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RESEARCH ARTICLE

Fermented Ethanolic Extract of *Moringa oleifera* leaves with *Lactobacillus plantarum* FNCC 0137 as Immunomodulators on *Salmonella typhi*-Infected Mice

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ABSTRACT:

The aim of this study was to determine the immunomodulator of fermented and non-fermented of ethanolic extract of *Moringa oleifera* leaves (MOL) on immune responses in *Salmonella typhi* infected mice. This research employed ten groups of female mice. Group of negative control (NC) received distilled water without bacterial infection, group of positive control (PC) received distilled water and infected by bacteria and six groups of bacteria infected mice that pre-treated with three different doses (14, 42, and 84 mg/kg BW/day) of fermented or non fermented extract of MOL for 21 days. Bacteria are intraperitoneally injected on the day 21th. The administration of extract was continued for one week after injection. The lymphocyte cell isolated from lymph nodes was analyzed with flow cytometry. Statistical analysis was performed using SPSS 17 ANOVA ($p<0.05$) and followed by Duncan's test. Mice dosed with fermented or non-fermented extract of MOL (14 and 42 mg/ kg BW/day) showed an increasing number of CD11c $^+$ IL-6 and CD8 $^+$ IFN-γ, CD8 $^+$ TNF-α. However, at a dose of 84 mg/kg BW/day those cells number is decreased indicating as immunosuppressant. Fermented ethanolic extract of MOL is more effective as immunomodulatory agent as compared to non-fermented extract of MOL.

KEYWORDS: Fermentation, Immunomodulator, Immunosuppressant, *Lactobacillus plantarum*, *Moringa oleifera* , *Salmonella typhi*.

INTRODUCTION:

Typhoid fever is an infectious disease that infects the small intestine caused by *Salmonella typhi,* also attacks the humoral and cellular immune system[1]. *Salmonella typhi* that enters the body will be recognized by the Antigen Presenting Cell (APC) leading to cellular immune cell activation[2]. The T cells are responsible for cellular immunity which only recognizes peptide fragments from protein antigens and is commonly referred to as the Major Histocompatibility Complex (MHC).

The CD8$^+$ T cells can be activated directly by infected cells, which APC presents for the MHC class I groups[3]. In this case, CD8$^+$ T cells will secrete pro-inflammatory cytokines, such as TNF-α$^+$ and IFN-γ$^+$. This agent is responsible for sending mediators as the host's cell defense and proteins in the blood to the infected location[2].

Typhoid fever can be cured by consuming herbal supplements. In recent decades, there have been many studies that the consuming of drugs lead to increasing the resistance of bacterial infections[4,5]. *Moringa oleifera* leaves are plants that are widely used as immunomodulators[6], immunostimulatory[7], anti-inflammatory[8] and also have antibacterial activity[9]. The Red Cultivar *Moringa oleifera* Leaves contains higher Fe, calcium, and β-carotene[10] than the White cultivar.

Calcium plays an active role in increasing the production of IL-2$^+$ in CD11c$^+$ cells[11] and production of IL-2$^+$ that can accelerate the proliferation of T cells[12]. Including the experience of[1,13], *Moringa oleifera* leaves (MOL) of water extract can be used as an immunostimulant at dose 14 and 42mg/kg BW but also as an immunosuppresant at dose dose 84mg/kg BW with the for threating the *Salmonella typhi* infected mice.

Fermentation of cereal dough with *L. plantarum* can increase the bioavailability of antioxidant bioactive compounds, Fe, magnesium and zinc compounds because of phytate degradation[14]. Fermentation of MOL with *Lactobacillus plantarum* can reduce phytic acid[15,16]. Besides, *L. plantarum* can inhibit the growth food born pathogens[17,18] and also capable of killing the growth of pathogens in the intestine with decreasing the environment pH[19]. The growth of *L. plantarum* was better in the medium added with MOL extracted with ethanol 95% than with MOL extracted with acetone or water[20].

This study was aimed to determine the immunomodulatory effects of non-fermented and fermented of MOL ethanolic extract using *L. plantarum* in female mice infected with *Salmonella typhi*. The immunomodulatory res1ponse was determined by measuring CD11c$^+$ IL-6$^+$, CD8$^+$ IFN-γ^+ and CD8$^+$ TNF-α^+ on lymph nodes cell.

MATERIALS AND METHODS:

Materials:

Red cultivar of *Moringa oleifera* leaves (MOL) are obtained from Pamekasan, Madura, East Java, Indonesia. MOL were taken from the Moringa tree aged 3-12 months. *Salmonella typhi* was obtained from the Laboratory of Microbiology, Faculty of Medicine, University of Brawijaya. *Lactobacillus plantarum* FNCC 0137 was obtained from the Food and Nutrition Study Center (PSPG), Gajah Mada University. A marker (Biolegend, USA) for immune analysis using flow cytometry was obtained from the Laboratory of Animal Physiology, University of Brawijaya.

Extraction:

Moringa oleifera leaves (MOL) were air-dried for three days and continued to dry at 40°C for 3 h in the oven. MOL powder of 200 g was macerated in 2000ml 70 % ethanol at 28±2°C for 72 h. Every 24 hours, the mixture was shaken at 120rpm for 60min[21]. After 72 h, the mixture was filtered using Whatman No.1 paper. Ethanol was evaporated using a rotary evaporator (IKA RV10).

Fermentation:

Lactobacillus plantarum strain FNCC 0137 culture stock was prepared using MRS broth medium and incubated at 37°C for 72 h. The culture was centrifuged at 6500rpm for 20 min at 4°C[22]. Extract of MOL was inoculated with 10^8 CFU/g of *Lactobacillus plantarum* with a ratio of 1:10 (culture: MOL extract) and then incubated at 37°C for 120 h[12]. The fermented MOL extract was mixed with 10% sucrose and 0.5% NaCl and then freeze-dried[23].

Mineral and Antinutrients Analysis:

Calcium analysis was analyzed using AOAC (1995), Analysis of Total tannin was referred to AOAC (1995), Oxalate analysis refers to Chamjangali (2006)

Calcium Content:

Concisely, 5g sample was dried in the oven, then burn on the electric stove for 30 minutes. Furnish the ash sample for 8 hours at 450C. Sample was added with 2ml HNO_3 0.2M and 20ml of distilled water, then homogenized and filtered using Whatman no. 1 paper. Sample was analyzed using Atomic Absorption Spectroscopy methode (Shimadzu AA – 6300).

Total Tannin Content:

Briefly, 1ml of clear filtrate, 0.5ml of 10% Folin Ciocalteau reagent and 1ml of Na_2CO_3 6% were mixed and homogenized and incubated for 30 min. Absorbance was measured at 760nm on UV Vis Spectrophotometry (Thermo, Genesys 10S UV/VIS). The total tannin value obtained using standard tannic acid curves.

Oxalate Content:

Shortly, 1ml of filtrate was added with 1ml of acetate buffer, 0.5ml of Fe (II) 7mg/L, 1ml of KI 0.12 M, and 1 ml of KBr 0.1 M then homogenized. The absorbance of the mixture was measured at 352nm using a UV Vis spectrophotometer Spectrophotometry (Thermo, Genesys 10S UV/VIS)[24].

Animal Experimens:

Female *Mus musculus* (mice) aged six weeks and weighing of 20-25g were obtained from the Bioscience Institute, University of Brawijaya, Indonesia. Mice were randomly divided into ten groups, seven mice in each group. Group of NC (a negative control) and PC (a positive control) were orally tampered with distilled water every day. Treated group of animals (6 groups) were orally tampered with non-fermented or fermented extract of MOL at dose of 14, 42, and 84mg/kg of body weight/day (BW). Groups of PC and treated were injected intraperitoneally with *Salmonella typhi* (0.5 ml/10g BW, 10^7 cfu/ml) on the day 21th. The administration of extract was continued for one week after bacteria injection. At the end of the experiment, the animal were killed by cervical dislocation. The lymph nodes obtained from the neck, base of the armpit and groin were taken for Flow cytometry analysis. The animal experiment in this research has been approved by the Research Ethics Committee of University of

Brawijaya (Center for Animal Care and Use) with ethic number 829-KEP-UB.

Confirmation test of Salmonella typhi:

Confirmation test was carried out on the 22th day, one day after injection with *Salmonella typhi*. Mice serum of 1ml was taken from the tail, then added with 450μL sterile physiological NaCl. Serum was then planted in Luria Broth media and incubated at 37°C and 120rpm for 24 hours. The results of incubation on Luria Broth media were then inoculated on *Salmonella* selective media, Xylose-Lysine-Deoxycholate (XLD) media. Positive results showed *Salmonella typhi* formed colonies marked with a black core[1,13].

Lymph Nodes Isolation and Flow Cytometry Analysis:

The lymph node was taken and washed using phosphate buffer saline (PBS) then crushed to obtain homogeneous sample. The homogenate obtained was then transferred to a propylene tube and PBS was added to the volume reached 3ml, then centrifuged at 2500rpm for 5 min at 10°C. The supernatant is removed, and the pellet obtained is added with 1 ml PBS, then resuspended using the vortex to homogenize. Homogenate of 50μL was taken and put into a 1.5ml tube containing 500μl PBS and anti-TNFα-PE antibodies, anti-IL6-PE and anti-IFNγ-Cy5 as much as 50μL for extracellular incubation. The mixture was resuspended and incubated (4ºC, 20 min, in the dark). For the anti-CD11c-FITC and anti-CD8-FITC staining was added with 100μL of the cytofix-cytoperm solution and incubated (4ºC, 20 minutes, in the dark). The remaining solution was removed, and a 500μL BD washperm solution was added then centrifuged (2500 rpm, 10ºC, 5 minutes). The pellets obtained were stained using specific antibodies for intracellular staining and incubated (4ºC, 20 minutes, without light). Samples after incubation extracellular and intracellular staining were then added with 400μL PBS. The mixture was resuspended then transferred into cuvettes and ready to be analyzed using flow cytometry (BD FACS Calibur, USA)[25].

Statistical Analysis:

The data were presented as mean ± SD (standard deviation) of the means. Data obtained were analyzed based on analysis of variance (ANOVA) with 95% confidence interval using SPSS 17 software. The significant differences were tested based on Duncan Test ($p > 0.05$).

RESULTS:

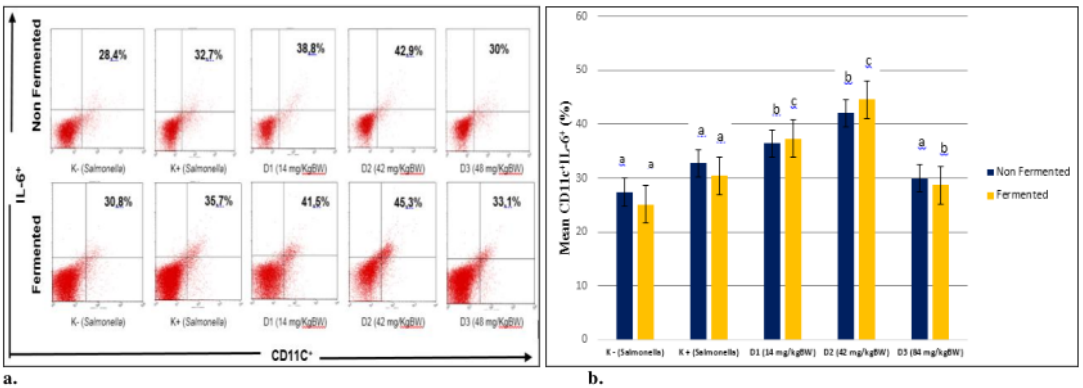
Characteristic of the Fermented and Non-fermented Extract of MOL:

Characteristic of the fermented and non-fermented MOL is presented in Table 1. The calcium, oxalate and tannin acid were different ($p<0.05$). The calcium content for MOL extract (261± 13,9mg/g DB) was significantly lower than fermented MOL (297±7,5mg/g DB). Decreasing oxalate after fermentation (14,23±1,23mg/g DB) compared with MOL extract (24,46±1,47mg/g DB). Total tannin acid of MOL extract (1,48±0,1mg/g TAE DB) was significantly higher than fermented MOL (1,1 ± 0,1mg/g TAE DB). Values are means of triplicate samples.

Table 1. Characteristic of the extract and Fermented MOL

Chemical Compositions		Treatment	
	MOL Extract	Fermented Extract	Freeze-Dried Fermented Extract
Calcium (mg/g) db	261 ± 13.9[a]	297 ± 7.5[b]	323 ± 11.8[c]
Oxalate (mg/g) db	24.46 ± 1.47[a]	14.23 ± 1.23[b]	17.2 ± 1.13[c]
Tannin (mg/g TAE) db	1.48 ± 0.1[a]	1.1 ± 0.1[b]	1.23 ± 0.08[b]

Note: Values are means of triplicate samples. Different notations in the same row shows a significance different at p-value <0.05

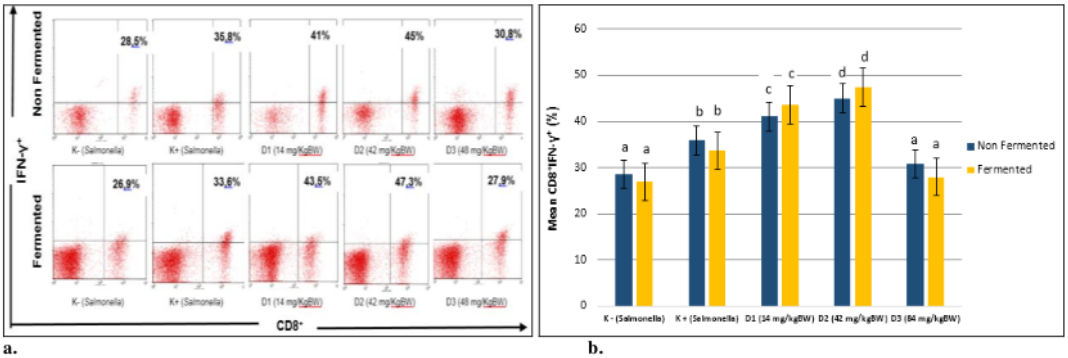


a. b.

Figure 1. The Flow cytometry results showed that at a dose of 14 and 42 mg/kg BW/day, fermented and non-fermented extract of *Moringa oleifera* leaves were able to increase the relative average number of CD11c$^+$ IL6. However, at a dose of 84 mg/kg BW/day, fermented and non-fermented extract of *Moringa oleifera* leaves decreased the relative average number of CD11c$^+$IL-6$^+$.

a. Flow cytometry analysis result

b. Average data of 4 mice each group, Different notations on the same graph colors showed significance values with p-value <0.05



a. b.

Figure 2. Flow cytometry results show that treatment using 14 and 42 mg/kg BW/day of fermented and non-fermented extract of *Moringa oleifera* leaves could increase the relative average of CD8 $^{+}$IFN-γ^{+}. However, at a dose of 84 mg/kg BW/day fermented and non-fermented extract of *Moringa* leaves decreased the relative average number of CD8^{+} IFN-γ^{+}.
a. Results of Flow cytometry analysis

Cell Population of CD11c^{+} IL-6^{+}:

Result of statistical analysis presented a significant difference ($p<0.05$) between fermented and non-fermented MOL. Based on Duncan's test, the dose 42 mg/kg BW was the highest percentage of CD11c^{+} IL-6^{+}, 42.9% in the group of non-fermented and 45.3% in fermented MOL extract (Fig. 1). The treatment of 14 and 42mg/kg BW have significant difference with the dose 84mg/kg BW. Increasing mean number of CD11c^{+} IL-6^{+} at 14 and 42mg/kg BW.

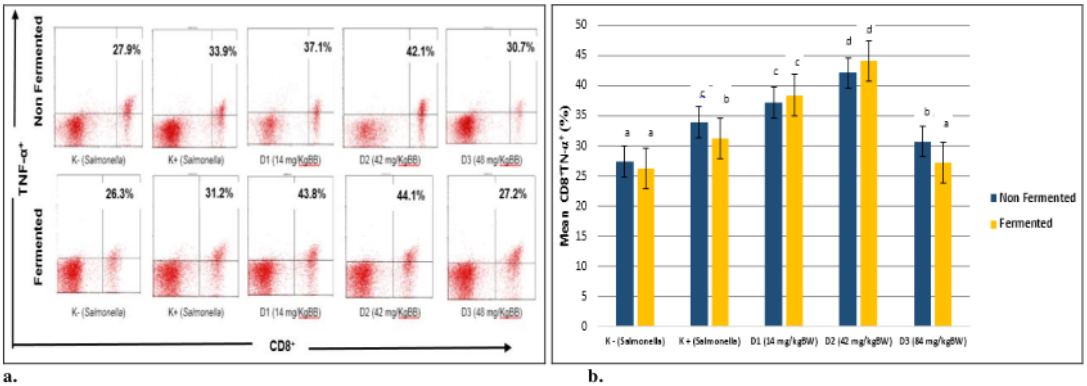
Cell Population of CD8^{+} IFN-γ^{+}:

Administration of experimental mice with fermented and non-fermented extract of MOL at dose of 14 and 42 mg/kg BW/day gave a significant effect (p-value<0.05) on the relative amount of CD8 $^{+}$ IFN-γ^{+} compared to negative and positive control. The highest percentage of CD8 $^{+}$ IFN-γ^{+} was found in the treatment dose of 42 mg/kg BW, 45% in the group of MOL extract and 47.3% in fermented MOL extract. However, at a dose of 84 mg/kg BW/day of fermented extract of MOL showed no significant difference compared to negative control (Figure 2).

b. Average data of 4 mice each group, Different notations on the same colors indicates significance values with p-value <0.05

Cell Population of CD8^{+} TNF-α^{+}:

The percentage of CD8 $^{+}$ TNF-α^{+} cells in the lymph node in extract of MOL group was significantly lower than fermented MOL ($p<0.05$). The group administrated with extract and fermented MOL at dose of 42 mg/kg BW was significantly different than 14 mg/kg BW, 42.1% in the group of non-fermented and 44.1% in fermented MOL extract. And the group that treated with dose 84 mg/kg BW was not significantly different from negative control group (K-) at fermented MOL group. Based on (Fig. 3), we can see that at dose 84 mg/kg BW the percentage of CD8 $^{+}$ TNF-α^{+} was lower than 14 and 42 mg/kg BW



a. b.

Figure 3. The Flow cytometry results showed that treatment with 14 and 42 mg/kg BW/day of fermented and non-fermented extract of *Moringa oleifera* was able to increase the relative average number of CD8 $^{+}$ TNF-α^{+}. However, at 84 mg/kg BW/day treatment of fermented and non-fermented extract of *Moringa oleifera* was decreased the relative average number of CD8^{+} TNF-α^{+}.
a. Results of Flow cytometry analysis
b. Average data of 4 mice each group, Different notations on the same color graph indicates a significance value with p-value <0.05

DISCUSSION:

Oxalate and tannin in fermented extract of MOL is lower than non-fermented. Fermentation of MOL extract using *L. plantarum* could increase calcium levels due to phytase enzyme activity. *L. plantarum* can produce phytase enzyme[14] which can decompose phytic acid (binding of several minerals, such as Ca and Fe) to phosphorus and inositol[26]. Increasing calcium also occurred in cereals and pulses after fermentation using *Lactobacillus spp*, those accompanied by decreasing phytic acid after fermentation[27,28].

Decreasing oxalate level with lactobacilli caused by catabolism of oxalic acid: formyl-CoA transferase which activates an oxalate molecule to oxalyl-CoA; which decarboxylates the oxalyl-CoA molecule to formyl-CoA[29]. This reaction generates a proton that contributes the generation of one ATP molecule when it's couple with oxalate transport[30]. Decreasing of substrate activates oxalate decarboxylase enzyme which plays a role in catalysis oxalate into CO_2 and formic acid[31]. It causes the oxalate content after fermentation becomes lower than non-fermented extract of MOL. The decrease tannins contents after fermentation are caused by the ability of *L. plantarum* to produce the tannase enzyme which can break galloyl ester bonds tannin[32].

Increasing $CD11c^+$ $IL6^+$ in mice given fermented and non-fermented MOL leaves with doses of 14 and 42 mg/kg BW can be caused by calcium contents in the sample. Calcium is a mineral that can increase the production of $IL\text{-}2^+$ cytokine via Nuclear factor of activated T-cell (NFAT) pathway[14]. $IL\text{-}2^+$ cytokines are needed for dendritic cells for the T cell proliferation process. The low $IL\text{-}2^+$ profduced by dendritic cells will result in decreased ability to activate $CD8^+$ T cells[33]. Besides calcium, *L. plantarum* also played a role in the distribution of dendritic cells by increasing Batf3. The Batf3 acts as a Presenting Antigen (AP-1) transcription factor in dendritic cells. Thus, dendritic cells will shorten the activation time of $CD8^+$ T cells[34], increasing dendritic cells as APC can also increased by IFN improvement[35].

Fermented and non-fermented MOL were as an immunomodulatory at a dose of 14 and 42mg/kg BW, however, at a dose of 84mg/kg BW showed as immunosuppressant. The decrease in $CD11c^+$ $IL\text{-}6^+$ cells thought to be due to the flavonoids in the leaves of MOL. Fermented and non-fermented extract of MOL contain total flavonoid 44.15±1.05 (mgQE/g) db and 97.49±0.50 (mgQE/g) db, respectively[36]. Quercetin at a dose of 50μM can act as immunosuppressant by inhibiting the production of $CD11c^+$ $IL\text{-}6^+$ in vitro using DC'c from mice's bone marrow[37]. Genistein suppression of $IL\text{-}6^+$ production in dendritic cells can be caused by the capability of flavonoids to suppress maturation of LPS in monocyte-derived dendritic cells, and reduce NF-kB expression in mice bone marrow's cells at the dose 6.25μM[38]. NF-kB is a transcription factor that is activated by TLR and expression promoter of various cytokines and endothelial adhesion molecules (LPS) as well as stimulants for the production of antiviral cytokines[3].

The other results indicated that the administration of 14 and 42mg/kg BW/day doses of fermented and non-fermented MOL can increase $CD8^+$ $TNF\text{-}\alpha^+$ and $CD8^+$ $IFN\text{-}\gamma^+$. $CD8^+$ T cells which are activated by stimulation from APC will release pro-inflammatory cytokines ($TNF\text{-}\alpha^+$ and $IFN\text{-}\gamma^+$)[2]. There is an increase in the number of IFN- γ^+ and $TNF\text{-}\alpha^+$ in $CD8^+$ T cells that occur due to the treatment using fermented and non-fermented red *Moringa* leaves. Increased $IL\text{-}2^+$ through the NFAT pathway caused by extracellular calcium metabolism which plays a role in various aspects of T cell function, such as the regulation of cytokines, i.e., $IL\text{-}2^+$, $IL\text{-}4^+$, $IL\text{-}10^+$, $IFN\text{-}\gamma^+$ and $TNF\text{-}\alpha^+$ [11,39]. Also, calcium can also stimulate T cell receptors for antigen (TCR), $CD4^+$ co-receptor, and $CD8^+$ which together recognize peptide antigen complexes and MHC molecules in antigen presenters[40]. The increase in the mean number of $CD8^+$ $TNF\text{-}\alpha^+$ and $CD8^+$ $IFN\text{-}\gamma^+$ can be caused by fermentation. Pure culture of *L. plantarum* can increase the expression of Toll-Like Receptors, such as (TLR)2, TLR4, and TLR9 which can activate transcription factors, so it stimulates the excretion of cytokines, such as $IFN\text{-}\gamma^+$ and $TNF\text{-}\alpha^+$ in the male and female mice's lamina propria after seven days[41].

Fermented and non-fermented MOL is an immunomodulatory at a dose of 14 and 42 mg/kg BW, however, at dose of 84mg/kg BW was as immunosuppressant. The decrease in $CD8^+$ $TNF\alpha^+$ and $CD8^+$ $IFN\text{-}\gamma^+$ cells is thought to be due to the flavonoids in the leaves of MOL. The administration of 12.5μM flavonoids of epicatechin, quercetin and tiliroside isolated from *Waltheria indica* can reduce the number of TNF-α in infected macrophage cells from female mice using *Escherichia coli* LPS[42]. According to[43] at dose of 200μM flavonoid of luteolin, epigenin, quercetin, and kaempherol act as immunosuppressants by inhibiting $TNF\text{-}\alpha^+$ production activities which activated by LPS via the TLR-4 pathway. The TLR can reduce regulation of activation of NF-kb and Mitogen-Activated Protein Kinase (MAPK) which can trigger the secretion of IL-10 and IL-4 cytokines. $IL\text{-}10^+$ plays a role in suppressing Th1 cells so that it can suppress $IFN\text{-}\gamma^+$ production[44].

In conclusion, fermented of MOL have better quality than non- fermented based on the calcium contents, also lower oxalate and tannin content. Administration of

fermented and non-fermented MOL at dose of 14 and 42 mg/kg BW on *Salmonella typhi* infected mice can modulate the CD11c $^{+}$ IL-6^{+} cells, CD8 $^{+}$ TNF-α^{+} and CD8^{+} IFN-γ^{+}. However, at a dose of 84 mg/kg BW both extract suppressed CD11c $^{+}$ IL-6^{+}, CD8 $^{+}$ TNF-α^{+} and CD8 $^{+}$ IFN-γ^{+}.

CONFLICT OF INTEREST:

The authors declare that there are no conflicts of interest.

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