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Submission date: 08-Dec-2022 09:40AM (UTC+0700)
Submission ID: 1974855230
File name: Jurnal_RJPT_Red_Moringa_-_Maria_Wuryandari.pdf (593.67K)
Word count: 4301
Character count: 23567

ISSN 0974-3618 (Print)
0974-360X (Online)

www.rjptonline.org

RESEARCH ARTICLE

Red *Moringa oleifera* leaf fermentation extract protecting Hepatotoxicity in Balb/C mice injected with *Salmonella typhi* through Nrf-2, HO-1, and SOD-2 signaling pathways

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ABSTRACT:

Oxidative stress can occur due to an imbalance between increased free radical production and a decrease in antioxidant capacity. *Reactive Oxygen Species* (ROS) excess will react with biological macromolecules either proteins or DNA and potentially damage hepatocytes. Nrf- 2 activations can trigger the expression of anti-oxidative enzymes, such as HO-1 and SOD-2, which have important role in preventing inflammation. The purpose of this study was to look at the expressions of Nrf-2, HO-1, SOD-2, and histopathological features of Balb/C mice injected with *Salmonella typhi* after fermented extract administration of red *Moringa oleifera*. Data were analyzed by SPSS 22.0 ANOVA ($p < 0.05$) and followed by Duncan's Multiple Range Test. The results showed that fermented extract of red *Moringa oleifera* leaves could act as an immunomodulatory agent characterized by increased expressions of Nrf-2, HO-1, and SOD-2. Here, we also showed the histology improvement in Salmonella- injected mice after received fermented extract of red *Moringa oleifera*.

KEYWORDS: Nrf-2, HO-1, SOD-2, necrosis, immunomodulator, *Moringa oleifera, Salmonella typhi*.

INTRODUCTION:

Salmonellosis is one of the most common bacterial infections in the world. It is responsible for a variety of clinical syndromes, including enteric fever, which is usually caused by typhoid or paratyphoid species, enterocolitis, bacteremia, and severe local infections[1]. *Salmonella typhi* (*S.typhi*) is a cause of systemic typhoid fever in humans, which is characterized by fever, enlarged spleen and liver (hepatosplenomegaly)[2,3]. *Salmonella typhi* is a facultative intracellular microorganism that can live and even it multiplies in the macrophage. It is resistant to lysosomes, can to prevent and inhibit phagolysosome fusion. Therefore, it is difficult to dispatch[4]. One way to remove these germs is by stimulating the function of macrophages with immunostimulants. Immunostimulants will stimulate the function of macrophages for killing through respiratory bursts. Activated macrophages will release various metabolites such as *Reactive Oxygen Species* (ROS). They are characterized by increased ROS. This substance is a crucial mediator of inflammation, microbicides, and tumoricidal. ROS plays important role in the killing and as one *chemical lethal* it can kill and eliminate bacteria. However, bursts of ROS can suppress the action of antioxidants which are responsible for blocking the excess ROS production, thereby, it causes oxidative stress in the liver[5]. Excessive ROS will react with biological macromolecules, proteins, and DNA and it can cause damage to hepatocytes[6]. Oxidative stress results from an imbalance between increased free radical production and decreased antioxidant capacity[7]. ROS is a major cause of oxidative stress. It can be in the form of

superoxide (*O2), hydroxyl (*OH), peroxyl (ROO*), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), nitric oxide (NO*), nitric peroxide (ONOO*), hydrochloric acid (HOCl), and fat oxidation results[8]. In physiological conditions, oxidative stress will trigger the up-regulation of endogenous antioxidants and cytoprotective proteins to prevent or limit tissue damage. This process is mediated by Nrf-2 activation, which will activate the gene transcription rate of various antioxidant and detoxification enzymes[7,9] and anti-inflammation[10]. Various external stimuli, including plants containing polyphenol through the expression of *nuclear factor-erythroid-2 related factor 2* (Nrf-2), will inject Heme Oxygenase-1 (HO-1) and Superoxide Dismutase-2 (SOD-2)[11,12,13,14,15]. Among the plants which can induce the activation of Nrf-2, there is *M. Oleifera* plant. *M. oleifera* leaves have the activities of antioxidant, hypolipidemic, anti-inflammatory[16,17] and anti-microbial[18] capable of stopping free-radical chain reactions[19,20]. *L. plantarum* can ferment the red *M. Oleifera* leaves and increase total phenol by producing β-glucosidase enzymes. The enzymes have an important role in biotransformation processes such as modifying primary and secondary metabolites which can add bioactive components such as polyphenols[21]. Total phenol in moringa leaves after 48 hours fermentation increased from 18.2±0.1 to 24.0±0.1 (mg/100g of extract)[22].

MATERIAL AND METHOD:

Materials:

Dark green red *M. oleifera* leaves were obtained from Pamekasan, Madura, East Java, Indonesia. *S. typhi* bacteria were obtained from the Microbiology Laboratory, Faculty of Medicine, Brawijaya University, Malang, Indonesia. *Lactobacillus plantarum* FNCC 0137 was obtained from the Center for Food and Nutrition Studies (OSPG) of Gajah Mada University Yogyakarta, Indonesia. Balb/C mice were obtained from the Biosciences Laboratory of Brawijaya University, Malang. Anti-superoxide dismutase 1 antibody [EP1727Y] (Abcam), anti-NRF- 2 [EP1809Y] (Abcam), anti-oxygenase 1 [EP1391Y] (Abcam), Ms mAb to Rb IgG [2A9] -FITC (Abcam), cytometry flow analysis were conducted at the Laboratory of Animal Physiology, Structure and Development, Brawijaya University.

Preparation of red *Moringa oleifera* leaf fermentation extract:

Red *M. oleifera* leaves were dried in the air for 3 days, then put them in an oven at 40°C for 3h. They were stored at room temperature before further analysis. The dried *M. oleifera* leaves were ground with a blender and sieved 100 mesh. *M. oleifera* powder was macerated with 70% ethanol for 72 h. The maceration results were then filtered with Whatman paper size No. 1. *M. oleifera* solution was evaporated to dryness in a rotary with a temperature of 50°C.[23] The concentrated extract was inoculated with 10^8 CFU/g *L. plantarum* and the evaporator was subsequently incubated at 37°C for 120h[24]. The fermented *M. oleifera* leaf extract was added by 10% sucrose and 5% NaCl and then it was put into a freeze dryer[25].

Preparation of experimental design:

This type of research is an *exploratory* study. It used 35 female white mice Balb/C strain with a bodyweight of about 20grams, ± 6 weeks of age obtained from Gadjah Mada University, Jogjakarta. Mice were grouped into five and each group consisted of 7 mice. There was positive control group (mice injected with *S.typhi* fed and drinking), negative control group (mice fed and drinking), and P1 treatment group (given fermented extract of *M.oleifera* leaves in a dose of 14mg/kg BW), P2 treatment group (given the fermentation of *M. oleifera* leaves at a dose of 42mg/kg BW), the P3 treatment group (given the fermentation of *M.oleifera* leaves at a dose of 84mg/kg BW). The treatment group was given *M. oleifera* fermentation extract for 28 days. On day 29 they were infected with *S. typhi* using a dose of 10^7 CFU/mL intraperitoneal (0.5mL/10g BW). After day 36 of post-treatment mice were neck dislocated, then surgery and cell isolation were performed.

Treatment of Animal Testing:

Six-week-old female mice (25-30g) were placed in the control room (25°C, RH 60%). They were given food and drink every day. After 1 week of acclimatization, they were randomly divided into 5 groups (7 individuals/group). Normal controls (K-) and positive controls (K+) were given orally distilled water every day. The test animal groups (3 groups) were each treated with a multilevel dose of *M. oleifera* fermentation extract (14, 42 and 84mg/kg BW/day) for 28 days before being injected by *S. typhi*. The administration of *M. oleifera* leaf fermentation extract was continued for 36 days after injection. *S. typhi* was intraperitoneally (0.5 mL/10g BW) with a concentration of 10^7 CFU/mL (except group K-). The animal testing protocol was approved by the ethics committee on animal experiments from Brawijaya University, Malang, Indonesia (No: 829-KEP-UB).

Test Confirmation of *Salmonella typhi* in the Blood:

In the group of mice infected by *S. typhi*, on day 30, a confirmation test was conducted to determine the success of *S. tyhpi* in infecting mice. The test was done by cutting off the tail to take the mice's blood. The blood was then tested for *pour plates* and catalase tests. The pour plate test was carried out in xylose lysine deoxycholate agar (XLD agar) media while the catalase test used hydrogen peroxide (H_2O_2)[26].

Cell Isolation in the liver:

After day 36 of post-treatment, mice were neck dislocated, then 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 transferred to a propylene tube and given PBS until the volume reached 3mL, then it was centrifuged at 2500rpm for 5 min at 10°C. The supernatant was removed and the obtained pellet was added with 1mL of PBS, then it was resuspended by using a vortex. Homogenates were mixed with 50µL of buffer and then they were incubated at 4°C for 20 min in the dark. Then, we added 200 mL of buffer. Homogenate was mixed well and centrifuged at 10°C at 2500rpm for 5 min. The pellets were added with 50µL of intracellular antibodies and incubated at 4°C for 20 min in the dark. Then the sample could be analyzed with flow cytometry (BD FACS Calibur, USA)[27].

Hematoxylin and Eosin (HE) Staining:

The liver tissues of mice were fixed in 10% neutral phosphate-buffered formalin solution, then dehydrated and embedded in paraffin to make conventional paraffin sections. The sections were cut into 4µm thickness and stained with Hematoxylin and Eosin (HE). The histopathological changes of liver tissues were observed under the optical microscope

Flow Cytometry analysis:

Flow cytometry analysis was performed to detect cell populations expressing HO-1, SOD-2, and Nrf-2. *Flow cytometry* was set to the acquiring state and parameter settings were analyzed. After incubating with the appropriate antibody, the sample was added with 500 mL of PBS and transferred to the flow cytometry cuvette for analysis. Then the acquire menu was selected and the *flow cytometry* counted the total cell count and detected fluorescents which were conjugated with antibodies. The obtained results were analyzed with BD cell quest Pro™.

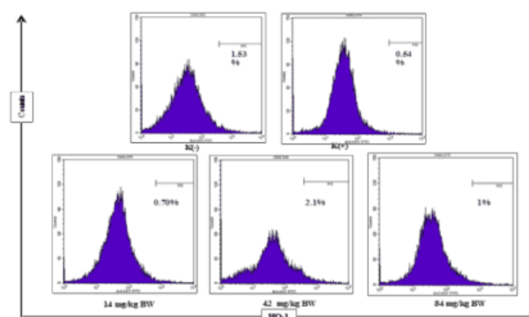
DATA ANALYSIS:

The number of cells (%) expressing HO-1, SOD-2, and Nrf-2 were analyzed by *flow cytometry* and the obtained results were tested for normality. Furthermore, the obtained data were tested by ANOVA with SPSS 16.0 for windows, followed by Duncan Multiple Range Test (DMRT) using p-value significance values <0,05. Hepatic preparations stained with HE were analyzed by a microscope to determine the profile of hepatocyte cells.

RESULT:

Analysis of HO-1 expressions:

From each treatment group, the average percentage of HO-1 showed the result that a dose of 14 mg/kg BW did not provide a significant difference when compared to control (+). Meanwhile, a dose of 84 mg/kg BW gave increasing results but they were not significant. However, a dose of 42 mg/kg BW gave significant results compared to a dose of 14 mg/kg BW and a dose of 42 mg/kg BW.



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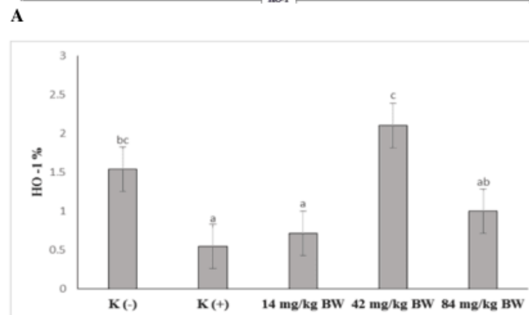
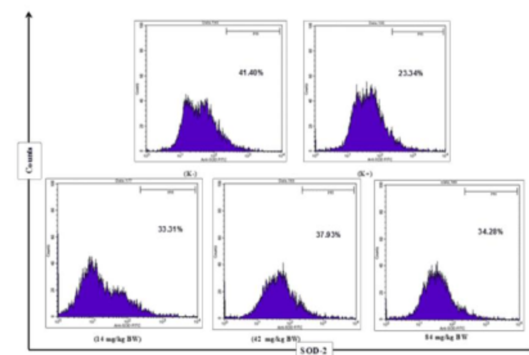


B

Figure 1. Flow cytometric analysis of liver cells. a. HO-1 representative expression in liver cells. b. Increase of HO-1 percentage in liver cells for 36 days after treatment. Results were considered significant at $p < 0.05$.

Analysis of SOD-2 Expression:

The average percentage amount of SOD-2 from each treatment group showed that fermentation extract of red *M. oleifera* leaves at a dose of 42 mg/kg BW had significant results than those of 14 mg/kg BW and 84 mg/kg BW. However, the treatment dose of 14 mg/kg BW was not significantly different from the dose of 42 mg/kg



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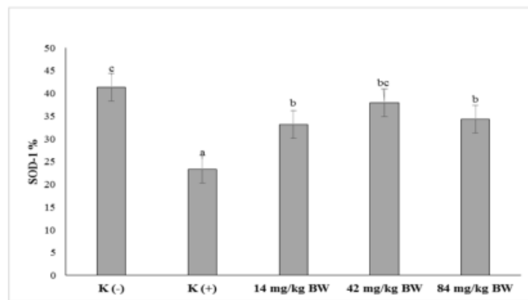
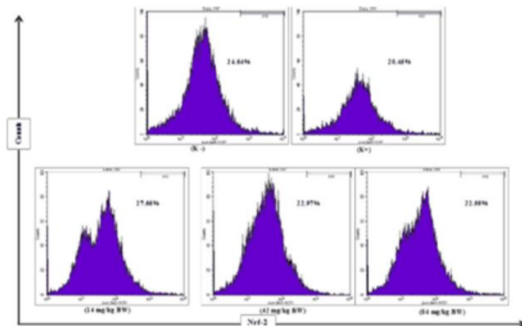


B

Figure 2. Flow cytometric analysis of liver cells. a. SOD-2 representative expression in liver cells. b. Increased percentage of SOD-2 in liver cells for 36 days after treatment. Results were considered significant at *p <0.05*.

Analysis of Nrf-2 expression:

Statistical test results showed a significant difference (p-value < 0.05) between treatments. The mean percentage of Nrf-2 from each treatment group showed that the dose of 42mg/kg BW was not significantly different from the dose of 84 mg/kg BW. However, the administration of fermented extract of red *M.oleifera* leaves at a dose of 14 mg/kg BW was significantly different with doses of 42 mg/kg BW and 84 mg/kg BW by 27.66 %.



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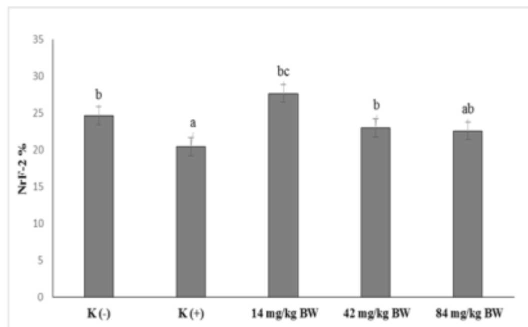

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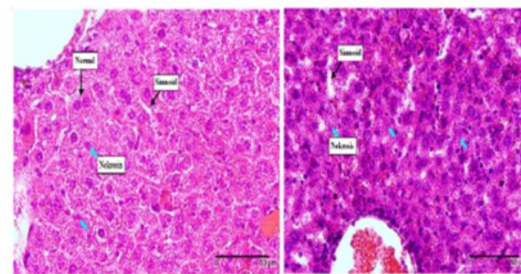
Figure 3. Flow cytometric analysis of liver cells. a. Nrf-2 **representative expression in the liver. b. Increased percentage of Nrf-2 in the liver for 36 days after treatment. Results were considered significant at *p <0.05*.**

Liver Histological Analysis:

Histological features in the treatment group showed a significant effect in preventing necrosis. In healthy controls (-) there were a few cells that experienced necrosis and dense hepatocyte cells were visible. It indicated that hepatocyte cells were in a normal state. Whereas in pain control (+), there were very many necrotic cells accompanied by sinusoids undergoing dilation. Treatment with a dose of 14mg/kg BW showed changes in liver cells and some normal cells were seen in some parts although necrosis cells were still visible. In some fields of view, fat degeneration appeared and sinusoidal widening was again seen. It was degenerating and necrotic by an average of 27.77%. Treatment with a dose of 42mg/kg BW showed improvement in hepatocyte cells and the sinusoid was not widened. Degeneration and necrosis appeared by an average of 26.89%. Treatment with a dose of 84mg/kg BW improved liver, but the cell damage was higher than the doses of 14mg/kg BW and 84mg/kg BW, in which the degeneration and necrosis were by an average of 30.88 %.



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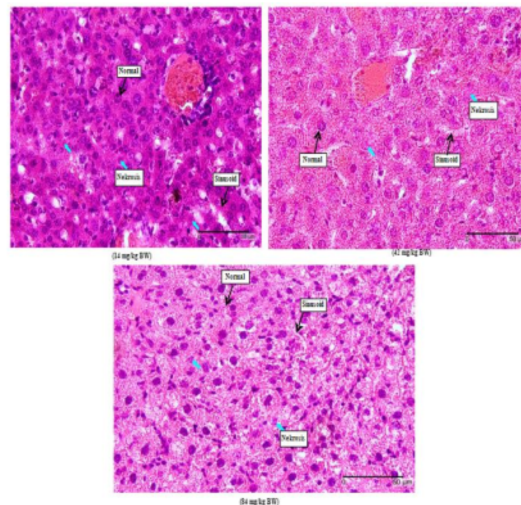

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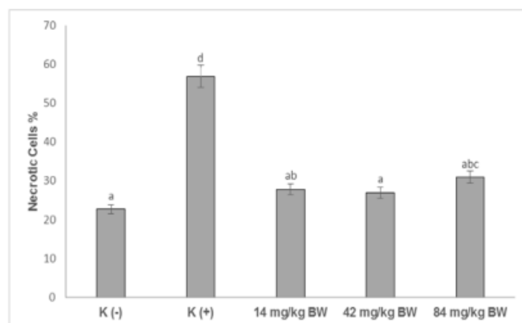

C

Figure 4. HE staining in liver preparations. a. (K-) indicated normal hepatocyte cells and (K+) indicated abnormal hepatocyte cells. b. Group dosage of 14 mg/kg BW, group dosage of 42 mg/kg BW and a group of 84 mg/kg BW. c. The percentage results and statistical analysis showed a significant difference ($P < 0.05$) between the treatment groups of fermented *M. oleifera* leaves extract. Results were considered significant at $p < 0.05$.

DISCUSSION:

The liver is an important organ that plays a central role in the metabolism of proteins, carbohydrates, fats, vitamins and detoxification functions. The liver can suffer damage caused by bacterial and viral infections, alcohol consumption, pollutants, and toxic chemicals[28]. *S. typhi* bacterial infections have been reported to cause liver enzymes to increase and their concentration is a marker of liver damage[29]. In *S. typhi* infection, the liver's histopathological evaluation usually shows mononuclear cell infiltration with a minimum focus of portal channel infiltration and focal necrosis areas[28]. Oxidative stress on cells is associated with increased expression and antioxidant enzyme activity[30]. This defense system is very important to detox ROS[31]. The appearing ROS is the *chemical lethal* which can kill and eliminate bacteria in phagocytic cells. ROS are the main causes of oxidative stress, including superoxide anions, hydroxyl radicals, and hydrogen peroxide[32]. Excessive ROS can cause oxidative stress in the liver, suppress the formation of antioxidant transcription pathways[33]. It will also react with biological macromolecules, proteins, and DNA leading to hepatocyte damage[5]. Nrf-2 is a transcription factor responsible for the transcription of antioxidants and cytoprotective genes[34]. The Nrf-2 pathway has an important role in acute liver damage[35]. HO-1 is effective to prevent various oxidative injuries. Many studies have confirmed that HO-1 has a protective effect on the liver with several pathological conditions, such as ischemia, reperfusion injury, chronic liver disease, etc[36]. Meanwhile, Superoxide dismutase 2 (SOD-2) is known as manganese-dependent superoxide dismutase (Mn-SOD). This enzyme plays an important role in the antioxidant defense system when dealing with oxidative stress[37]. SODs act first in defense of antioxidant enzymes against ROS and especially superoxide anion radical[38].

The results showed that anti-oxidative compounds derived from the extract fermentation produced a powerful antioxidant and contributed to the hepatotoxicity protection associated with the activation of Nrf-2/HO-1 and SOD-2 signaling pathways. SOD-2 works in such a way to capture and inhibit ROS production by delaying or preventing the occurrence of free radical reactions or by arresting the antioxidative radical compounds (radical scavenging) as initial protection against oxidative damage[39]. As an anti-oxidative stress regulator, Nrf-2 regulates the expression of antioxidant genes and phase II detoxification enzymes such as heme oxygenase-1 (HO-1), NAD (P) H quinone oxidoreductase 1 (NQO-1), and Glutamate Cysteine Ligase Catalytic subunit (GCLC) which counteracts oxidative stress by increasing the inhibition of ROS[40,41]. Uncontrolled chronic inflammation can cause disease. Therefore, anti-inflammatory agents from natural or synthetic sources are needed as therapeutic agents to prevent the disease. The increase in flavonoid total during fermentation is thought to be due to the activity of lactic acid bacteria during the fermentation process. Lactic acid bacteria produce enzymes that can free phenolic compounds in Moringa leaves so that they can add to the phenol group of flavonoid compounds[42].

In conclusion, it showed that anti-oxidative compounds derived from the fermented extract have a powerful antioxidant that acts as immunomodulatory agents that contribute to hepatotoxicity protection. The mechanism of action seems to be associated with the activation of Nrf-2/HO-1 and SOD-2 signaling pathways.

ACKNOWLEDGMENT:

The author would like to thank the members of Animal Anatomy and Physiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, and LPDP Ministry of Finance of Indonesia through the BUDI-DN scholarship for their supports in our research.

CONFLICT OF INTEREST:

All the authors have no conflict of interest.

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