Mature leaf concentrate of Sri Lankan wild type Carica papaya Linn. modulates nonfunctional and functional immune responses of rats

Chanika Dilumi Jayasinghe¹, Dinara S Gunasekera², Nuwan De Silva², Kithmini Kawya Mandakini Jayawardena³ and Preethi Vidya Udagama¹*

Abstract

Background: The leaf concentrate of Carica papaya is a traditionally acclaimed immunomodulatory remedy against numerous diseases; nonetheless comprehensive scientific validation of this claim is limited. The present study thus investigated the immunomodulatory potential of Carica papaya mature leaf concentrate (MLCC) of the Sri Lankan wild type cultivar using nonfunctional and functional immunological assays.

Methods: Wistar rats (N = 6/group) were orally gavaged with 3 doses (0.18, 0.36 and 0.72 ml/100g body weight) of the MLCC once daily for 3 consecutive days. Selected nonfunctional (enumeration of immune cells and cytokine levels) and functional (cell proliferation and phagocytic activity) immunological parameters, and acute toxic effects were determined using standard methods.

Results: Counts of rat platelets, total leukocytes, lymphocyte and monocyte sub populations, and BMCs were significantly augmented by oral gavage of the MLCC (p < 0.05). The highest MLCC dose tested herein significantly reduced proinflammatory cytokines, Interleukin 6 (IL-6) and Tumor Necrosis Factor α (TNF α) levels of rats (p < 0.05). The in vivo phagocytic index of rat PMs significantly increased by oral gavage of all three doses of the MLCC (p < 0.05). In vitro phagocytic activity of rat PMs were enhanced by the MLCC and triggered a Th1 biased cytokine response. The MLCC at low concentrations elicited ex vivo proliferation of BMC (31.25 μg/ml) and SC (31.25 and 62.5 μg/ml) respectively. Conversely, high concentrations (500 and 1000 μg/ml) exhibited cytotoxicity of both BMC and SC with significant modulation of cytokines. Chemical profile of the MLCC revealed the presence of several immunomodulatory compounds. The oral gavage of the MLCC was found to be safe in terms of both hepatic and renal toxicities.

Conclusion: The present study established that the mature leaf concentrate (MLCC) of Carica papaya Sri Lankan wild type cultivar is orally active, safe and effectively modulates nonfunctional and functional immunological parameters of rats that unequivocally corroborate the traditional medical claims.

Keywords: Carica papaya, Sri Lankan wild type cultivar, Mature leaf concentrate, Immunomodulation, Immune cell counts, Phagocytosis, Cytokines

* Correspondence: preethi@zoology.cmb.ac.lk
¹Department of Zoology & Environment Sciences, Faculty of Science, University of Colombo, Colombo 3, Sri Lanka
Full list of author information is available at the end of the article

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Background
Pharmacological manipulation of the immune system is emerging as a novel approach in managing human diseases. There is a recent spurt in the interest of identification of immunomodulatory leads of herbal origin which are safe, efficient and economical in chronic usage.

The immune system is an ensemble of complex defense mechanisms functioning at different levels, from an individual cell to the whole organism [1]. Hence, imbalance of the immune homeostasis is implicated in the onset and progression of chronic ailments [2]. An overactive immune system is associated with autoimmunity, chronic inflammatory diseases, systemic vasodilatation and carcinogenesis [3, 4]. Conversely, immunosuppression increases the susceptibility to infection and is implicated in tumor development [5, 6].

The clinical concept of immunomodulation focuses on stimulating both innate and adaptive arms of immunity or suppressing excessive immune function using exogenous therapeutic agents [2]. Synthetic cytotoxic drugs (cyclophosphamide, methotrexate and 5-fluorouracil), recombinant cytokine therapy, and antibody therapy are established in allopathic medicine as immunotherapy against cancers and autoimmune diseases [7]. However, the occurrence of adverse events and high costs of these have generated barriers to successful therapeutic applications [8]. In this regard, plant based immunomodulators are advantageous considering their pleotropic activity, less side effects and cost effectiveness.

Since ancient times, medicinal plants have been used virtually in all cultures as sources of medicines to improve the immune system. Recently, advancements in immunological techniques enabled the characterization of medicinal plants in unprecedented detail. Numerous plant species such as Aloe vera, Withania somnifera, Allium sativum, Ocimum sanctum, Azadirachita indica and many others have been scientifically justified for their immunomodulatory activity together with possible mechanistics [9].

Carica papaya, (Common name- paw paw) belongs to family Caricaceae and is one of the most popular and economically important plants in the world as a food source and as a herbal medicine [10]. It is native to the tropics of the Americas but now is cultivated worldwide as a fruit crop [10].

Different parts of C. papaya has been traditionally claimed as therapeutics for a broad range of diseases [10]. Particularly the leaf extract/concentrate has been scientifically investigated for pharmacological properties such as anti-microbial, anti-parasitic (antimalarial) activity, anti-cancer, anti-inflammatory and membrane stabilization properties [10].

In Sri Lankan traditional medicine the leaf juice of C. papaya is claimed as a powerful remedy for modulating the immune system (Personal communication- Dr. Pathum Jayaweera, Sri Lankan traditional medical practitioner). Thus far several in vitro and in vivo experiments have been carried out to investigate the immunopharmacological properties of the leaf concentrate of C. papaya.

Our group for the first time, established the platelet and total white blood cell (WBC) increasing activity of the mature leaf concentrate of C. papaya of the red lady cultivar grown in Sri Lanka using hydroxyurea induced thrombocytopenic Wistar rat model as well as in normal counterpart rats [11]. Concurrently, we observed pronounced anti-inflammatory activity of this preparation indicating immunomodulatory potential [11]. Similarly, oral gavage of papaya leaf extract exhibited a significant platelet and WBC increasing activity in immunosuppressive rats using cyclophosphamide [12] and ansgrelide (thrombocytopenic drug) [13].

Dharmarathna et al. [14] observed a marked platelet count elevation in none thrombocytopenic mice following the oral treatment of C. papaya extract for seven days [14]. Another study conducted using non thrombocytopenic rats reported a marked increment in platelet counts, mean cell haemoglobin (MCH) and mean corpuscular volume (MCV) following the oral treatment with this leaf formulation for 7 days [15].

In addition to animal experimentation, the platelet increasing activity of papaya leaf extract has been validated in thrombocytopenia associated dengue patients. An open labeled randomized controlled trial carried out with 228 patients (Treatment = 111, Control = 117) with dengue fever (DF) and dengue haemorrhagic fever (DHF) revealed a significant increase of platelet counts after 40 and 48 h of oral administration of C. papaya leaf juice for 3 consecutive days [16]. A pilot study reported an increase of platelet and WBC counts of dengue patients after 24 h of administration of papaya leaf juice and patients recovered without hospital admission [17]. A similar effect was also observed in an open labeled randomized controlled trial with 30 dengue patients treated with a tablet (Caripill) prepared from C. papaya leaf extract [18]; A significant increase of platelet counts was observed following oral treatment with this tablet three times daily for five days and exerted fewer side effects and good tolerability.

Although, a wealth of information is available on platelet increasing activity of different extracts of C. papaya leaves, a comprehensive account on immunomodulatory potential is limited. Otsuki et al. [19] reported immunomodulatory potential of the aqueous extract of C. papaya leaf using in vitro culture of human peripheral blood mononuclear cells (PBMCs).

When PBMCs were treated in vitro with C. papaya aqueous extract, Interleukin 2 and 4 (IL-2 and IL-4) secretion were attenuated whereas that of Interleukin
Commercial grade chemicals, ethanol, isopropanol, hydrochloric acid, ammonium oxalate, acetic acid were purchased from Sigma Aldrich Co. Ltd. (St. Louis, MO, USA). For LC-MS analysis water was purified by a Milli-Q purification system from Evoqua Water Technologies.

Collection of plant material
Mature leaves (5th leaf from the apex) of Carica papaya (Local name: Sinhala: papoli, gaslabu; Tamil: pappali, pappayi; English: Paw paw, Papaya) of the Sri Lankan wild type cultivar were collected from a home garden in Kadawatha, Gampaha district in Sri Lanka (longitude-79°57′0″E,latitude- 7°4′0″N) during April 2014 to June 2015. The specimen was identified and authenticated by Dr. H Kathriarachchi of the Department of Plant Sciences of the University of Colombo, Sri Lanka. A voucher specimen (No- 120) was deposited at the Department of Zoology and Environment Sciences, University of Colombo, Sri Lanka.

Preparation of mature leaf concentrate
The mature leaf concentrate of C. papaya (MLCC) was prepared essentially following the procedure described in [11] with slight modifications. Briefly, fresh mature leaves of C. papaya (wild type cultivar) were thoroughly washed under running tap water, blotted dried and after removal of petioles and primary veins, leaf blades were pulverized using a mechanical juice extractor (HR,1861, Philips, Hong Kong) without adding water (at 10 g leaf blade/ ml of concentrate). The test animals were orally gavaged directly with the fresh MLCC at doses of 0.18, 0.36 and 0.72 ml/100g of body weight (BW) of rats representing low, mid (human equivalent) and high doses, respectively [11]. For the in vitro assays, the extract was filtered (0.22 μm Millipore filter, 290 Concord Rd, Billerica, MA, USA) and concentrations were calculated by extrapolating the dry weight of the MLCC.

Detection of endotoxins
The fresh MLCC was screened for endotoxin contamination using the Limulus Amebocyte Lysate (LAL) gel-clot test according to the manufactures instructions (the Pyrosate® Kit, Associates for Cape Code Incorporated,124 Bernard E.Saint Jean Drive.E.Falmouth, MA,02536 USA).

Experimental animals
Healthy, adult male and female Wistar rats (180–230 g of weight) purchased from the Medical Research Institute, Colombo, Sri Lanka were used in this study. All animals received humane care. They were housed in plastic cages in the animal house of the Department of Zoology and Environment Sciences, University of Colombo under standard animal house conditions (temperature; 28–31 °C, photoperiod; approximately 12 h natural light per day,
Effects of the MLCC on nonfunctional immunological parameters of rats

Enumeration of immune cells
Four separate groups of adult male Wistar rats (N = 6/group) were orally gavaged with distilled water (DW) as the control, three doses of the MLCC at 0.18, 0.36 and 0.72 ml/100g BW of rats, once daily for three consecutive days. On day 3 post treatment, rats were anesthetized under high dose of ether and blood was collected by cardiac puncture and dispensed into ethylenediaminetetraacetic acid (EDTA) containing tubes. Platelets, and total and differential white blood cell counts were established according to [22]. Femur and spleen were aseptically excised from sacrificed rats and placed in glass vials containing 5 ml of phosphate buffered saline (PBS). Spleen was macerated in PBS to release the splenocytes (SC), and bone marrow cells (BMC) were separated by flushing PBS through the femur; SC and BMC counts were made using a Neubauer’s improved haemocytometer (B.S 748, Weiber, England) [22].

Plasma cytokine levels
Two separate groups of rats (N = 6/group) were orally administrated with the highest dose (0.72 ml/100g BW) of the MLCC and DW as the control once daily for three consecutive days. On day 3 post treatment, blood was collected by heart puncture and dispensed into ethylenediaminetetraacetic acid (EDTA) containing tubes. Platelets, and total and differential white blood cell counts were established according to [22]. Femur and spleen were aseptically excised from sacrificed rats and placed in glass vials containing 5 ml of phosphate buffered saline (PBS). Spleen was macerated in PBS to release the splenocytes (SC), and bone marrow cells (BMC) were separated by flushing PBS through the femur; SC and BMC counts were made using a Neubauer’s improved haemocytometer (B.S 748, Weiber, England) [22].

Effects of the MLCC on functional immunological parameters of rats

Neutral red dye uptake assay
Functional immunological test based on phagocytic activity was measured using neutral red dye uptake assay [22]. In brief, peritoneal macrophages (PMs) were aspirated from the rats treated with the three doses of the MLCC (low: 0.18 ml, mid: 0.36 ml and high: 0.72 ml/100 g BW) into 10 ml of PBS [22]. The cell suspension was centrifuged at 500 x g for 5 min at RT and the resultant cell pellet was dissolved in 1 ml of PBS. Two drops of 1% Neutral Red (in PBS) were added to the cell suspension and PMs with ingested red dye particles were counted using a Neubauer improved haemocytometer (B.S 748, Weiber, England). The phagocytic index was calculated as follows [22]:

\[
\text{Phagocytic index} = \frac{\text{Number of active PM cells}}{\text{Number of total PM cells}} \times 100\%
\]

In vitro phagocytic activity and cytokine profile of rat peritoneal macrophages

Preparation of rat peritoneal macrophages (PMs)
Five milliters of FBS was injected intraperitoneally into rats as a stimulant to elicit PMs [23]. Three days later, the peritoneal exudate was collected by peritoneal lavage with 10 ml of complete RPMI 1640 (CRPMI) medium supplemented with 50 μM 2-mercaptoethanol.

NBT dye reduction assay
In vitro phagocytic activity of rat immune cells was established by the nitroblue tetrazolium (NBT) reduction assay [23]. Twenty microliters of complete RPMI 1640 (CRPMI; Control), different concentrations of the MLCC (31.25, 62.5, 125,250, 500 and 1000 μg/ml), 20 μl of the PM suspension and 40 μl of CRPMI medium were added to wells of a 96-well plate (Corning, Sigma,USA). After incubation for 24 h at 37 °C in 5% CO₂ humidified atmosphere, 20 μl of a heat inactivated yeast (Saccharomyces cerevisiae) suspension (5 × 10⁷ particles/ml) and 20 μl of 1.5 mg/ml NBT in PBS were dispensed and the mixture was further incubated under the same conditions.

After incubation for 60 min, the adherent cells were rinsed vigorously with CRPMI medium and washed four times with 200 μl methanol. After air-drying, 120 μl of 2 M potassium hydroxide (KOH) and 140 μl of DMSO was added. The absorbance was measured at 570 nm using a micro plate reader (Microplate reader 680, Bio-Rad, USA) and the percentage of NBT reduction representing phagocytic activity was calculated using the following equation [23]:

\[
\text{Phagocytic activity} (\%) = \frac{\text{OD sample}-\text{OD negative control}}{\text{OD negative control}} \times 100
\]

Cytokine profiling of PM cell cultures
Briefly, 40 μl CRPMI (Control), different concentrations of the MLCC (31.25, 62.5, 125,250, 500 and 1000 μg/ml), 20 μl of the PM suspension and 40 μl of CRPMI medium were cultured in wells of a flat bottom 96-well plate (Corning, Sigma, USA). After incubation for 24 h at 37 °C in 5% CO₂ humidified atmosphere, 20 μl of a heat inactivated yeast (Saccharomyces cerevisiae)
suspension (5 × 10^7 particles/ml) and 40 μl of CRPMI were added and incubated at the same condition for another 1 h. Cultures were centrifuged at 1000 x g at 4 °C for 10 min and the resultant supernatants were analysed for IFN-γ and IL-10 using rat sandwich ELISA kits according to the manufacturer's instructions (BD Bio science, Torreyana Rd., San Diyeo, CA 92121). Viable cells of each concentration were calculated using trypan blue dye exclusion assay [20] and cytokine levels were normalized to the viable cell count.

**Ex vivo proliferation and cytokine profiling of rat immune cells**

**Preparation of rat bone marrow cells (BMC) and splenocytes (SC)**

Femurs and spleens were aseptically excised from scari-
fied Wistar rats. Bone marrow cells were obtained by flushing PBS through the femur bone cavity [22]. Spleens were collected into PBS and the SCs were gently released. Both cell suspensions [22] were centrifuged at 300 x g, at 25 °C for 10 min [24]. The erythrocytes in the cell suspensions were then lysed by hypotonic solution (0.2% NaCl) and the cells were resuspended in 1.6% NaCl to restore the isotonicity [23]. Cell suspensions were washed twice with CRPMI medium supplemented with 10% heat-inactivated FBS, 100 U penicillin, and 100 μg/l streptomycin [23, 24]. The cell numbers were adjusted to 10^6 cells/ml using a Neubauer's improved haematocytometer (B.S 748, Weiber, England). Trypan blue dye exclusion assay was performed to assess the viability of both cell types [20].

**MTT based cell proliferation assay**

The in vitro cell proliferation assay was carried out using 4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay without mitogenic stimulation [24, 25]. Briefly, 20 μl of CRPMI as the control and various concentrations of the MLCC (31.25, 62.5, 125, 250, 500 and 1000 μg/ml) and 2 μg/ml Lipopolysaccharides (LPS) as a positive control were added to 20 μl of BMC or SC cell suspensions (10^6 cells/ml). Another 40 μl of CRPMI was added to the 96- well plate (Corning, Sigma,USA) and incubated at 37 °C in a humidified 5% CO2 atmosphere for 48 h. Subsequently, 20 μl of MTT (5 mg/ml) in PBS and 40 μl of RPMI was added. The culture medium was removed by aspiration and 100 μl of 0.04 M hydrochloride acid (HCl) in isopropyl alcohol were added to lyse the cells. Following the addition of 100 μl of distilled water absorbance was measured at 570 nm using a micro plate reader (Microplate reader 680, Bio-Rad, USA) and the % cell proliferation was calculated using the following equation [23, 24]:

\[
\text{Cell proliferation (\%) = } \frac{\text{OD of treated-OD of control}}{\text{OD of control}} \times 100
\]

**Cytokine profiling of rat BMC and SC cultures**

Briefly, 40 μl of BMC or SC suspension was cultured with 40 μl of CRPMI (control) and different concentrations of the MLCC (31.25, 62.5, 125, 250,500 and 1000 μg/ml) and 120 μl of CRPMI in a 96 well plate for 48 h. Culture medium of each well was collected, centrifuged at 1000 x g at 4 °C for 10 min and the supernatants were subjected to cytokine analyses. Cytokine levels were quantified using rat sandwich ELISA kits according to the manufacturer's instructions (BD Bio science, Torreyana Rd., San Diyeo, CA 92121). Viable cells of each concentration were calculated using trypan blue dye exclusion assay [20] and cytokine levels were normalized to the viable cell count.

**Acute toxicity of the MLCC on rats**

Hepato and nephro toxic parameters were measured in rats (n = 6) treated for three consecutive days with the highest dose (0.72 ml/100g BW) of the MLCC compared with the control group. Serum parameters such as aspartate transaminase (AST), alanine transaminase (ALT), urea, blood urea nitrogen (BUN), creatinine levels were determined using Randox kits (Randox Laboratories Ltd., Co. Antrium, U.K) and the spectro-
photometer (JASCO V560, Jasco Corporation, Tokyo, Japan) as per manufacturer's instructions [26].

**Chemical profiling of the MLCC**

**LC-MS analysis**

LC-MS analysis were performed on a Waters e2695 instrument equipped with a photodiode array (PDA) detector (model 2998,). The separations were carried out on a reversed phase column (Waters Spherisorb 5 μm ODS2, 4.6x250mm) maintained at 20 °C. The sample was scanned from 200 to 800 nm with a reso-
lution of 1.2 nm.

The mobile phase consisted of 50% of solvent A (0.1% v/v of formic acid in water) and 50% of solvent B (0.1% v/v of formic acid in methanol) at a flow rate of 500 μl/min for 30 min under isocratic conditions.

**GC-MS analysis**

GC-MS studies were carried out using Agilent 7890B GC system and MS of 5977A Mass Selective Detector (MSD). The separations were carried out on Agilent HP-5 ms ultra inert column (30 m × 250 μm × 0.25 μm). Initial temperature for the GC analysis was 50 °C (held for 5 min), which was raised to 230 °C (ramp 20 °C/min). The 230 °C temperature was maintained for 10 min. The sample(1 μl) was injected using split-less mode with an
inlet temperature of 260 °C. Helium was used as the carrier gas with a flow rate of 2 ml/min. MS transfer line temperature was maintained at 260 °C. The assignment of the peaks is based on the National Institute of Standards and Technology (NIST) reference database.

Statistical analyses
Data were expressed as Mean ± SEM (Standard error of means). The in vitro experiments were examined in three individual experiments, performed in triplicate for each concentration. One-way Analysis of variance (ANOVA) followed by post-hoc Tukey HSD Calculator were used for multiple comparison.

In vivo experiments entailed 6 rats per group and statistical comparisons were determined using the Mann Whitney U test.

SPSS –20 (IBM, US) statistical package was used for data analysis and $p < 0.05$ and $\ast p < 0.01$ was considered as indicative of significance as compared to the control group.

Results
Detection of endotoxin contamination of the MLCC
Adding the LAL reagents to the MLCC did not result in a firm clot which was visible in the positive control of the test kit indicative of absence of the endotoxins.

Effect of the MLCC on nonfunctional immunological parameters of rats
Enumeration of immune cells; platelet, white blood cell (WBC), bone marrow cell (BMC) and splenocyte (SC) counts of rats
Compared with the control, platelet counts were significantly increased by 42, 59 and 68% in rats treated with low, mid and high doses of the MLCC ($p < 0.05$), respectively (Fig. 1a). The mid and high doses of the MLCC significantly increased the total WBC by 15 and 19% ($p < 0.05$), respectively (Fig. 1a). Similarly, BMC counts significantly increased by the mid (25%) and high doses (49%) ($p < 0.05$) (Fig. 1a). Differential WBC count of the MLCC treated rats recorded a marked amelioration of monocyte and lymphocyte counts compared with the control ($p < 0.05$); Monocyte and lymphocyte counts were significantly increased by mid (43.23%, 7.5%) and high (44.67%, 10%) doses ($p < 0.05$), respectively, of the MLCC (Fig. 1b).

Plasma cytokine levels
The oral administration of the highest dose of the MLCC compared with the control significantly reduced pro inflammatory cytokines, TNFα (by 39.09%) and IL-6 (by 55.06%) as depicted in Fig. 2 ($p < 0.05$). Although IFNγ levels were also reduced in treated groups compared to controls, the reduction was not significant ($p > 0.05$) (Figure 2). Plasma levels of IL-10 of both control and treated rats were below the detection level of the ELISA kit used.

Effect of the MLCC on functional immunological parameters of rats
Phagocytic activity of peritoneal macrophages (PM) of rats
The functional assay based on phagocytic activity was significantly and dose dependently ($r^2 = 0.99$) increased in rats of all treated doses (low: by 43% mid: 79% and high: 109%) compared with the rats of the control group ($p < 0.05$) (Figure 3).

In vitro phagocytic activity of rat peritoneal macrophages (PMs)
As represented in Fig. 4A the MLCC markedly enhanced in vitro phagocytic activity of rat PMs at MLCC concentrations of 62.5, 125, 250, 500 and 1000 μg/ml by 112.5, 168.75, 189.58, 139.5 and 72.91%, respectively ($p < 0.05$). The exception was the lowest concentration of MLCC tested (31.25 mg/ml) that had no effect on the phagocytic activity of PMs.

Cytokine profile of peritoneal macrophage (PM) culture
Compared with the control, the MLCC at 62.5, 125, 250, 500 and 1000 μg/ml concentrations significantly enhanced both IFNγ and IL-10 from cells in PM culture ($p < 0.05$) (Fig. 4b). However, the control and 31.25, 62.5, 125 and 250 μg/ml of MLCC exhibited a Th1 (T helper 1) biased cytokine response (Th1/Th2 < 1) while higher concentrations 500 and 1000 μg/ml concentrations exerted a Th2 biased response (Th1/Th2 < 1) (Fig. 4c).

Ex vivo proliferation of rat BMCs and SCs
Compared with the control the MLCC at 31.25 μg/ml concentration significantly increased the proliferation of BMCs by 63.2% in the absence of mitogens ($p < 0.01$). Conversely, the MLCC at high concentrations of 500 and 1000 μg/ml exerted a significant cytotoxic effect with 36.76 and 37.09% cell inhibition of BMCs ($p < 0.05$), respectively (Fig. 5a).

Similarly, significant cell proliferation of 39.62 and 33.96% was observed in SC cultures treated with MLCC at 31.25 and 62.5 μg/ml concentrations, respectively ($P < 0.01$). Similar to that of the BMCs the highest tested concentrations of 500 and 1000 μg/ml manifested 17.82% and 19.08% cell inhibition respectively, indicative of significant cytotoxic activity ($p < 0.05$) (Fig. 5c).

The MLCC at 125 and 250 μg/ml concentrations had no significant effect on both BMC and SC and the cell viabilities were comparable to that of the control.
LPS (2 μg/ml), a known mitogen, elicited a significant enhancement of both BMC (158.8%) and SC (127.88%) proliferation, respectively as presented in Figs. 5a, c ($p < 0.01$).

Cytokine profile of rat BMC and SC culture supernatants
IFNγ (Th1 cytokine) levels of BMCs treated with MLCC with the exception of 31.25 μg/ml concentration were significantly higher compared to that of the control (Fig. 5b) ($p < 0.05$). However, IL-10 level of BMCs was only increased by the 62.5 μg/ml MLCC treatment ($p < 0.05$) (Fig. 5b).

All tested concentrations of the MLCC significantly stimulated the secretion of IFNγ from the SCs compared significantly higher compared to that of the control (Fig. 5b) ($p < 0.05$). However, IL-10 level of BMCs was only increased by the 62.5 μg/ml MLCC treatment ($p < 0.05$) (Fig. 5b).
with the control ($p < 0.05$). Conversely, the MLCC at 31.25, 62.5 and 125, 250 and 500 μg/ml concentrations significantly inhibited the secretion of IL-10 from SCs compared with the control ($p < 0.05$) (Fig. 5d). While SCs treated with 1000 μg/ml was comparable to that of the control ($p > 0.05$) (Fig. 5d).

The BMCs treated with LPS (2 μg/ml) significantly increased the IL-10 ($p < 0.05$) level while IFNγ was comparable ($p > 0.05$) to that of the control. Conversely, SCs treated with LPS significantly elevated IFNγ level and inhibited the IL-10 level compared to that of the control ($p < 0.05$) (Figs. 5b, d).

**Acute toxicity of the MLCC on rats**
Acute oral treatment of the highest dose (0.72 ml/100g BW) of the MLCC elicited neither hepato nor renal toxicities. Serum levels of liver functional parameters (ALT and AST) were not significantly different in the treated group compared to the control ($p > 0.05$) (Fig. 6). Similarly, renal parameters (urea, BUN and creatinine) were unaltered in the test group compared to the control (Fig. 6).

**Chemical profile of the MLCC**
Figure 7a represents the major peaks obtained for the MLCC from the PDA detector. Retention times and wavelengths of major peaks observed are summarised in Table 1.

The GC-MS chromatographic peaks are presented in Fig. 7b and the identified compounds are lists in Table 2.

**Discussion**
The present study for the first time established, that the mature leaf concentrate of the Sri Lankan wild type cultivar of *C. papaya* modulates both nonfunctional and functional immune responses of Wistar rats.

The oral administration of MLCC for 3 consecutive days had significantly ameliorated rat immune cell counts i.e. platelets, WBC and BMCs, where platelet increasing activity was pronounced. Increase of both platelets and WBCs were consistent with previous studies established with non-thrombocytopenic [14, 15] as well as thrombocytopenic murine models [12, 13].

The immunostimulatory potential of the MLCC is well illustrated in the numerical increment of total WBCs as
well as of sub populations of monocytes and lymphocytes. Significant increase in the percentage of circulating mononuclear cells (lymphocytes and monocytes) indicated the effectiveness of the MLCC on both innate and adaptive arms of the immune system [1]. Previously, aqueous leaf extract of *C. papaya* treated peripheral blood mononuclear cells (PBMCs) elicited an up regulation of 23 genes mainly including monocyte chemo-attractant protein-1, 2 and 3 (MCP-1, MCP-2 and MCP-3) [19]. These proteins regulate the migration and infiltration of monocytes/macrophage [27]. Thus, activation of monocytes in numbers and function by the papaya leaf constituents raise the possibility of enhancing immunological surveillance.

The mechanism of the papaya leaf constituents induced platelet and leukocyte increment continues to be explored. Augmentation of bone marrow hematopoiesis is the most debated mechanism in literature [28]. Tham et
al., [30] demonstrated that plasma levels of IL-6 were significantly reduced in rats treated with the MLCC. Although, papaya leaf constituents may follow this type of dose response. Previously, upregulated synthesis of IL-6, a major thrombopoietic cytokine, was observed in human peripheral blood lymphocytes and stem cells from exfoliated deciduous teeth following the treatment with unripe papaya extract rich in papain [30]. This finding prompted us to investigate the effects of the oral gavage of MLCC on rat cytokine levels which may regulate the immune responses.

The present study established, in contrast to Aziz et al. [30], that plasma levels of IL-6 were significantly reduced in rats treated with the MLCC. Although, papaya based extracts are known to induce IL-6 levels in in vitro stem cell cultures, oral administration of the MLCC had reduced the systemic IL-6 levels. This paradox may be due to different experimental systems and pleiotropic activity of IL-6 [31].

Similar to that of IL-6, TNFα levels were significantly reduced by the oral treatment of MLCC. Conversely, the reduction of IFNγ levels was not significant. Since, IL-10 levels were lower than the detection levels of the ELISA kits used, the influence of MLCC on IL-10 cannot be predicted. However, inhibition of TNFα indicated anti-inflammatory property of the MLCC which was established previously [11, 32].

The MLCC administered orally to rats enhanced phagocytosis of peritoneal macrophages and suggested a modulation of functional immunity of rats. Consistent with our findings, Tomar et al. [21] in 2012 demonstrated that oral treatment of an alcohol extract of C. papaya leaf significantly increased carbon clearance by the rat reticular-endothelial system that provided evidence for enhanced phagocytosis [21].

The in vitro phagocytic activity based on NBT dye reduction assay, revealed an enhancement of phagocytosis by the MLCC treatment. However, precise dose dependency was not observed. Increased phagocytosis in both in vitro and in vivo experiments signifies the immunostimulatory potential of the MLCC.

Increased phagocytic activity of the PMs was correlated with Th1 type cytokine response. IFNγ enhances phagocytosis and increases protection towards pathogens [33]. The MLCC mediated IL-10 may stimulate the anti-inflammatory responses [34] and may lessen the consequences of inflammation.

The functional immunological assays based on ex vivo proliferation provide more concrete evidence for immunomodulatory potential of the MLCC. The proliferation ability of the MLCC was compared with LPS, a bacterial endotoxin that revealed paradoxical activity i.e. low concentrations of the MLCC stimulated the proliferation while higher concentrations exhibited cytotoxic activity. Such a biphasic dose responsive effect is termed “hormesis” [35]; A comparable response pattern was observed in in vitro membrane stabilization potential of papaya leaf extract [36]. Thus, we assume bioactivities of the papaya leaf constituents may follow this type of dose response.

The significant proliferation of BMCs and SCs by the low concentration of the MLCC may be attributed to the presence of single or several types of mitogens or growth factors. Plant mitogens such as lectins are glycoproteins that nonspecifically bind to cell surfaces and stimulate cells to undergo mitosis [37]. However, these are selective in triggering T or B cell populations. Previously, several mitogens such as Phytohaemagglutinin(PHA), concanavalin A (Con A) and Pokeweed Mitogen (PWM) have been identified from plants and these mostly impacted on the proliferation of T lymphocytes [37]. Conversely, polysaccharides, polynucleotides or lipoproteins from bacterial cell wall favor B cell proliferation. There is a high possibility that fresh plant extracts
Fig. 7 Chemical Profiling of the MLCC. a LC-MS chromatogram, b GC-MS chromatogram of the MLCC. Molecular weights, peak retention times and wave length of the peaks are given.
such as the MLCC may contain bacteria and their products (endotoxins) which may provide false positive results. To eliminate such contaminations the MLCC was filtered using 0.22 μm filter and furthermore, contamination of bacterial endotoxins was ruled out due to negative results obtained from the LAL test, confirming that the mitogenic activity indeed resulted due to phyto-constituents of the MLCC.

Previously, human PBMC cultures treated with C. papaya aqueous leaf extract revealed an enhanced production of cytokines, such as IL-12p40, IL-12p70, IFN-γ and TNF-α without growth inhibition [19]. A similar pattern of IFNγ release was observed in both rat cell types (BM and SC) we tested, but in contrast to Ostuski et al. [19] we observed significant cell inhibition at high concentrations (500 and 1000 μg/ml) of the MLCC. This disparity may be due to different types of preparation of the papaya leaf, different papaya cultivars used, and cell types used in these two studies.

IFNγ has been specifically reported as a hematopoietic inhibitor as it attenuates human bone marrow colony formation and inhibits CD34+ bone marrow cells [38]. Thus, IFNγ may have played a major role in the cell inhibition observed in high concentrations of the MLCC (500 and 1000 μg/ml). Similarly, elevated level of IFNγ was observed in SCs upon treatment with the MLCC and higher IFNγ levels were correlated with the inhibition of SCs counts. Thus, MLCC induced higher IFNγ may contribute to the inhibition of both BMCs and SCs under in vitro conditions.

Both in vivo and in vitro murine models verified the remarkable immunomodulatory potential of the MLCC. In vivo immunomodulation indicated that the active constituents of the MLCC are indeed bioavailable and effective against the ethno-pharmacologically accepted oral route [39].

The ethno-pharmacological significance of the MLCC was further emphasized by its safety. Though, the MLCC exerted in vitro cytotoxicity at higher
concentrations against cultured cells it was well tolerated by rats showing no overt signs of toxicity, stress, aversive behavior or behavioral changes. Further, hepatotoxicity and renal toxicity were also ruled out. Also, the MLCC failed to alter the body weights and the weights of vital organs of the test rats.

The chemical profile data obtained from LC-MS and GC-MS chromatograms revealed the presence of several bioactive compounds. The LC-MS profile exhibited several peaks and it is presumed these peaks resemble both mitogenic and anti-inflammatory compounds. Among them phenolics and flavonoids may be prominent as previous studies reported phenolic and flavonoids were abundant in the papaya leaf [40]. Further, studies are required to identify the compounds obtained from the LC-MS analysis.

Several potent anti-inflammatory compounds such as Azelaic acid [41], 1-Hexadecanoic acid [42], antioxidant compounds; 2,4-Di-tert-butylphenol [43], were elucidated in the GC-MS analysis. Hence, reported immunomodulatory properties of the MLCC could be attributed to these compounds. In addition, the GC-MS profile revealed the presence of several cytotoxic compounds such as 9-Octadecenoic acid methyl ester [44], 2,4-Di-tert-butylphenol [43] and Benzyldihydrazone [45]. Thus, these compounds may have contributed to the MLCC induced cytotoxicity observed in higher MLCC concentrations in in vitro cultures of PM, BMC and SCs and warrant the investigation of anticancer activity of the MLCC.

The remarkable ability of differential modulation of the immune system was previously reported for a few plants such as *Echinacea angustifolia* and *Pelargonium sidoides Echinacea* [39]. However some preparations are active only in in vitro systems. Nevertheless, the MLCC is an orally active, safe, and readily available preparation which has the potential to develop a therapeutic lead, with immense benefit to individuals suffering from immune disorders such as infections, autoimmune diseases and cancers.

**Conclusion**

The present study in toto established that the MLCC effectively modulates the nonfunctional and functional immune responses such as stimulation of immune cell proliferation, increase of phagocytosis activity and modulates cytokine responses. Several active secondary metabolites with immunomodulatory properties were identified.

Collectively, when administered orally, the MLCC is safe (non-toxic) for a period of 3 days and is orally active, effectively modulates the immune response and inhibits pro-inflammatory cytokines which overly justify claims of traditional medicine. Hence, the MLCC may be a potential candidate for further research leading to the development of a herbal therapeutic agent for modulating the immune system in numerous diseases.

**Abbreviations**

ALOX-12: Arachidonate 12-Lipoxigenase; ALT: Alanine transaminase; AST: Aspartate transaminase; BALB: Bagg Albino laboratory bred; BMC: Bone marrow cells; BUN: Blood urea nitrogen; BW: Body weight; Con A: Concanavalin A; CDX: Cyclooxygenases; CRPMI 1640: Complete Roswell Park Memorial Institute; DF: Dengue fever; DHF: Dengue haemorrhagic fever; DMSO: Dimethyl sulfoxide; DM: Distilled water; EDTA: Ethylenediaminetetraacetic acid; ELISA: Enzyme Linked Immunosorbent Assay; FBS: Fetal bovine serum; GC-MS: Gas-chromatography- mass spectroscopy; HA: haemagglutination antibody; HCl: Hydrochloric acid; IFNy: Interferon gamma; IL-10: Interleukin 10; IL-6: Interleukin 6; KOH: Potassium hydroxide; LAL: Limulus amebocyte lysate; LC-MS: Liquid chromatography- mass spectroscopy; LLOX: Lipooxygenases; LPS: Lipopolysaccharides; MAb: Monoclonal antibody; MAb: Monoclonal antibody; MAPKs: Mitogen-activated protein kinases; MCH: Mean cell haemoglobin; MCP: Monocyte chemo-attractant protein; MCV: Mean corpuscular volume; MLCC: Mature leaf concentrate of *Carica papaya*; MSD: Mass Selective Detector; MT: 4S-dimethylthiazole-2-yl-2,5-diphenyloxetrazolium bromide; NBT: Nitrobluetetrazolium; NIST- National Institute of standards and Technology (NIST); OECD: Organization for Economic Co-operation and Development; PBMC: Peripheral blood mononuclear cells; PBS: Phosphate Buffered Saline; PDA: photodiode array; PHA: Phytohaemagglutinin; PMs: Peritoneal macrophages; PNM: Pekeweite Mitogen; RPMI 1640: Roswell Park Memorial Institute 1640; SC: Splenocytes; SEM: Standard error of means; Th cells: T helper cells; TNF α: Tumor Necrosis Factor α; WBC: white blood cells.

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**Availability of data and materials**

The supporting materials can be obtained upon request via email to the corresponding author.

**Authors’ contributions**

PVU and CDJ contributed towards the conception, analysis, and manuscript writing. CDJ and KMWJ conducted the laboratory experiments. DSG and NDS conducted the LC-MS and GC-MS analyses of the MLCC and data interpretation. PVU made contributions to acquisition of data, interpretation of data and intellectual content. All authors read and approved the final manuscript.

**Authors’ information**

PVU, a Professor in Zoology at the University of Colombo, Sri Lanka, obtained her PhD in Immuno-parasitology from the Faculty of Medicine, University of Colombo. She was awarded with visiting & postdoctoral fellowships from NSERC, Canada. PVU has many peer reviewed journal publications and is currently supervising a research project on “Hematological, anti-inflammatory potential and toxicity of mature leaf concentrate of *Carica papaya*” (AP//3//2012//CG//29).

CDJ obtained an MSC in Epigenetics from the University of Osaka, Japan and is a Senior Lecturer at the Open University of Sri Lanka. She is currently reading for her PhD at the department of Zoology and Environment Sciences, University of Colombo. CDJ is the research assistant of the research project “Hematological, anti-inflammatory potential and toxicity of mature leaf concentrate of *Carica papaya*” (AP//3//2012//CG//29).

DSG is Senior Research Scientist at SUNTEC and obtained MSc (Organic Chemistry) from University of Minnesota, USA and PhD from University of Purdue, USA.

NDS is a Research Scientist at SLINTEC (Material Chemistry). NDS obtained his PhD from the University of Mississippi State University, USA. KMWJ was an undergraduate at the Department of Biotechnology, Faculty of Agriculture & Plantation Management, Wayamba University of Sri Lanka.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.
Ethics approval
Ethical approval for the laboratory animal study was obtained from the Institute of Biology, Sri Lanka (Ethical approval number -OBSL 111 05 20). Hence, all experiments conducted were in compliance with the Organization for Economic Co-operation and Development (OECD) guidelines.

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Author details
1Department of Zoology & Environment Sciences, Faculty of Science, University of Colombo, Colombo 3, Sri Lanka. 2Sri Lanka Institute of Nanotechnology, Mahenwatte, Pitipana, Homagama, Sri Lanka. 3Department of Biotechnology, Faculty of Agriculture & Plantation Management, Wayamba University of Sri Lanka, Wayamba, Sri Lanka.

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