# Immunomodulatory effect of Red Moringa oleifera Leaves Fermentation Extract on Il-21 And Il-22 Expressions in Balb/C Mice Exposed to Salmonella typhi

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*RESEARCH ARTICLE*

# Immunomodulatory effect of Red *Moringa oleifera* Leaves Fermentation Extract on Il-21 And Il-22 Expressions in Balb/C Mice Exposed to *Salmonella typhi*

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**ABSTRACT:**
Interleukin-21(IL-21) and interleukin-22(IL-22) expressions in the substantial intestine increase when inflammation occurs. They can also induce the production of pro-inflammatory cytokines and *Matrix Metallo Proteinases* (MMP) in subepithelial fibroblasts. To evaluate at IL-21 and IL-22 expressions in BALB/c mice after administering red *M. oleifera* leaf fermented extracts exposed to *Salmonella typhi*. The expression of IL-21 and IL-22 were evaluated for immunomodulatory effect was analyzed by flowcytometer. Data were analyzed by one-way ANOVA and continued with the Tukey test ($p<0.05$). The results showed that the fermented extract of red *M. oleifera* leaves could act as an immunosuppressor characterized by decreased levels of IL-21 and IL-22 both on CD4 T and CD8 T cells in mice injected with *Salmonella typhi* ranging from 14 mg/Kg BW to 42 mg/Kg BW and Pyrex analysis explains that the cathecin compound has the same active side as 8MR in forming bonds with MMP-9. The results of this study provide is the fermented extract of red *M. oleifera* leaves decreasing the expression of $CD4^+IL21^+$, $CD8^+IL21^+$ and $CD4^+IL22^+$, $CD8^+IL22^+$ through inhibition of MMP-9

**KEYWORDS:** IL-21, IL-22, Fermentation, *M. oleifera*, *Salmonella typhi*, *Lactobacillus plantarum*.

## INTRODUCTION:

*Salmonellosis* is still a substantial disease burden since it causes high mortality rates globally in both animals and humans[1]. *Salmonella* causes three primary types of diseases in humans. They are *enteric fever (typhoid fever)*, bacteremia with focal lesions, and enterocolitis[2]. Humans can be infected by *Salmonella typhi (S. typhi)*, which comes from contaminated food or water. In addition, the amount of *S. typhi* in the human body can affect the severity of the infection and is also primarily determined by the relationship mids microbes and hosts[3].

*S. typhi* enters the upper digestive tract leading to the small intestine. TH17 produces IL-17, IL-17F, IL-21,

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and IL-22, which will be needed to control various bacterial, and fungal infections on the mucosal surface[4]. IL-21 and IL-22 expressions in the colon increase under the inflammatory condition[5]. The IL-22 level, during *S. typhi* infection, increased to 10,000 times[6]. Even so, IL-22 is a cytokine with dual functions, which can be protective and pro-inflammatory; thus, it is entitled 'wolf in sheep's clothing'[7].

IL-22 expression increases in inflammatory bowel conditions and it can directly cause the production of pro-inflammatory cytokines and *matrix metalloproteinase* through subepithelial fibroblasts[8], whereas IL-21 can exicite non-immune cells to synthesize certain inflammatory cytokine molecules[9]. IL-21 increases the production of *matrix metalloproteinases 2* (MMP-2) and MMP-9 (but not MMP-1, MMP-3, or MMP-7), which donate to mucosal ulceration and epithelial damage[10]. Microbiota balance in the intestines is very important and influences host immunity. The use of probiotics in the fermented extract of red *M. oleifera* leaves is the essential strategy for preventing intestinal bacterial infections[11]. Fermentation using *Lactobacillus plantarum* and other lactic acid bacteria is sufficient to increase the concentration of phenolic components in fermented foods using the β-glucosidase enzyme[12].

This study was conducted to understand the immunomodulatory mechanism of red *M. oleifera* leaves fermentation extract using *Lactobacillus plantarum* against the expressions of $CD4^+IL21^+$, $CD8^+IL21^+$ and $CD4^+IL22^+$, $CD8^+IL22^+$. Furthermore, we applied molecular modeling to gain further insight into the relationship of flavonoid (catechin) types in inhibiting MMP-9.

## MATERIAL AND METHODS:

**Materials:**

Red *M. oleifera* leaves were obtained from Pamekasan, Madura, East Java. *S. typhi* bacteria were obtained from the Microbiology Laboratory, Faculty of Medicine, Brawijaya University Malang. *Lactobacillus plantarum* FNCC 0137 was obtained from the Center for Food and Nutrition Studies (OSPG) of Gajah Mada University Yogyakarta. Balb/C mice were obtained from the Laboratory of Biosciences, Brawijaya University Malang. Antibodies used are PerCP/Cy5.5 anti-mause IL-22 (clone: Poly5164), FITC anti-mouse CD4 (clone: GK1.5), FITC anti-mouse CD8a (clone: 53-6.7) purchased from Biolegend, USA. PE anti-IL-21 (RM0268-6G5G [PE], Novus Biological. In this experiment, the number of cells was adjusted at $2x10^6$; antibodies were applied at a concentration of 0.005 mg/100 µL. Flow cytometry analysis was carried out at the Animal Physiology, Structure and Development Laboratory, Brawijaya University.

**Preparation of experimental design:**

The group of research was an experimental study with 35 mice from the University of Gadjah Mada, Jogjakarta. They were divided into five groups. Each group consisted of 7 mice, positive control group/K (+) (mice injected with *S. typhi* fed and drink), normal control group/K (-) (mice fed and drink), and treatment group EF1 (fermented red *M. oleifera* leaves extract at a dose 14mg/kg BW of mice), and treatment group EF2 (fermented red *M. oleifera* leaves at dose 42mg/kg BW of mice). The treatment group EF3 was given red *M. oleifera* leaves fermentation at dose 84 mg/kg BW of mice for 28 days. Then the treatment group on the 29nd day was infected with *S. typhi* by using a dose of $10^7$ CFU/mL intraperitoneal (0.5mL/10g BW). After the 36th post-treatment day, the mice were neck dislocated, then surgery was performed and cell isolation was carried out.

**Red *M. oleifera* leaves fermentation extract preparation:**

The collected red *M. oleifera* leaves were dried in the air for three days then dried in the oven at the temperature of 40°C for 3h. They were borne at room healer before further analysis. Then they were grounded with a blender and sieved 100 mesh. Red *M. oleifera* pollen was macerated with 70% ethanol to 72hours. The maceration result was then filtered with Whatman paper size No. 1. Red *M. oleifera* solution was evaporated to dryness in a rotary at 50°C[13]. The concentrated extract was inoculated with $10^8$ CFU *Lactobacillus plantarum* and the evaporator was then incubated at 37°C for 120 hours[14]. The fermented red *M. oleifera* leaves extract was added by 10% sucrose and 5% NaCl and then dried[15].

***Animal Treatment Try:***

Six-week-old female mice (20-30g) were placed in the control room (25°C, RH 60%). They were fed and given water every day. After 1 week of acclimation, mice were randomly divided into 5 groups (7 animals/group). The normal control (K-) and positive control (K+) groups were orally given distilled water every day. The tested animals (three groups) were each treated with multilevel dosages *of red M. oleifera* fermentation extract (14, 42 and 84mg/kg BW/day) for four weeks before being injected with *S. typhi*. The administration of red *M. oleifera* leaves fermentation extract was continued for one week after intraperitoneal injection of S. typhi (0.5 mL/10g BW) with a concentration of $10^7$ CFU/mL (except the K-group). The ethics committee approved the animal testing protocol on animal experiments from Brawijaya University, Malang, Indonesia (No: 829-KEP-UB).

**Test Confirmation of *Salmonella typhi* in the Blood:**
On the 30nd day, the group of mice infected with *S. typhi* performed a confirmation test to decide the success of *S. typhi* in infecting mice. The test was done by taking the blood of mice through cutting the tail. The taken blood was under pour plate and catalase tests. The pour plate test was carried out by using xylose-lysine-deoxycholate agar (agar XLD) while the catalase test used hydrogen peroxide ($H_2O_2$)[16].

**Cell Isolation in the spleen:**
After the 36th post-treatment day, the mice were slain by neck dislocation. Then, the surgery was performed to remove liver organs. The obtained liver organ was crushed, filtered, and suspended with phosphate-buffered saline (PBS). The obtained homogenate was then transferred to a propylene tube and given PBS until the volume reached 3mL, then centrifuged at 2500rpm for 5 min at 10°C. The supernatant was secluded and the obtained pellet was added with 1ml PBS, then suspended with a vortex to homogenize. Homogenates were divided into two analyses, intracellular and extracellular analysis. A 50µL homogenate was taken and put in a 1.5ml tube containing 500mL PBS. Homogenates were stained with FITC anti-mouse CD4 (clone: GK1.5) or FITC anti- mouse CD8a (clone: 53-6.7). They were mixed with 50µL buffer and then incubated 4°C for 20 min in the dark, and added with 500mL of buffer. The compound was well confused and centrifuged at 10°C at 2500rpm for 5min. The pellet was added with 1µL of intracellular antibody (anti-IL-21 or anti-IL-22) and incubated at 4°C for 20 min in the dark. Those samples were ready to be analyzed by flow cytometry (BD FACS Calibur, USA)[17].

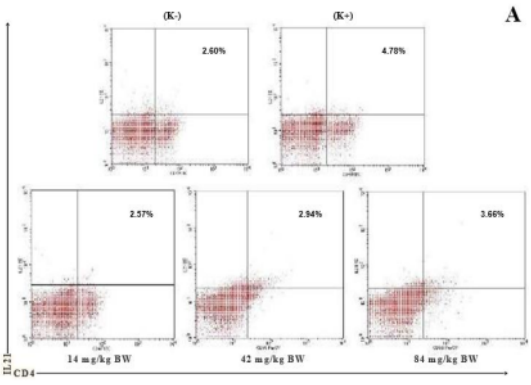
***Flow cytometry analysis:***
Flow cytometry analysis was performed to detect cell populations that expressed $CD4^+$ $IL21^+$, $CD8^+IL21^+$, $CD4^+IL22^+$ $CD8^+IL22^+$. Then, it was connected to the computer, and flow cytometry was set to the acquiring state and parameter procedure were adjusted. After incubation, the sample was added with 500ml PBS and transferred to the cuvette and the sample is run. After that, the acquire was selected and flow cytometer would count the total cell and the number of cells was detected by the antibody label. The obtained results were then processed with BD Proquest TM cell quest.

***Docking Analysis:***
The selected compounds from fermented extracts were Catechin (CID:9064), Luteolin (CID:5280445), Quercetin (CID:5280343), Naringenin (CID: 932), Apigenin (CID:5280443) obtained from PubChem and 8R (CID:5280343), Naringenin (CID: 932), Apigenin (CID:5280443) obtained from PubChem and 8R (CID: 5280343), Naringenin (CID:932), Apigenin (CID: 5280443) -4,4-Difluoro-3- [(4-Methoxyphenyl) Sulfonyl] Butanoic ACID). MMP-9 protein was downloaded from Bank Data (GDP). First, all water molecules from MMP-9 were removed by PyMOL (Schrödinger Inc., LLC) and ligand energy was minimized by Open Babylon at PyRx 0.8 (The Scripps Research Institute, California) before docking simulation. Auto grids for docking ligands for MMP-9 were prepared in the same box size at 30 and coordinates were set at x = - 1,1958; y = 9,0149; z = 19.7598 by AutoDock Vina at Pyrx 0.8. Docking visualization used PyMOL.

**Data Analysis:**
Data were stated as mean standard deviation (SD). The total of cell (%) was from $CD4^+IL21^+$, $CD8^+IL21^+$ and $CD4^+IL22^+$, $CD8^+IL22^+$ and the obtained results were tested for normality. Furthermore, the obtained data were tested with ANOVA with SPSS 16.0 for windows, followed by Duncan Multiple Range Test (DMRT) using the value of p-value significance <0.05.

## RESULT:

**The fermented extract of red M. oleifera leaves decreasing the expression of $CD4^+IL21^+$ and $CD8^+IL21^+$** The fermentation extract of red *M. oleifera* leaves with doses of 14 mg/kg BW and 42 mg/kg BW could significantly reduce $CD4^+IL21^+$ cytokine cell expression compared to the positive control group ($p<0.05$). Whereas the dose of 84 mg/kg BW could reduce $CD4^+IL21^+$ expression but the results were not much different from positive control (Fig.1c) and $CD8^+IL21^+$ cell expression could be reduced by the fermentation extract of red *M. oleifera* leaves at doses of 14 mg/kg BW and 42 mg/kg BW significantly ($p <0.05$), whereas the dose of 84 mg/kg BW with $CD8^+IL21^+$ expression decreased. However, it was not much different from positive control results (Fig.1c).



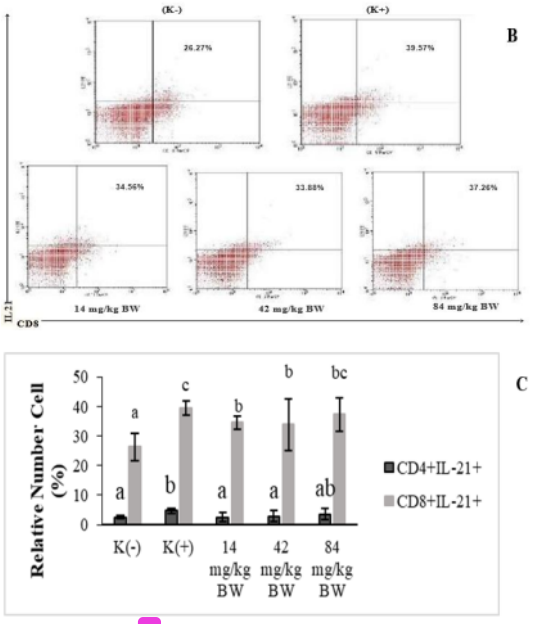
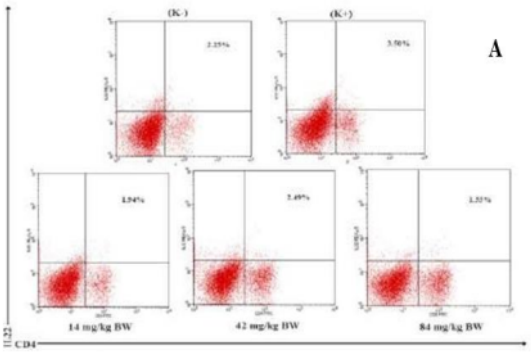



**Figure 1: Expression of immunomodulatory marker.** A. The fermented extract of red *M. oleifera* leaves decreasing the expression of $CD4^+IL21^+$; B. The fermented extract of red *M. oleifera* leaves decreasing the expression of $CD8^+IL21^+$. C. Relatif Number Cells Percentage of $CD4^+IL21^+$ and $CD8^+IL21^+$. Results that not same letters in the same graphic are significantly different by ANOVA followed by a Tukey's test ($p < 0.05$). $CD4^+IL22^+$ and $CD8^+IL22^+$ in the picture above showed the results that at doses of 14mg/kg BW and 24mg/kg BW they could significantly reduce levels of IL- 21 compared to the positive control group ($p < 0.05$). BW: body weight.

**The fermented extract of red M. oleifera leaves decreasing the expression of $CD4^+IL22^+$ and $CD8^+IL22^+$** $CD4^+IL22^+$ was decreased significantly ($p < 0.05$) at doses of 14mg/kg BW, 42mg/kg BW and doses of 84mg/kg BW compared with positive controls (Fig. 2a). Meanwhile, $CD8^+IL22^+$ cell expression was also decreased significantly ($p<0.05$) at doses of 14mg/kg BW and 42mg/kg BW (Fig. 2b), whereas at a dose of 84mg/kg BW it could decrease but not significantly compared to positive control. (Fig. 2c).



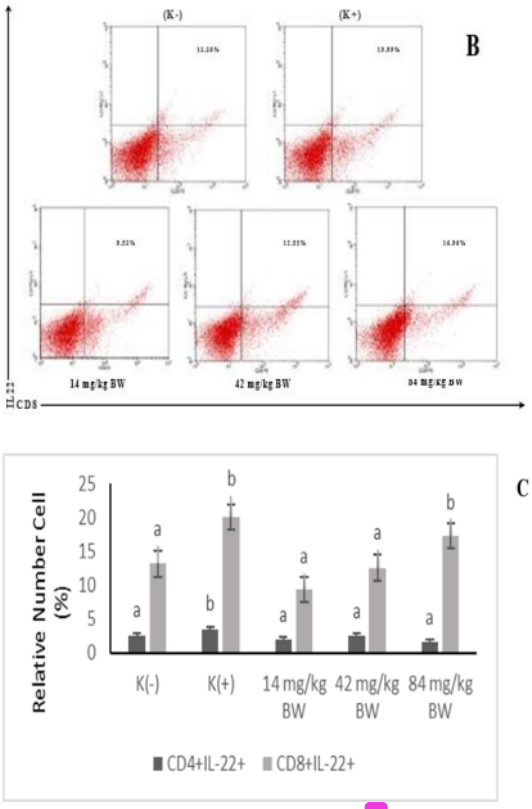




**Figure. 2. Expression of $CD4^+IL22^+$ and $CD8^+IL22^+$.** In the picture above showed the results that at doses of 14mg/kg BW and 24 mg/kg BW they could significantly reduce levels of IL- 22 compared to the positive control group ($p < 0.05$). A. The fermented extract of red *M. oleifera* leaves decreasing the expression of $CD4^+IL22^+$; B. The fermented extract of red *M. oleifera* leaves decreasing the expression of $CD8^+IL22^+$. C. Relatif Number Cells Percentage of $CD4^+IL22^+$ and $CD8^+IL22^+$. Results that not same letters in the same graphic are significantly different by ANOVA followed by a Tukey's test ($p < 0.05$). $CD4^+IL22^+$ and $CD8^+IL22^+$ in the picture above showed the results that at doses of 14 mg/kg BW and 24 mg/kg BW they could significantly reduce levels of IL- 21 compared to the positive control group ($p<0.05$). BW: body weight.

**Analysis of inhibitor molecular docking for MMP-9:** Illustrated the results of Catechin, Luteolin, Apigenin, Quercetin, Kaemferol docking molecules and 8MR (control) to bind MMP-9 in which all of these compounds had a strong bond to MMP-9 compared to 8MR control. Based on the binding affinity value of the docking analysis, it was known that Catechin had high potential as an MMP-9 inhibitor of -9.7, compared to another bioactive.

**Table 1: Result Inhibitor molecular docking for MMP-9**

| Bioactive | PUBCHEM ID | Binding Affinity (kkal/mol) |
|---|---|---|
| Naringenin | 932 | -9.6 |
| Catechin | 9064 | -9.9 |
| Luteolin | 5280445 | -9.7 |
| Quercetin | 5280343 | -9.7 |
| 8MR | (3R)-4,4-Difluoro-3-[(4-Methoxyphenyl) Sulfonyl] Butanoic Acid | -7.3 |

In addition, we found that there were similarities in certain types of required amino acids to bind MMP-9 between controls and ligands. Our docking results suggested that Catechin had high potential as an MMP-9 inhibitor, compared to another bioactive, and had the same amino acid residue as control.

**Table 2: Bond Hydrophobic and Hydrogen**

| Compound | Hydrophobic Bond | Hydroge n Bond | Binding Distance (Angstrom) |
|---|---|---|---|
| Kontrol (8MR) | GLN402, PRO421 | HIS401, HIS405 | HIS401: 3.03 |
| | HIS190, TYR423 | ALA189, LEU188 | HIS405: 3.32<br>HIS411: 3.09 |
| | VAL398, LEU418 | HIS411 | LEU188:3.33<br>ALA189:3.20 |
| Cathecin | GLY186, LEU187 **PRO421, HIS401 LEU418**, TYR423 TYR420, MET422 | **GLN402, ALA189, LEU188** | LEU188:2.81<br>ALA189:3.03<br>2.89<br>GLN402:2.86 |
| | LEU397, **VAL398** | | |

The same amino acid residues between control and ligands consisted of Pro421, His401, Leu418, Val398, Gln402, Leu188 and Ala189 (Figure.3) and had the same binding position with the control. (Figure.5).

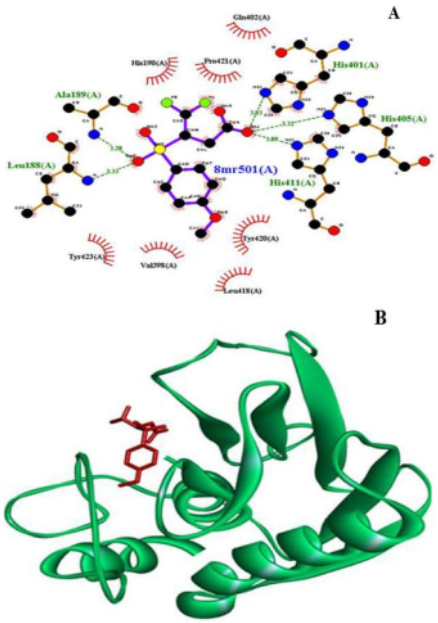



**Figure. 3: Molecular docking analysis between 8MR (control) with MMP-9.** A. 8MR ligplot results with MMP-9. B. 3-Dimensional structure of 8 MR with MMP-9.

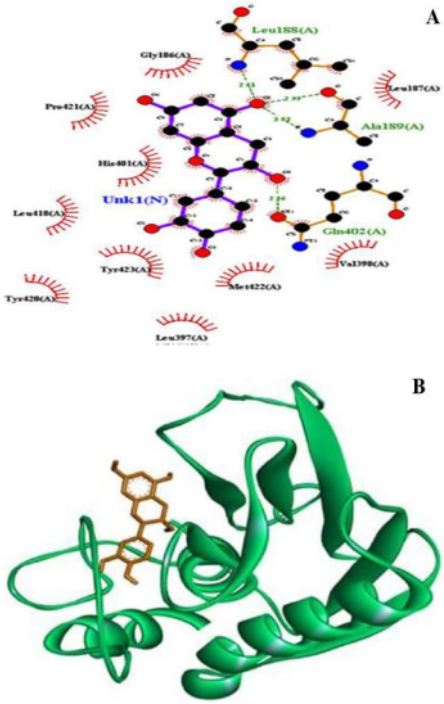



**Figure. 4: Molecular Analysis of Catechin Docking with MMP-9.** A. Catechin Ligplot Results with MMP-9. B. 3-dimensional structure of 8 MR with MMP-9

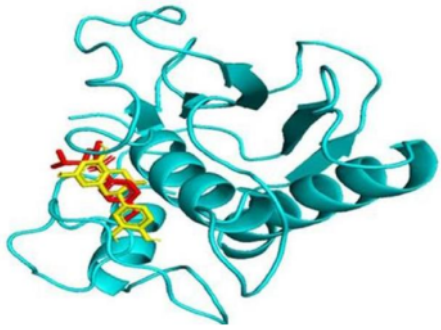


**Figure 5: Docking Visualization Results of 3-dimensional MMP-9, Yellow (Catechin), and Red (Control/8MR) have the same bond.**

## DISCUSSION:

*Salmonellosis* is a disease, which can cause considerable death in humans and animals throughout the world. After infection, manifestations begin from gastroenteritis to enteric fever[18]. The pathogenic *Salmonella* serotype attacking the mucosa is *S. typhi*, which colonizes the digestive tract, and it can cause severe inflammatory diarrhea[19]. In our study, it was proven that the treatment results of red *M. oleifera* leaf fermentation extract as a preventive effort could be as a suppressor marked by decreasing IL-21, and IL-22

levels in a condition where mice infected with *S typhi*[20]. It has only recently been said that IL-22 is a cytokine protecting the intestinal mucosal surface from intracellular bacteria and it injects beta defensor 2, and lipocalin 2 antimicrobials, which bind to enterochelin siderophore limiting the iron availability in the intestine[21]. However, IL-22 is also referred to as double-headed interleukin or wolf-haired sheep[22]. During *S. typhi* infection, IL-22 is a cytokine, which is regulated to be around 10,000 times[23]. IL-22 induces the expression of antimicrobial proteins during *S. typhi* infection. Pathogens avoid this response with specific virulence mechanisms[24]. If lipocalin-2 and calprotectin limit the availability of iron and zinc, the acquisition of thalassin and zinc through the ZnuABC transporter will cause an increase in the competitive advantage of *S. typhi* in the intestine[25]. IL-22 also induces pro-inflammatory chemokine production and neutrophils recruitment into inflammatory regions[26].

Neutrophils are the primary contributors to MMP-9. They are stored in granules and they can be released in intestinal inflammation[27]. IL-22 can also promote the secretion of pro-inflammatory cytokines in chronic inflammation[28]. Moreover, MMP-9 also activates IL-1β[29], as inflammatory mediators produced by macrophage[30].

Interleukin-21 is excessively produced in inflammatory diseases within the gastrointestinal tract[31]. It can maintain chronic inflammation, and it can be involved in tissue damage by promoting the recruitment of immune cells in inflamed tissue, autoreactive T cell expansion[32], and the synthesis of extracellular *matrix metalloproteinases*[33]. IL-21 can increase the production of MMP-1, MMP-2, MMP-3, and MMP-9 in gastric epithelial cells[34]. Macrophage infiltration appears to be a significant source of MMP-8, MMP-9, and MMP-10 in human IBD conditions and rat colitis models[35].

The fermented extract of red *M. oleifera* leaves using *Lactobacillus plantarum* is known to be competent to produce α-D galactosidase enzyme for hydrolyzing raffinose, for reducing raffinose levels by 31%[36]. *Short Chain Fatty Acids* (SCFA) synthesized from carbohydrate fermentation is an essential ingredient for colonocytes in the colon, it can induce IL-12 and IL-10, and it strongly inhibits the proliferation activity of T lymphocytes, decreases IL-4, IL-5, IFN-ɤ and maintains sufficient levels of IL-10, by suppressing colonic inflammation and carcinogenesis by blocking the activation of NFkB pathway[37]. Thus, it allows the oral tolerance and homeostasis of probiotic gastrointestinal tract, as well as can overcome gastrointestinal infections. Fermentation using *Lactobacillus plantarum* and other *lactic acid* bacteria is sufficient to increase the concentration of the phenolic component in fermented foods using the β-glucosidase enzyme[38]. With the increase of flavonoid total in fermented extracts due to the flavonoid conversion, glycosides become the form of aglycone by β-glucosidase from *Lactobacillus plantarum*, which is easily absorbed by the intestine[39].

By looking at the potential of flavonoid compounds contained in the fermentation content of red *M. oleifera* leaves, researchers tried to analyzed through the insilico test to determine flavonoid types, which were inhibitors for MMP-9[40]. Catechin is a group of polyphenol products, especially those found in natural plants[41]. It acts as an anti-inflammatory, microvascular, anti-cancer, and antioxidant, catechin peroxidase and catechins are the primary polyphenols in many foods, and they can be a direct antioxidant against scavenging reactive oxygen species, they can also suppress IL-1β production[42]. From results of the LigPlot test between the control and the ligand, it was found that there were same amino acid residues in the hydrophobic bond, namely Pro-421, Leu-418, Val-398[43]. Meanwhile, the hydrogen bond in the ligand was the amino acid Leu-188, in which the distance was 2.81 Å and at control, it was 3.33 Å[44]. As for the Ala-189 amino acid, it had a bond distance of 303 Å while in control it was 3.33 Å[45]. Therefore, the catechin compound can act as an inhibitor of MMP-9[46].

## CONCLUSION:

The fermented extract of red *M. oleifera* leaves decreasing the expression of $CD4^+IL21^+$, $CD8^+IL21^+$ and $CD4^+IL22^+$, $CD8^+IL22^+$ through inhibition of MMP-9

## CONFLICT OF INTEREST:

Not conflict of interest with the data in the the manuscript

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