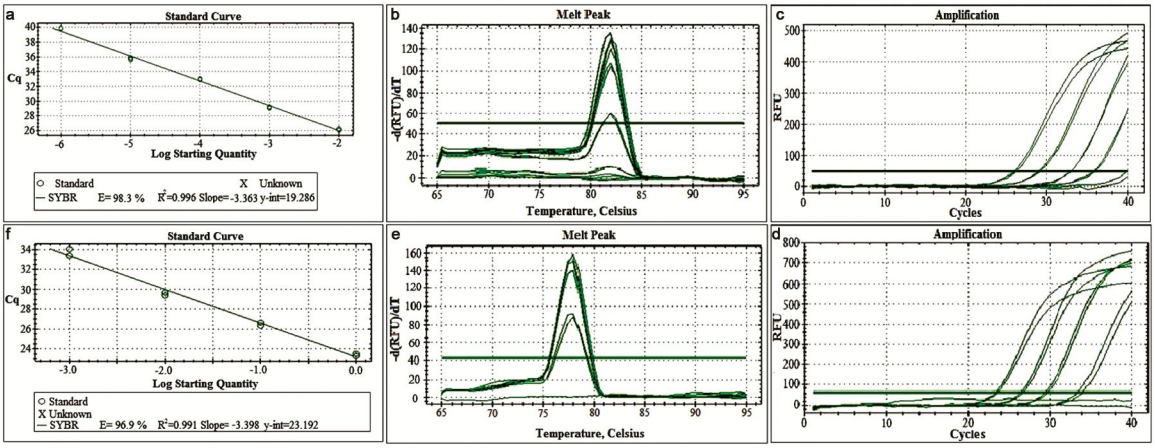

Fig. 3 — Real time data generated by machine for genes β-actin and IL-2, Clockwise from top left: **a-** Standard curve for β-actin gene- standard curve was plotted with cycle threshold (C_T) on the Y-axis against concentration of RNA on X-axis. The Y intercept in figure reflects the theoretical crossing threshold (C_T) value (C_T =19.286) at one copy of input RNA, while the slope (*S*) of the linear regression curve correlates with the efficiency (*E*) of the PCR reaction according to the formula: $E=[10^{-1/slope}-1]$. The calculated PC R efficiency for this assay, based on the slope value of −3.363, was 98.3%.The correlation coefficient, R^2, for the standard curve was 0.996; **b-**Melt curve of β-actin gene with the Tm at approx. 82 ℃ for the specific product; **c-**Relative amplification of β-actin gene with the Cq representing different concentrations of RNA; **d-**Relative amplification of IL-2 gene with the Cq representing different concentrations of RNA; **e-**Melt curve of IL-2 gene with the Tm at approx. 78 ℃ for the specific product; **f-** Standard curve of IL-2 gene - standard curve was plotted with cycle threshold (C_T) on the Y-axis against concentration of RNA on X-axis. The Y intercept in figure reflects the theoretical crossing threshold (C_T) value (C_T =23.192) at one copy of input RNA, while the slope (*S*) of the linear regression curve correlates with the efficiency (*E*) of the PCR reaction according to the formula: $E=[10^{-1/slope}-1]$. The calculated PC R efficiency for this assay, based on the slope value of −3.398, was 96.9 %.The correlation coefficient, R^2, for the standard curve was 0.991.

Table 3 — Expression of target gene (IL-2) in presence of 250, 500 µg/mL HAE of *N. cadamba* with respect to IL-2 of untreated rat (control)

S.N	Gene	Mean Cq value/ C_T value	Fold Change of target gene (IL-2) = $2^{-\Delta\Delta CT}$ $\Delta\Delta CT$ =(Target gene –Target internal control)-(Untreated control gene-Untreated internal control)
1.	IL-2 control rat	28.12	-
2.	IL-2, HAE treated rat (250 µg/mL)	22.25	28.84
3.	IL-2, HAE treated rat (500 µg/mL)	17.23	330.84
4.	β-actin control rat (internal control)	26.42	-
5.	β-actin, HAE treated rat (250 µg/mL)	25.4	-
6.	β-actin, HAE treated rat (500 µg/mL)	23.9	-

cadamba with reference to cytokines induction. The present study was carried out with the objective to validate the use of this plant as immunomodulators.

Maximum increase in white blood cell count with 500 mg/kg b.wt. of HAE of *N. cadamba*[25] and significant increase ($p < 0.01$) in lymphocyte count (%) accounts for its inflammatory activity, thereby indirectly influencing the immunostimulatory effect.

Immunomodulatory efficacy of HAE of *N. cadamba* leaves on humoral immune responses has been studied and a dose dependent significant elevation ($p < 0.05$) in serum antibody titer (Fig. 1) was recorded in rats fed orally with different concentrations of HAE (125 mg/kg, 250 mg/kg and 500 mg/kg) of *N. cadamba* leaves, which is in affirmation with similar findings on *Brahmi*, *Guduchi* and *Barringtonia acutangula*[35,36]. Enhanced antibody titer in presence of HAE reflects the stimulation and proliferation of B-lymphocytes, plasma cells and memory cells, since it is evident that B lymphocytes with / without participation of T helper cell play a vital role in antibody production[37,38]. Marked enhancement in skin thickness during type IV hypersensitivity reaction through 1-fluro 2,4-dinitrochlorobenzene (DNCB) was recorded in previous study[39]. These findings depict the effectiveness of HAE in upregulation of the immune responses, thus helpful to improve the adaptive immune system of host. The augmented humoral and cell mediated immune response corroborates to the fact that the HAE of *N. cadamba* leaves facilitates the proliferation and activation of both B and T lymphocytes.

Keeping the clinical significance of cytokines in mind, immunomodulatory activity of HAE of *N. cadamba* has been analyzed by *in vitro* experiments using Con A induced splenocyte cell culture. In this study, levels of IL-2 in HAE treated rat splenocyte cell culture supernatants were determined by an enzyme linked immune sorbent assay (ELISA) using commercially available kit. A Dose dependent significant augmentation ($p < .01$) in level of IL-2 cytokine in comparison to control was observed (Table 2). These results have a definite bearing to re-iterate the stance that the ability of IL-2 in the direct induction of B lymphocytes[40] as well as its vital role in activation of Th1 subsets in its fight against intracellular pathogens[41,42]. HAE extract of *N. cadamba* leaves might have a potential to enrich humoral immune response against various infections through stimulating the B lymphocytes under the influence of IL-2 inducing Th1 lymphocytes.

The possible role of IL-2 in boosting the immunity has also been reported based on the previous research conducted in AIDS patients, where IL-2 therapy has improved the functional efficacy of immune system by promoting IFN-γ (Th1 cytokine) secretion[43,44]. In view of this, US food and Drug administration (FDA) has given a positive consent to inculcate IL-2 as immunotherapeutic agent for the treatment of malignant melanoma, renal cancer and metastasis[45,46]. Upregulation of IL-2 in splenocyte culture in presence of different concentrations of HAE was further confirmed by qRT-PCR analysis. Fold change in the expression of target gene IL-2 was 28.84 and 330.84 at 250, 500 µg/mL HAE of *N. cadamba* concentrations respectively in comparison to control (untreated) IL-2 gene (Table 3), suggesting the increased expression of m-RNA of IL-2 cytokine gene in presence of varying doses of HAE extract (250 µg/mL and 500 µg/mL). The quantification of mRNA is an indirect way of measuring a cellular biological activity. The best possible way to compare these intermediate molecules is RNA-sequencing based Transcriptome analysis and qRT-PCR. Here the authors report the differential expression of IL-2 gene using qRT-PCR in various groups of *N. cadamba* HAE treated rats against untreated controls. The biological events at the cellular level can be captured through the RNA transcripts, which act as a vital information in deciphering the host response to a particular stimuli. Here the host response is according to the treatment with varied doses of HAE of *N. cadamba*.

The current study suggests alternative therapy using medicinal plants which modulate various biomolecules thereby aiding in positive biochemical reactions influencing the immune system and balancing the homeostasis of the body. These desired effects acts as an impetus to apply such plant ingredients to play a future role in the immunomodulation, beneficial metabolomics and the best alternative ways to boost the overall health. Present study also stated to draw parallels between naturally rewarding plants and modern medicines at molecular level. Results revealed further assessment of the potential use of *N. cadamba* not only as an immunomodulator, but also as a supportive strategy along with conventional therapy in prevention of infectious diseases.

Conclusion

Results obtained during this study indicates that the leaves of *N. cadamba* have immunomodulating potential as indicated by the antibody response in immunized animals. Increase in IL-2 expression at gene as well as at protein level clearly suggests its application in immune suppressed animals. To the best of our knowledge, the current study on the effect of leaves (HAE) of *N. cadamba* on its immunomodulatory effect will be the first documentation supported with data from scientific techniques including sandwich ELISA, qRT-PCR of IL-2 cytokine and TLC. Further well designed clinical studies following precise and standard methodology directed towards the restoration of immune-regulatory mechanisms may be conducted to assess and analyze the medicinal potential of *N. cadamba*.

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