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Original Research Article

Immunostimulant activity of standardised extracts of *Mangifera indica* leaf and *Curcuma domestica* rhizome in mice

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Abstract

Purpose: *To investigate the immunomodulating activity of Mangifera indica. (MI) and Curcuma domestica (CD) extracts in mice.*
Methods: *Mice were randomly divided into 4 groups, namely, vehicle, untreated, MI and CD groups (n = 6). They were treated with MI (160 mg/kg) or CD (200 mg/kg) extracts, and vehicle for control group, for 14 days and sacrificed on day 15. In innate immunity test, the mice were challenged with sheep red blood cells (SRBCs) antigen on day 8, while in adaptive immunity test, mice were immunized and challenged on days 7 and 14, respectively, with SRBC. White blood cells count (WBC), spleen index (SI), and haemagglutination (HA) titer, and delayed type hypersensitivity (DTH) responses were determined.*
Results: *For both plant extracts, adaptive immunity groups showed the highest response compared to innate groups. In adaptive immunity, the WBC count of MI and CD treated animals was significantly higher than in the untreated and vehicle treated groups ($p < 0.001$). Moreover, the SI of mice from MI and CD treated groups differed significantly that of the untreated group ($p < 0.01$ and $p < 0.05$, respectively). HA titer in CD (both non-challenged and challenged) groups was significantly higher than in the non-challenged vehicle group ($p < 0.001$). HA titer in MI group (non-challenged) was significantly lower than in non-challenged CD and challenged groups ($p < 0.01$ and $p < 0.001$, respectively).*
Conclusion: *MI and CD extracts, in appropriate doses, exerted immunostimulant effects in mice by enhancing both innate and adaptive immune systems via increase in WBC, SI, HA titer and DTH responses.*

Keywords: *Mangifera indica, Curcuma domestica, Immunostimulant activity, Spleen index, Haemagglutination, Hypersensitivity response*

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INTRODUCTION

The immune system contributes significantly to human health. Malfunctional immune system results in various diseases like arthritis, ulcerative colitis, asthma, allergy, cancer and infectious diseases [1]. Such diseases, caused by modulation of immune responses, have been of interest for many years. Substances which can affect the immune system are known as

immunomodulators. Immunomodulators can be immunostimulant or immunosuppressant [2].

Recently, a number of synthetic as well as natural immunomodulatory agents have been introduced in order to modulate non-specific and specific immune responses. Currently available chemotherapeutic agents mainly have immunosuppressive activity and most of them are cytotoxic and exert a variety of side effects. Therefore, the medicinal plants and their isolated bioactive components with immunomodulatory potential are gaining importance to discover alternative immunomodulatory agents. Several medicinal plants with established immunomodulatory potential have been documented by several researchers. *Viscum album*, *Panax ginseng* and *Tinospora cordifolia,* have been shown to alter the immune function [3]. The usage of medicinal plants with immunostimulatory effects in patients, reported less toxicity and side effects [4]. Researchers around the world are focusing to explore medicinal plant and plant derived substances which can alter certain immune responses. Thus, these natural substances can replace conventional chemotherapies for modulation of immune response, particularly in impaired host immune response. The current study was aimed to explore the immunomodulatory activities of methanol extracts of *Mangifera indica* leaves and *Curcuma domestica* rhizomes on innate and adaptive immune response in male albino ICR mice.

EXPERIMENTAL

Chemicals and antigenic materials

The chemicals and antigenic materials used in this study include methanol 99.5 % obtained from Merck KGaA, (Germany). While sheep red blood cells, and glutaraldehyde stabilized were purchased from Sigma Aldrich (M), (Malaysia). Glacial acetic acid (Nacalai Tesque Inc. Japan) disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, sodium chloride (R & M Marketing, Essex, U.K.), ammonium, chloroform ($CDCl_3$), dilute sulphuric acid, mayer reagent, ethanol, diethyl ether (Ranbaxy Fine Chemical Limited, New Delhi, India), 10 % sodium chloride, 5 % ferric chloride, 20 % sodium hydrochloride, 1 % hydrochloric acid, tragacanth powder and normal saline were also used in this study.

Plant material

Both plants, *Mangifera indica* and *Curcuma domestica* (coded as MI and CD respectively) were collected from Kuala Lumpur, Malaysia on January 2012. The botanical identification of the plant materials was confirmed by the botanist of herbarium Emeritus Professor Dato' Dr. Abdul Latiff Mohamad, Universiti Kebangsaan Malaysia and voucher specimen of *Mangifera indica* (no. UKM 30059) and *Curcuma domestica* (no. UKM 30060) were deposited at Herbarium Universiti Kebangsaan Malaysia (UKM), Bangi, Malaysia.

Preparation of plant extract

The air dried plant materials were powdered using a mechanical grinder. The dried plant powder sample was extracted with 99.5 % methanol three times by cold maceration method. The crude extracts were filtered with Whatman no. 1 filter paper. The solvent was evaporated using a rotary evaporator.

Phytochemical screening

Methanol extract of the plants were subjected to preliminary phytochemical screening using the methods of Malaysian Herbal Monograph 2009 for the detection of various plant constituents such as alkaloids, triterpenes, steroid, saponin, phenolics and flavonoids [5].

HPLC standardisation of *Mangifera indica* and *Curcuma domestica*

The HPLC analysis was carried out on *Waters* instruments*, (United States)* equipped with 600controller, 2707 auto sampler, 2998 photodiode array detector, 2475 multi fluorescence detector and in – line degasser AF. The HPLC analysis of MI and CD extracts basis on their reference compounds mangiferin, M3547 and curcumin, C1386 respectively was obtained on HyPURITY C18 (4.60 × 250 mm, 5 µm Thermo Scientific, U.S) column. Potassium dihydrogen 0.01 M orthophosphate solution pH 2.7 ± 0.2 (A) and acetonitrile (B) were used as mobile phase. Extracts and reference compounds were eluted isocratically with mobile phase A and B at a flow rate of 1.0 mL/min. MI and its reference compound was eluted with A and B at a ratio of 15:85 (v/v), while for CD and its reference compound the ratio of A and B was 40:60 (v/v). The detection was carried out at 254 nm. Data acquisition and peak analysis were performed using Empower Pro System with a small modification.

Validation procedure for HPLC analysis

Parameters like linearity, precision, limits of quantification (LOQ) and detection (LOD) were determined for the validation of reversed phase

HPLC procedure. LOD was determined by calculating the standard deviation (σ) of the responses of the lowest concentration in calibration curve of six runs (n = 5) and the slope of the calibration curves of external standards was used to calculate LOQ. Linearity was determined by linear calibration analysis, while calibration curves were used to calculate the correlation coefficient (R^2). The precision of the method was determined by intra-assay and inter-assay validation. Calibration standard curves for mangiferin and curcumin were prepared at a concentration of 15.625 to 1000 µg/mL and 0.3 to 1 mg/mL respectively. A graph was plotted for area versus concentration of the corresponding compound. Extract and reference compound at concentration of 10 mg/mL and 1 mg/mL respectively, were separately injected three times in one day and on three different days. RSD and slope (S) of the calibration curves were used to calculate LOD and LOQ using the following equations: LOD = 3.3 × (RSD/S) and LOQ = 10 × (RSD/S).

Experimental animals

Male Albino ICR mice (*Mus musculus* L) weighing between 20 - 25 g was used in the study, and were obtained from the Laboratory Animal Resource Unit, Faculty of Medicine, UKM. Animals were allowed to acclimatize to laboratory condition for one week before performing the experiment. The animals were housed in clean and appropriate cages and kept at constant temperature of 25 ± 2 °C with humidity (78 % ± 2 °C) environment under 12 h light and 12 h dark cycles. The mice were provided with standard mice pellet diet and water *ad libitum*. The animal studies were approved by Universiti Kebangsaan Malaysia Animal Ethics Committee (approval no. FF/2012/ENDANG/23-MAY/435-MAY-2012-FEBRUARY-2013) and in accordance with international guidelines for animal studies [6].

Preparation of sheep red blood cell (SRBC) suspension antigen

SRBCs were used as antigenic material. SRBC suspension of 20 % in phosphate buffered saline was used for both immunization and challenge of the animals throughout the study.

Animal studies

Innate immune response test

The mice were randomly divided into eight groups, with six animals in each group. Group I: vehicle (tragacanth 1 %), Group II: vehicle with challenge, Group III: untreated, Group IV: untreated with challenge, Group V: MI extract (160 mg/kg), Group VI: MI extract with challenge, Group VII: CD extract (200 mg/kg), Group VIII: CD extract with challenge. The animals were treated orally with plant extracts and vehicle control daily for 14 days. On day 8, mice in Group II, IV, VI and VIII were challenged with 0.1 mL of 20 % SRBCs suspension intraperitoneally (i.p). On day 15, the mice were sacrificed by cervical dislocation method with anesthesia.

Adaptive immune response test

The mice were divided into four groups. Group I: vehicle control, Group II: untreated, Group III: MI extract and Group VI: CD extract. The animals were treated orally with plant extracts and vehicle control daily for 14 days. On day 7, mice from all groups were immunized with 0.1 mL 20 % SRBCs intraperitoneally (i.p) and on day 14 the mice were challenged by injecting 0.03 mL of 2 % SRBCs subcutaneously (s.c) in the right front of the foot pad region and then paw thickness due to edema was measured. On Day 15, mice were sacrificed by cervical dislocation method with anesthesia.

White blood cell (WBC) count test

Mice were anesthetized and blood was collected from retro-orbital plexus on the last day of the experiment. A 1-in-20 dilution of blood was prepared by the addition of 0.02 mL of whole blood to 0.38 mL of 2 % acetic acid. The suspension formed was mixed for two to three minutes to ensure complete RBC lyses than WBC were counted using Neubauer haemocytometer. The counting of WBC was performed in duplicate and the final cell count for each group of mice was expressed as the number of white blood cells per milliliter and calculated as was described by Rodak *et al* [7].

Spleen index (SI) test

Mice from all groups were sacrificed on day 15 by cervical dislocation method after anesthesia. The spleen was isolated and weighed. The results were expressed as organ index using the formula described by Tripathi *et al* [8].

Determination of haemagglutination antibody (HA)

The serum samples were collected from individual mice of innate immune group to determine antibody titer using haemagglutination method. 25 µL of 1 % SRBC were added to two-fold dilutions of serum samples in PBS in V-bottomed micro-titration plates. The plates were

incubated in carbon dioxide (CO_2) incubator for one hour at 37 °C. They were allowed to settle at room temperature for 60 min until the plates showed a small button formation (negative pattern) [9]. Positive haemagglutination result was determined by the appearance of diffused color and absence of button shape formation at the bottom of well. Graded manner was used for the expression of the antibody titers, moreover minimum dilution (1/2) was ranked as 2. The reciprocal of the highest dilution of the test serum giving haemagglutination was calculated as the antibody titer [10].

Determination of delayed type of hypersensitivity (DTH) response

On day 14, all immunized mice in the adaptive immunity group were challenged by injecting 0.03 mL of 2 % (v/v) SRBCs suspension subcutaneously (s.c.) in sub-plantar region of tright hind paw. Baseline values for paw thickness of the mice were taken just after injecting SRBCs antigen. Paw thickness of the mice was measured after 30 min, 1, 2, 3, 4, 5 and 24 h of SRBC challenge. DTH response was expressed as a mean percent increase in paw thickness due to edema and calculated as described by Jafarian *et al* [11].

Statistical analysis

Data are expressed as mean ± SEM and were statistically analyzed using one-way ANOVA test and GraphPad Prism 5 software. $P < 0.05$ was considered significantly different.

RESULTS

Phytochemical profile

Table 1: Phytochemical profiles of MI and CD methanol extracts

Phytochemical constituent	MI	CD
Alkaloids	-	++
Triterpene	-	+++
Steroids	++	-
Saponin	-	-
Phenolics (tannin)	+	+
Flavonoids	-	+

Note: -: Absent; +: less present; ++: moderate present; +++: high present

The results of the qualitative preliminary phytochemical screening analysis of the MI and CD methanol extracts are presented in Table 1. The analysis showed that methanol extract of MI contained steroids and phenolics (tannins), CD methanol extract revealed the positive result for the presence of alkaloids, phenolics (tannins), triterpenes and flavonoids.

HPLC profile of *Mangifera indica* and *Curcuma domestica* extract

Figure 1 & 2 shows the HPLC chromatogram of the MI and CD methanol extract respectively. The mangiferin and curcumin standards were detected at 2.959 and 4.289 min respectively, while the retention time of mangiferin and curcumin in the extract were 2.947 and 4.247 min, respectively. The concentration of mangiferin in *MI extract* was 172 mg/g and 80 mg of curcumin was found in 1 g of CD extract. The mangiferin calibration curve plotted was linear over the concentration range of 15.625 to 1000 µg/mL with a correlation coefficient (r^2) of 0.993, On the other hand a correlation coefficient (r^2) for curcumin was 0.957 from the concentration range of 0.3 to 1 mg/mL of standard curcumin.

White blood cell (WBC) count

The effect exerted by both plant extracts and control groups on WBC count of mice in both innate and adaptive immunity tests are shown in Figure 3.

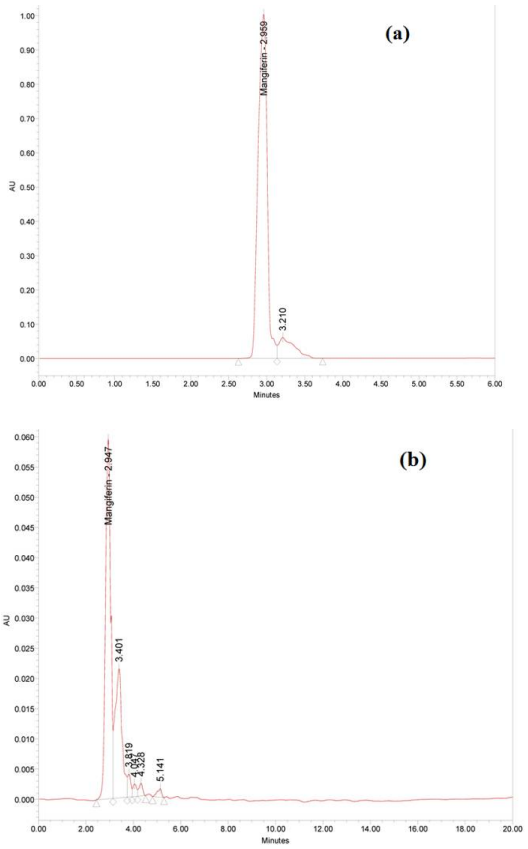

Figure 1: Representative HPLC chromatogram of mangiferin (a) in *Mangifera indica* extract (b).

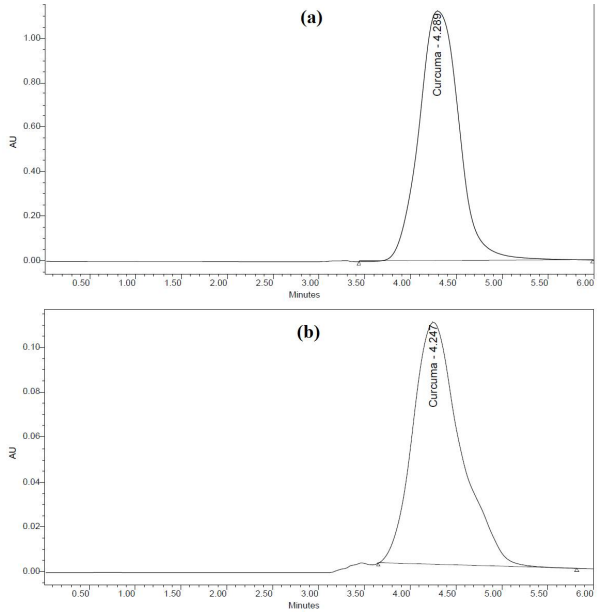

Figure 2: Representative HPLC chromatogram of (curcumin (a) in *Curcuma domestica* extract (b).

The WBC counts of mice treated with both plant extracts in adaptive immunity were significantly higher ($p < 0.05$) as compared to extracts treated groups (both non-challenge and challenge) in innate immunity responses. . However, for untreated and vehicle groups no significant difference was observed in innate and adaptive immune responses. In adaptive immunity, WBC count of MI (75.20 x 10^6 ± 11.14 cells/mL) and CD (77.40 x 10^6 ± 11.76 cells/mL) were found to be significantly higher than untreated group (26.80 x 10^6 ± 1.60 cells/mL) and vehicle group (36.75 x 10^6 ± 1.28 cells/mL), $p \leq 0.001$.While, WBC count of MI in adaptive immunity (75.20 x 10^6 ± 11.14 cells/mL) was significantly higher compared with MI in innate immunity non-challenge (29.8 x 10^6 ± 7.71 cells/mL) and challenge (30.6 x 10^6 ± 6.99 cells/mL), $p < 0.001$ for both. Similarly WBC of CD in adaptive immunity (77.40 x 10^6 ± 11.76 cells/mL) was significantly higher as compared with CD in innate immunity non-challenge (26.20 x 10^6 ± 1.49 cells/mL) and challenge (25.80 x 10^6 ± 4.72 cells/mL), $p < 0.001$ for both. Activities of MI and CD extracts in stimulating of WBC count in both innate and adaptive immune responses were comparable, $p > 0.05$.

Spleen index (SI)

Results in Figure 4 show the effects of both plant extracts on spleen index (SI) on both innate and adaptive immunity systems. The plants showed no significant difference in SI of adaptive with their own innate immune responses, $p > 0.05$. In adaptive immunity, SI of MI (4.82 ± 0.40 mg/g) and CD (4.54 ± 0.23 mg/g) showed a significant difference with an untreated group (1.97 ± 0.35 mg/g), $p < 0.01$ and $p < 0.05$ respectively.

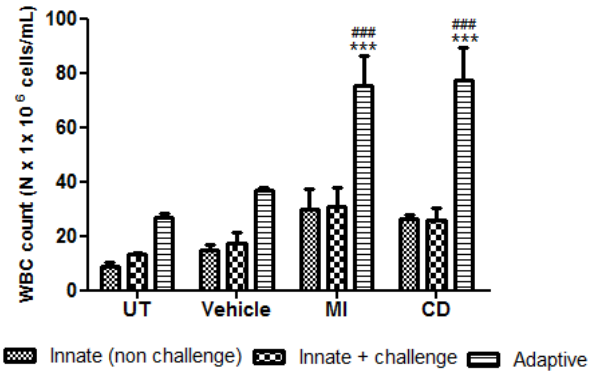

Figure 3: Effect of plant extracts on WBC count of mice in innate and adaptive immunity test (n = 6**). ***:** significantly different, ($p < 0.001$), with untreated (UT) and vehicle groups in adaptive immunity. ###: significantly different with MI or CD innate non-challenged and challenged groups, $p < 0.001$

Furthermore, increasing SI activities of MI (2.54 ± 0.15 mg/g) and CD (3.04 ± 0.96 mg/g) in innate immune responses were comparable, as well between MI (4.82 ± 0.40 mg/g) and CD (4.54 ± 0.23 mg/g) in adaptive immune responses $p > 0.05$.

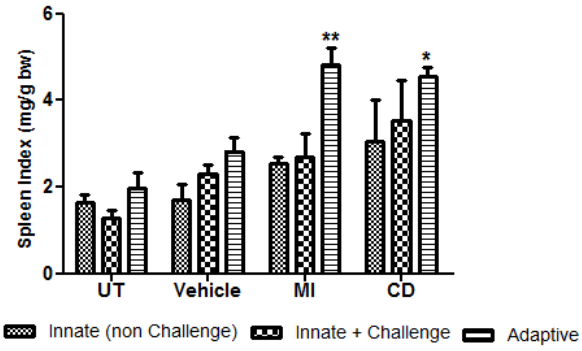

Figure 4: Effect of plant extracts on spleen index in innate and adaptive immunity test (n = 6). **, *: significantly different with untreated group $p < 0.01$ and $p < 0.05$ respectively.

Haemagglutination antibody (HA) titer

Figure 5 shows the effect on HA titer in both challenge and non-challenge animal groups, of the plant extracts. The HA titer in CD non-challenge group (28.80 ± 3.20) and CD challenge group (38.40 ± 6.40) were significantly higher compared with vehicle non-challenge group (4.33 ± 1.20), $p < 0.001$ for both and they are also significantly higher compared with vehicle challenge group (5.20 ± 2.73), $p < 0.01$ and $p < 0.001$ respectively. MI non-challenge group (8.33 ± 2.55) was significantly lower compared with CD

non-challenge group (28.80 ± 3.20) and CD challenge group (38.40 ± 6.40),$p < 0.01$ and $p < 0.001$ respectively. MI challenge group (13.33 ± 3.96) showed HA titer significantly lower $p < 0.001$ compared with CD challenge group (38.40 ± 6.40) in inducing humoral immunity.

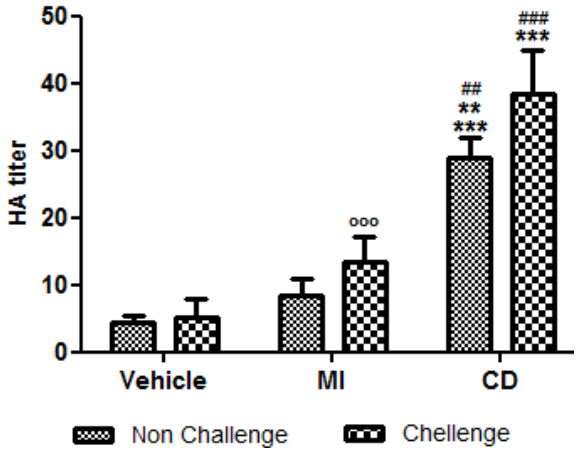

Figure 5: Effect of plant extracts on HA titer of mice in innate immunity test (n = 6). ***: CD challenge is significantly different, $p < 0.001$, with vehicle non-challenge and challenge groups. ***, **: CD non-challenge is significantly different to vehicle non-challenge and challenge groups $p < 0.001$ and $p < 0.01$ respectively. ^{ooo}: MI challenge group is significantly lower compared to CD challenge group with $p < 0.001$

Delayed type hypersensitivity (DTHR)

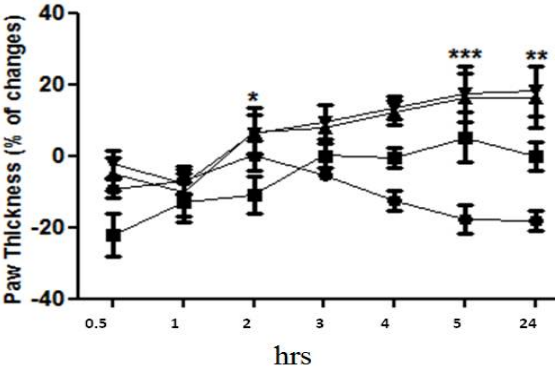

Figure 6: Effect of plant extracts in increasing delayed type hypersensitivity (DTHR) on mice foot edema. Symbols represent, ● untreated, ■ vehicle, ▲ MI and ▼CD groups. ; ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$, are significantly different compared to untreated groups

Specifically, Figure 6 refers to the percentage (%) increase in paw thickness of the mice treated with MI, CD, vehicle and untreated group. The results revealed that SRBC injection increased paw thickness in MI and CD treatment groups with a maximum response of 16.38 ± 6.75 % (at 5 h) and at 18.15 ± 7.01 % (at 24 h), respectively. The onset of action of both plant extracts were at 2 h, it was shown by significant increase in paw thickness $p<0.05$ compared with untreated group. After 2 h, there was a significant increase in paw thickness in animals which received plant extracts until 24 h. Paw thickness in mice treated with both plant extracts were significantly higher compared with the untreated group, $p<0.001$ for 5h and $p<0.01$ for 24 h treatment.

DISCUSSION

According to the phytochemical screening study conducted by two researchers from Nigeria [12], the methanol extract of MI contain saponins, steroids, tannins, and flavonoids. The previous study conducted by Jyoti and Rajeshwari [13] in India had also reported the presence of steroids and flavanoids in the methanol crude rhizomes extract of CD. Environmental factors and habitat influence the phytochemical contents present in plants. So the difference in the phytochemical constituents of methanol extract of the CD rhizome with those reported in previous studies is supported by the fact that the phytochemical contents of the same plant collected from different locations are influenced by the environmental factors and habitat [14].

It was observed that, WBC count of extract treated and control groups in the adaptive immune study was significantly higher compared to those groups in innate immunity test that received same treatment. Another researcher who conducted WBCs test on mice treated with aqueous extract of *Phyllantus amarus* and *Xylopia aethiopica* reported that increase in WBC count suggests that there is a high probability that the extracts of the plants contain agents that have the ability to stimulate the production of leucocytes [15]. Degenerative body systems are strengthened and harmonize with immune boosters which provide assistance to the immune system to combat foreign invading agents like bacteria and viruses.

Balekrar *et al* revealed that mangiferin (a naturally occurring xanthone-C-glucoside) at a dose of (15 mg/kg) has the ability to enhance the total WBC and lymphocyte count in blood; Furthermore, it increases its splenic index [3]. These findings demonstrate the possibility of plant involvement in the first line of defense via immunomodulation of lymphoid cell. Antony *et al* reported that the administration of CD extract in Balb/c mice resulted in an increase in total WBC count [16]. The main factor that contributes to the increased in WBCs count might be due to immune stimulating activity of curcumin, which is the active ingredient extracted from CD. The

significant increase in WBC counts in extracts treated animals, suggests that the extracts may exert immunological properties, thereby boosting the defense system against antigen introduced or infection from the surrounding environment.

Besides, there was no significant difference in WBC counts of both plant extracts in innate and adaptive immunity group. This can be best explained that both plant extracts exerted the same effect as an immunostimulant agent.

The spleen index test revealed that the administration of MI and CD result in higher spleen index as compared to vehicle and untreated groups. Kannan & Singh reported that the spleen plays a significant role in both humoral and cellular arms of immune system. Thus, the study suggested that an increase in spleen weight of an organism showed that there is an increase in the immune status of that particular organism [17]. Besides, another study, which tests of the immunostimulant ability of several plant extracts, proved that the bigger immunity index represents the stronger immunity capability of the organism against infection or foreign materials [18]. An individual will be prone to infection when the spleen is damaged or removed. This fact is important to reflect that spleen acts as a suitable parameter for monitoring immune system function [8].

It was observed that the HA titer for animal groups which were treated with plant extracts and challenge were the highest as compared to vehicle group. Generally, when the test animals were being introduced with the antigen for the first time, it takes about four days to four weeks for an antibody to be detected in the serum. Then there will be an increase in antibody titer of the animals, which reaches its maximum by three days to three months [19]. A study that was carried out by Ghule *et al* reported that an increase in the antibody titer in mice represent the improvement of the humoral immune response to SRBCs antigen [20]. Thus, the results indicate that there was an augmentation in responsiveness of B lymphocyte subsets, which are actively involved in the production of antibody molecules.

HA titer test was performed in order to evaluate the humoral immune responses. Generally, humoral immune system initiates upon the interaction of B-cells with the antigen, which is then followed by their subsequent proliferation and differentiations into antibody producing plasma cells. Antibody binds to antigen either by neutralization or formation of clusters that are promptly being ingested by phagocytic cells. The role of antibodies as an effective component of the humoral response can be clearly shown based on its interactions. An increase in antibody titer value indicates the increase in humoral immune response of the animals [21]. The humoral immune responses of the test animals which were treated with plant extracts were assessed based on the interaction of antibodies with SRBCs. The animal groups which received MI and CD treatment showed an increase significantly in the haemagglutination titer, which reflect a general stimulation of humoral immune response.

Generally, DTHR is an important immune system in an organism. It was usually used in order to assess the effect of plant extracts on cell mediated immunity response of an organism in immunostimulant test [22]. DTHR reaction can be quantified by measuring the paw thickness after the tested animals were injected with the antigen [11]. In the present study, the animal groups which were treated with plant extracts shown significantly higher percentage of increase in paw edema compared with the vehicle and untreated groups. The increase in DTHR response to SRBC shows the activation of adaptive immune system, which plays a significant role in the treatment of diseases. SRBC serves as T-dependent antigen revealed the increase in cell mediated immunity, thus poses a possibility of stimulatory effect of plant extracts on T-cells [23].

DTHR is involved in a protective response towards numerous intracellular infectious micro-organisms, particularly those which cause chronic diseases. The reaction involving DTHR effect is initiated by the recognition and activation of T-lymphocytes prior to exposure to a specific antigen that was SRBC. The activation of DTHR results in proliferation and release of cytokines by T lymphocytes. These cytokines in turn cause an increase in vascular permeability, induction of vasodilation, accumulation and activation of macrophage cells. These events prompt improve phagocytosis and lytic catalysts fixation, which all the more successfully kill the antigen [21].

CONCLUSION

Mangifera indica leaves (160 mg/kg) and *Curcuma domestica* rhizomes (200 mg/kg) methanol extract act as potent immunostimulant agents by enhancing both innate and adaptive arms of the immune system by increasing cell mediated and humoral immune responses. The findings also reveal that *Curcuma domestica* rhizome has stronger immunostimulant activity than *Mangifera indica* leaves. Besides these,

adaptive immunity groups showed the higher response than challenge and non-challenge group with regard to innate immunity.

DECLARATIONS

Acknowledgement

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Conflict of interest

The authors declare that there is no conflict of interests with regard to this study.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. EK developed the idea, conceptualized the study hypothesis and coordinated the research activities. SNAI and SMAS performed the experiments and carried out statistical analysis. EK and WA planned the experimental phase and analyzed the data. The manuscript was written by SNAI AND SMAS and reviewed by EK and WA. All authors have read and approved the manuscript.

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