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Immunostimulatory Activity of Sri Lankan Wild Type *Carica papaya L.* Mature Leaf Concentrate in a Rat Model

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ABSTRACT

Modulation of the immune system by plant based immunomodualtors is well accepted. The present study evaluated the immunomodulatory activity of the mature leaf concentrate (MLC) of *Carica papaya* Sri Lankan wild type variant, to fill an existing knowledge gap. Separate groups of albino Wistar rats (N=6) were orally administered with 3 doses (low: 0.18, mid: 0.36 and high: 0.72 ml/100 g body weight) of the MLC and distilled water as the control for 3 consecutive days. Selected nonfunctional and functional immunological parameters were determined at the 3rd day post treatment using standard methodology. Liver and kidney functional parameters were determined to assess the acute hepato and renal toxicities, respectively. The MLC elicited significant immunostimulatory activity for both nonfunctional and functional immunological assays. Rat platelet count was significantly (P<0.05) increased by all three doses at post treatment while total WBC, monocyte, lymphocyte and bone marrow cell counts were significantly (P<0.05) increased by mid and high doses of the MLC. Conversely, no significant (P>0.05) difference was observed for splenocyte counts in treated rats. The phagocytic activity based on functional assay was significantly (P<0.05) increased by all three doses compared to the control. Further, the oral administration of the MLC neither provoked acute hepato or renal toxicities. Thus, the present study confirmed that mature leaf concentrate (MLC) of *Carica papaya* Sri Lankan wild type variant is orally active and effectively stimulates the nonfunctional and functional immunological parameters tested with no acute liver and kidney toxicities.

KEYWORDS*: Carica papaya*, Mature Leaf Concentrate, Immunomodulation, Immunostimulatory Activity

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INTRODUCTION

The Immune system is an elaborate system which protects our body from a variety of pathogens including viruses, bacteria, fungi and parasites (Goldsby *et al*., 2002). It mounts immediate responses to pathogens by the activation of immune cells, cytokines, chemokines and also releasing inflammatory mediators (Sharififar *et al.*, 2009). Dysfunction of the immune system makes our body more susceptible to infections and can lead to chronic immune disorders (Kumar *et al.*, 2011).

Alleviation of various diseases by immune modulatory agents has been of interest for many years (Kumar *et al.*, 2011). Although, allopathic medicine has succeeded in developing some immunostimulatory drugs (mostly steroids) these are mostly associated with undesirable side effects and are less effective in chronic immune disorders (Spelman *et al.*, 2006). Meanwhile, medicinal plants have gained renewed interest for their ability to modulate the human immune system, safely and cost effectively (Kumar *et al.*, 2011).

Carica papaya, of the family *Caricaceae* is a common fruit plant with significant medicinal importance (Otsuki *et al*., 2010). In traditional medicine, *C. papaya* has been claimed as a promising remedy against numerous diseases. Its effectiveness against viral, fungal and parasitic infections (Subenthiran *et al.*, 2013) and as an anti-inflammatory (Gammulla *et al.*, 2012) and anti-cancer (Otsuki *et al.*, 2010) agent have been scientifically justified. However, thus far its potential role of modulating the immune system has not been tested.

Thus, we for the first time undertook the present study to evaluate the immunomodulatory activity of the mature leaf concentrate (MLC) of *C. papaya* Sri Lankan wild type variant using both non-functional and functional immunological assays.

MATERIALS AND METHODS

Collection of Plant Material

Mature leaves (5th leaf from the apex) of *C. papaya* of the wild variety were collected from a land plot in Kadawatha, Gampaha district in Sri Lanka (longitude-79º57´0´´E,

latitude- 7°4´0´´N) during March to August, 2014. The specimens were identified and authenticated by the Department of Plant Sciences of the University of Colombo and voucher specimens were deposited in the Department of Zoology.

Preparation of Leaf Concentrate

Mature leaves of *C. papaya* (wild variety) were thoroughly washed with running tap water, blotted dry and after removal of petioles and primary veins, leaf blades were pulverized using a mechanical juice extractor (Philips 1861) without adding water (1 ml of concentrate was extracted from 10 g leaf blade).

Experimental Animals

Ethical clearance was obtained for animal studies (ERC IOBSL 111 05 29). Healthy, adult male and female albino Wistar rats (180-250 g) purchased from the Medical Research Institute, Colombo, Sri Lanka were housed in plastic cages in the animal house of the Department of Zoology, University of Colombo under standard animal house conditions as described in Gammulla *et al.* (2012).

Effect of the MLC on Total and Differential WBC, RBC and Platelet Counts of Rats

Three groups (N=6 / group) of Wistar rats (180-250 g) were orally treated with three doses (low: 0.18 ml, mid: 0.36 ml and high: 0.72 ml/100 g body weight) (Gammulle *et al.*, 2012) of the MLC for 3 consecutive days. The control group (N=6) received distilled water. On day 3 post treatment blood parameters: total and differential White Blood Cell (WBC) and Red Blood Cell (RBC) counts (Ghai, 2000), platelet counts (Brecker, 1950) were measured using Neubauer's improved Haemocytometer (B.S 748, Weiber, England).

Effect of the MLC on Spelenocytes and Bone Marrow Cell Counts

On day 3 post treatment rats were sacrificed and spleen and the left femur were collected into glass vials containing 5 ml of Phosphate Buffered Saline (PBS, pH 7.4). Spleen was macerated to take out the splenocytes (Hudson and Hay, 1989). Bone marrow cells were separated by flushing PBS through the bone (Hudson and Hay, 1989**).** The cell counts were taken using a Neubauer's improved Haemocytometer.

Effect of the MLC on Phagocytic Activity of Peritoneal Macrophages

Functional immunological assay based on phagocytic activity was measured using neutral red dye uptake assay (Hudson and Hay, 1989). In brief, peritoneal macrophages were aspirated from the rat peritoneal cavity (Hudson and Hay, 1989) into 10 ml of PBS. The cell suspension was centrifuged at 1,500 rpm for 5 min and the resulted cell pellet was dissolved in 1 ml of PBS. Two drops of 1 % neutral red were added. After 10 min, phagocytic counts were measured using a Neubauer's haemocytometer. The macrophages stained red–pink were considered as activated cells. The phagocytic capacity was calculated as follows.

$$\text{Phagocytic capacity} = \frac{\text{Number of active cells}}{\text{Total phagocytic cells}} \text{ X } 100$$

Preliminary Phyto and Biochemical Analysis of the MLC

Preliminary qualitative phyto and bio chemical Screening of the MLC was carried out according to standard protocols as described in Fransworth, (1993). Presence of carbohydrates, proteins, amino acids, alkaloids, flavonoids polyphenols, saponins and tannins,in MLC was determined.

Acute Toxicity Assay

Two separate groups of rats (N=6) were orally administered with the highest dose (0.72 ml/100 g) of MLC and distilled water was given to the control group (N=6) for three consecutive days. On day 3 post treatment, 1.5 ml of blood from each rat was collected by heart puncture. Serum parameters (Alkaline Phosphatase-ALP, Aspartate Aminotransferase -AST, urea, creatinine levels) were determined using Randox kits (RANDOX LABORATORIES Ltd., Co. Antrium, U.K) and the spectrophotometer (JASCO V560, Jasco Corporation, Tokyo, Japan) as per manufacturer's instructions**.**

Statistical Analysis

Data were described by mean and standard error of mean ± SEM. Effects of different MLC doses were analyzed using Mann-Whitney U-test. (Minitab, 14.0, Statistical package). The significance level was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

The present study examined the functional and non-functional immuno modulatory effects of the oral administration of freshly prepared, mature leaf concentrate of *C. papaya* Sri Lankan wild variety in adult Wistar rats.

Effect of the MLC on Platelet, Total WBC, and RBC Counts of Rats

Compared to the control, platelet count was significantly (P<0.05) increased by 42, 59 and 68 % in rats treated with low, mid and high doses, respectively (Figure 1). The mid and high doses of the MLC significantly (P<0.05) increased the total WBC by 15 and 19 %, respectively (Figure 1). Conversely, no significant (P>0.05) difference was observed for RBC counts between the treated and the control group (Figure 1).

The results revealed the constituents of the MLC enhance the cellular immune activity by increasing the platelet and WBC counts (Figure 1). Both WBCs and platelets play an important role in modulating the first line defense mechanism (Goldsby *et al.*, 2002). This increment has plausibly resulted from the release of cells from their sequestration sites such as spleen, thymus or following their synthesis in the bone marrow (Goldsby *et al.*, 2002). It is more likely that the cell counts were increased by the propagation of bone marrow progenitor cells (Sharififar *et al.*, 2009) as bone marrow cells were increased by the MLC.

The non-significant alteration of RBC indicates the MLC is not effective against the humoral regulator of RBC production (Yakubu and Afolayan, 2009).

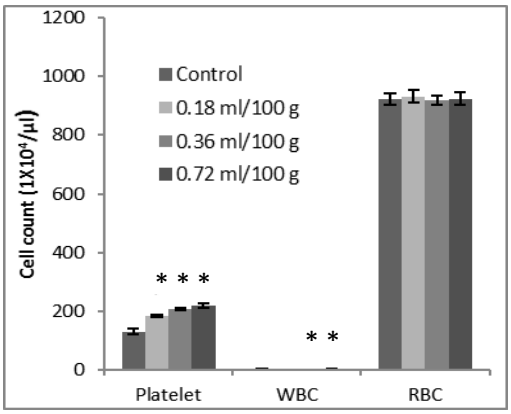

Figure 1. Platelet, White Blood Cells and Red Blood Cell counts of Adult Wistar rats after 3 days of consecutive oral treatment. (means±SEM) *P <0.05 as compared with the control (Mann Whitney U test)

Effect of the MLC on Differential WBC Counts of Rats

Differential WBC counts of the MLC treated rats indicated significantly increased both monocytes and lymphocyte counts. Monocyte count was significantly increased (P<0.05) by mid (43.23 %) and high (44.67 %) doses of the MLC compared to control (Figure 2). Similarly, significant (P<0.05) increase of lymphocyte count was observed for mid (7.5 %) and high (10 %) doses (Figure 1).

An increase of total WBC count is not always associated with increase of all types of WBCs (Yakubu and Afolayan, 2009). Marked increase of monocyte and lymphocyte counts by the MLC was observed while neutrophils and Eosinophil counts remained unaltered (Figure 2).

Striking increase of monocytes and lymphocyte counts by the MLC indicated that the constituents may either selectively stimulate the propagation of specific hematopoietic cells in bone or release from the sequestrations sites (Goldsby *et al*., 2002). Monocytes which are produced by the bone marrow are normally stored in the spleen and when released to tissues they develop into macrophages (Goldsby *et al.*, 2002). Thus, the increase of monocytes in blood stream indicates an enhancement of macrophages at the tissue sites.

Lymphocytes are the effector cells of the adaptive immune system which are produced in the bone marrow and stored in primary or secondary lymphoid organs (Goldsby *et al*., 2002). It is rational to anticipate an enhancement of adaptive immune system by the MLC, due to the increment of lymphocyte in treated rats.

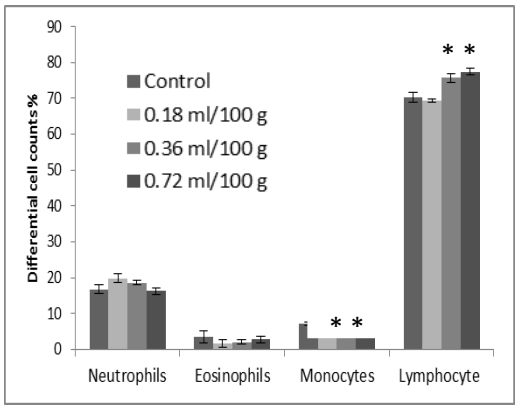

Figure 2. Differential white blood cell counts of Adult Wistar rats after 3 days of consecutive oral treatment. (means±SEM) *P < 0.05 as compared with the control (Mann Whitney U test)

Effect of the MLC on Bone Marrow and Splenocyte Counts of Rats

As shown in Figure 3, the bone marrow cell count was significantly (P<0.05) increased by the mid (25 %) and high doses (35 %) compared to control. However, there was no significant (P>0.05) difference of splenocyte counts between the test and control groups (Figure 2).

Bone marrow is the site where RBCs, WBCs and platelets are produced (Goldsby *et al.,* 2002). The profound increase of bone marrow cells may be responsible for the increase of platelets and WBCs. However, no effect on RBCs point out that the MLC selectively stimulates the progenitor cells in the bone marrow cells. Similar pattern of stimulation has also been observed for the aqueous extract of the stem of *Buldine natalensis* (Yakubu and Afolayan, 2009).

The splenocyte counts remained unaltered in treated rats indicating the other immune linked organs such as lymphoid organs, liver and bone marrow (Gomes *et al.,* 2014) are involved in the MLC induced stimulation of immune cells.

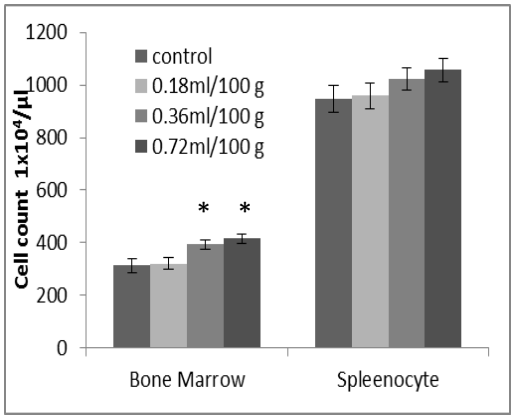

Figure 3. Splenocyte and bone marrow cell counts of Adult Wistar rats after 3 days of consecutive oral treatment. (means±SEM) *P<0.05 as compared with the control (Mann Whitney U test)

Effects of the MLC on Phagocytic Activity of Peritoneal Macrophages

The functional assay based on phagocytic activity was significantly (P<0.05) increased by all three doses (low: 43 % mid: 79 % and high: 109 %) compared to the control. The results obtained after 3 days of consecutive treatment with 3 doses of the MLC are summarized (Figure 4).

According to the results the cellular activation of the MLC was further strengthened by the increased phagocytotic activity of macrophages. Macrophages are found in virtually all organs and tissues (Goldsby *et al.,* 2002). These cells are strategically placed where pathogens are abundant such as body cavity and internal surface of respiratory and digestive systems (Goldsby *et al.,* 2002). Therefore, the MLC induced activation of macrophages in the peritoneal cavity of rats has profoundly increased the first line defense mechanism of the host body. However, the exact mechanism of phagocytic activation is yet to be investigated.

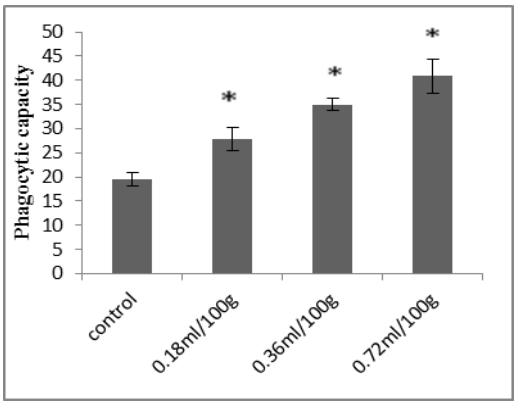

Figure 4. Phagocytic capacity of adult Wistar rats after 3 days of consecutive oral treatment with the MLC (means±SEM) *P < 0.05 as compared with the control (Mann Whitney U test)

Acute Toxicity Assay

Acute oral treatment of the highest dose (0.72 ml/100 g body weight) of the MLC did not elicit hepato and renal toxicities. Serum levels of liver enzymes (ALT and AST) were not significantly (P>0.05) different in the treated group compared to the control (Figure 5). Similarly, renal parameters (urea and creatinine) were unaltered in the test group compared to the control. Our results agree with the previous report on sub chronic toxicity study of red lady variety of *C. papaya* (Gammulle *et al*., 2012). This study reiterated that the MLC is orally active and a safe choice of a plant based immunostimulant

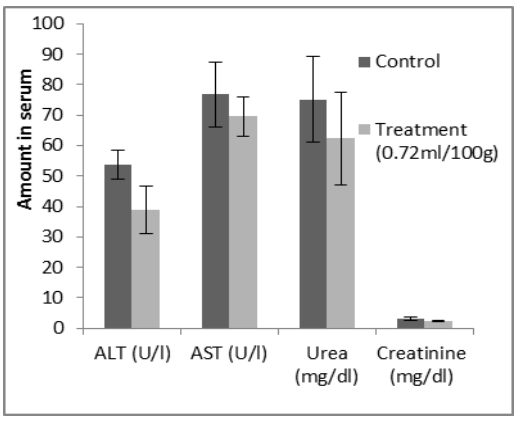

Figure 5. Liver and kidney functional parameters of Wistar rats after 3 days of consecutive oral treatment with the high dose of MLC. (means±SEM) P < 0.05 as compared with the control (Mann Whitney U test

Preliminary Phyto and Biochemical Analysis of MLC

The MLC established the presence of polyphenols, flavonoids, tannins, saponins, alkaloids, carbohydrates, proteins and amino acids. Polyphenols and flavonoids were relatively high in the MLC compared to other constituents. Recent findings suggest flavonoids and saponins play an important role in immunomodulation (Gomes *et al*, 2014). Several types of flavonoids are known to increase the peripheral blood leukocyte proliferation (Sharififar *et al.,* 2009). Some reports suggest certain flavonoids can directly enhance the lymphocyte activation (Sharififar *et al.,* 2009). It could be proposed that the high content of flavonoids in the MLC may have played a role in increasing the lymphocyte counts.

The active constituents possessing antioxidant properties have been reported to induce immunostimulatory effects (Sharififar *et al*., 2009). Strong antioxidant properties of the MLC reported previously (unpublished data) is likely to enhance the immune system.

Further, some polysaccharides are known to induce macrophage activity such as polysaccharides present in green tea (Gomes *et al.,* 2014). The constituents of the MLC either singly or several constituents synergistically may play a role in stimulating the immune system. However, further studies are required to identify the active constituent/s responsible for the above activity.

CONCLUSIONS

This study established that the mature leaf concentrate (MLC) of *Carica papaya* Sri Lankan wild type variant is orally active, non-toxic, and possesses immunostimulatory properties in a rat model.

ACKNOWLEDGEMENTS

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