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The hepatoprotective effect of Cheral as anti-oxidant and anti-inflammation on mice (*Mus musculus*) with breast cancer

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ABSTRACT

Introduction: Recent studies have reported that breast cancer may affect the physiology of other organs, including oxidative stress in the liver. On the other hand, some agents such as white turmeric (*Curcuma longa*) and Meniran (*Phyllanthus niruri*) seem to maintain redox stability and immunomodulation. Both of them are combined into Cheral potion. This study was aimed to investigate the Cheral efficacy in modulating oxidative stress based on Nuclear factor erythroid 2-related factor 2 (Nrf2), HEME OXIGenase (HO), and superoxide dismutase (SOD) levels as well as pro-inflammatory cytokines under breast cancer condition *in vivo*.
Methods: Nrf2, HO, and SOD from hepatocytes, and tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) from splenocytes were measured by flow cytometry after 14 days of Cheral administration.
Results: The results showed that mice model for breast cancer underwent oxidative stress denoted by high levels of HO, and SOD accompanied by increased levels of TNF-α and IFN-γ in the cancer group compared to normal healthy group ($P<0.05$). In contrast, Cheral treatment was able to modulate redox balance by declining levels of HO, SOD, TNF-α, and IFN-γ, but not Nrf2, compared to cancer group ($P<0.05$).
Conclusion: The results showed that breast cancer could alter the host's physiology, including liver oxidative stress. The levels of TNF-α and IFN-γ might contribute to regulation of redox balance in the liver. However, Cheral has potency as an alternative therapeutic agent to reduce oxidative stress in the liver under breast cancer condition.

Implication for health policy/practice/research/medical education:
Cheral showed to be a good therapeutic alternative to prevent oxidative stress and inflammation during breast cancer progression. Hence, it might be beneficial in patients with these conditions, especially the ones with breast cancer.
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Introduction

Breast cancer triggers the most mortality to women worldwide (1). Indonesia is a country with a high number of breast cancer cases. More than 36 per 100 000 of the total population suffer from breast cancer (2). Generally, treatments for breast cancer patients are surgery, radiotherapy, and chemotherapy, but these procedures often lead to resistance resulting in death (3). In addition, breast cancer also affects physiological conditions that worsen the condition of the patient. One of the physiological disorders is oxidative stress in the liver (4,5). Furthermore, certain disease can emerge due to chemotherapy treatment such as cisplatin (6,7).

Oxidative stress in the liver under breast cancer conditions was confirmed by Hojo et al *in vivo* (8). Although the mechanism is still unresolved, most likely the occurrence of oxidative stress is caused by the body's immune response such as cytokine production during cancer progression. Breast cancer may uprise the levels of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in response to the cancer progression (9,10).

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Since the spleen is a key organ in systemic regulation of immunity, its condition represents the activity of immune response in the body (11). A recent study suggested that the levels of splenic TNF-α and IFN-γ were augmented in the mice model of breast cancer (12,13). On the other hand, the liver also has many immunocompetent cells, such as Kupffer cells, which can be activated by those cytokines. Activated Kupffer cells can synthesize reactive oxygen species (ROS) and reactive nitrogen species and induce elevation of oxidant levels leading to oxidative stress (14).

Liver is the organ responsible for detoxification and xenobiotic metabolism, so it has the highest risk of experiencing oxidative stress among other organs. To deal with this condition, the liver is adapted by producing several antioxidant proteins such as heme oxygenase (HO) and superoxide dismutase (SOD). Those proteins are regulated by transcription factor Nrf2, which will be bound to antioxidant responsive element in DNA to transcribe genes for HO and SOD proteins (15,16). Alteration levels of those proteins may represent the presence of oxidative stress in the liver.

Exploration of herbal compounds continues to be developed as a safe alternative medicine to treat breast cancer, including combining several herbal compounds to provide better efficacy (17). One of the results of the herbal compound combination is Cheral, which consists of White Turmeric (*Curcuma longa*) and Meniran (*Phyllanthus niruri*). Both *C. longa* and *P. niruri* have high antioxidant properties that can either eliminate oxidants directly or regulate signaling such as Nrf2 and induce HO and SOD (18-22). In addition, extracts from these or other species of their families have anti-inflammatory and hepatoprotective activities (23-25). Therefore, this study aims to evaluate the hepatoprotective and anti-inflammatory properties of Cheral to prevent liver oxidative stress during breast cancer condition *in vivo*.

Materials and Methods

Mice and breast cancer model

Female Balb/c mice (24 individuals, 6-8 weeks old) were purchased from the Institute for Research and Community Service (LPPM), Gadjah Mada University, Yogyakarta, Indonesia. After 1 week of acclimatization, mice were injected with 7,12-dimethylbenz[a]anthracene (DMBA) (Tokyo Chemical Industry, Japan) dissolved by corn oil with dose as performed by Jayakumar et al (26) with some modification. In general, DMBA was given at the dose of 0.015 mg/g subcutaneously in the mammary region once in a week for 6 consecutive weeks. The confirmation of tumor formation was performed by palpation and histological observation.

Experimental design and Cheral treatment

Mice were divided into six groups, i.e. normal (N), breast cancer (K), breast cancer with cisplatin treatment, 3 mg/kg (CISP) (27), breast cancer with Cheral treatment, 2,466 mg/kg (D1), breast cancer with Cheral treatment, 1,233 mg/kg (D2), and breast cancer with Cheral treatment, 4,932 mg/kg (D3). Cisplatin was given through intraperitoneal injection (27), while Cheral was given through oral administration for 14 consecutive days. Having been treated for 14 days, the mice were sectioned for hepatocyte and splenic cells isolation.

Isolation of hepatocyte and splenic cells

The mice were killed by neck dislocation and then dissected for liver and spleen isolation. Liver and spleen then rinsed with sterile phosphate buffer saline (PBS) and homogenized using syringe holder in a petri dish containing PBS. The cell suspension was transferred to the polypropylene tube and PBS was added until 10 mL volume then they were centrifuged (2500 rpm, 10°C, 5 minutes). The supernatant was exiled from pellets then the pelles were resuspended in 1 ml PBS sterile.

HO, Nrf2, SOD, TNF-α and IFN-γ detection

A total of 50 µL hepatocyte and splenic cell suspensions were divided into 1.5 mL microtube containing 400 µL of PBS, then they were centrifuged (2500 rpm, 10°C, 5 minutes). The pellet containing hepatocytes was added with fixation buffer (BioLegend, USA) and incubated for 30 minutes, then the mixture was added with intracellular staining perm wash buffer or wash-perm (BioLegend, USA) before they were centrifuged (2500 rpm, 10°C, 5 minutes). The pellet was added the primary antibody (anti-Nrf2, anti-HO, and anti SOD) (BioLegend, USA) and incubated for 30 minutes. Having been incubated, the mixture was rinsed with wash-perm and centrifuged again (2500 rpm, 10°C, 5 minutes). Then, the pellet portion was added the secondary antibody FITC (BioLegend, USA) and incubated for 20 minutes. After the sample was incubated, it was resuspended with PBS and analyzed using flow cytometer (BD FACSCalibur, USA).

TNF-α and IFN-γ levels were analyzed from splenic cells. In general, the pellets containing spleen cells were mixed with extracellular antibodies (BioLegend, USA) and incubated for 30 minutes. After the incubation process completed, the cell suspension was added the fixation buffer (BioLegend, USA) and incubated again for 30 minutes. Then, it was added the wash-perm (BioLegend, USA) and centrifuged (2500 rpm, 10°C, 5 minutes). The pellet portion was separated from the supernatant and added the anti-TNF-α, and IFN-γ (BioLegend, USA) then incubated for 20 minutes. After incubation, the cell suspension was resuspended with PBS and analyzed by flow cytometer (BD FACSCalibur, USA).

Data analysis

The result from flow cytometry was analyzed using

BD Cellquest Pro™ program. Statistical analysis was performed using one-way ANOVA with α = 5% followed by Tukey Honestly Significant Difference (HSD) test to evaluate the significant difference among treatments (SPSS, IBM Statistics, USA).

Results

The hepatoprotective role of Cheral to modulate oxidative stress during carcinogenesis was shown by declining level of HO compared to cancer group (K) ($P < 0.05$). The decrease in HO levels in the treated dose resembles normal healthy group conditions. This was indicated by the absence of the significant difference between the two groups, especially in the treatment of dose 1 (D1). Treatment of dose 1 tended to give a better effect than the cisplatin group, although there was no significant difference between them (Figure 1).

Cheral also had a modulation effect on hepatic oxidative stress as presented in SOD levels. The best dose to modulate SOD was dose 1, strengthened by similarity to normal conditions ($P > 0.05$). The cisplatin group, dose 2, and dose 3 had similar efficacy in modulating SOD statistically, but Cheral administration was able to give better results than cisplatin. Administration of cisplatin reduced SOD levels, although it was not significant when being compared to the cancer group. On the other hand, oxidative stress was shown in the cancer group with the highest SOD level among other groups ($P < 0.05$) (Figure 2).

The level of Nrf2 was relatively similar in all treatments ($P > 0.05$). Neither normal, cancer, nor Cheral dose treatment groups had significantly different Nrf2 levels. This result allows the possibility of HO and SOD regulatory mechanisms without going through the Nrf2 pathway (Figure 3).

TNF-α and IFN-γ levels were measured to confirm the protective role of Cheral in reducing oxidative stress in the liver through regulation of spleenic proinflammatory cytokines. As secondary lymphoid organ, spleen condition represents imunological occasions throughout the body. The effect of Cheral administration on TNF-α and IFN-γ levels is shown in Figure 4 and Figure 5, respectively. The breast cancer group showed the highest augmentation in TNF-α level among other groups (Figure 4). These results emphasize the role of TNF-α as oxidative stress inducer in mice under breast cancer condition. On the other hand, administration of cisplatin and Cheral can significantly downregulate TNF-α levels as well as normal healthy group. The cisplatin treatment group also showed a decrease in TNF-α levels compared to the cancer group ($P < 0.05$).

Cheral administration caused a similar result to IFN-γ as well as TNF-α levels. It was consistent with TNF-α, IFN- γ levels increasing significantly in the cancer group compared to the normal healthy group. Administration of cisplatin and some doses of Cheral could decrease the levels of IFN-γ. Significant decrease in IFN-γ level occurred in the cisplatin and dose 3 ($P < 0.05$) groups. In doses 1 and 2 groups, IFN-γ levels were also decreased but with less profound when being compared with the cancer

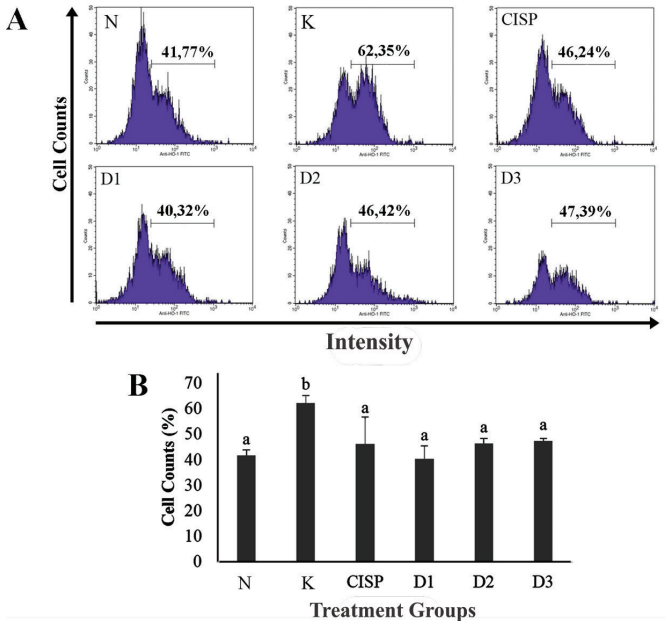

Figure 1. The effect of Cheral treatment on liver-derived Heme Oxigenase (HO) levels compared to normal healthy (N), cancer (K), and Cisplatin group (CISP). Cheral treatment could modulate HO level as well as normal healthy group compared with cancer group, shown by flow cytometric histogram (A) and statistical analysis (B). N: normal healthy mice, no-treatment; K: breast cancer mice, no-treatment; CISP: breast cancer mice, cisplatin treatment; D1: breast cancer mice, dose 2,466 mg/kg treatment; D2: breast cancer mice, dose 1,233 mg/kg treatment; D3: breast cancer mice, dose 4,932 mg/kg treatment. Results represent mean ± standart deviation (SD), n = 4. Different notation shows significant difference among groups based on Tukey HSD test ($P \leq 0.05$).

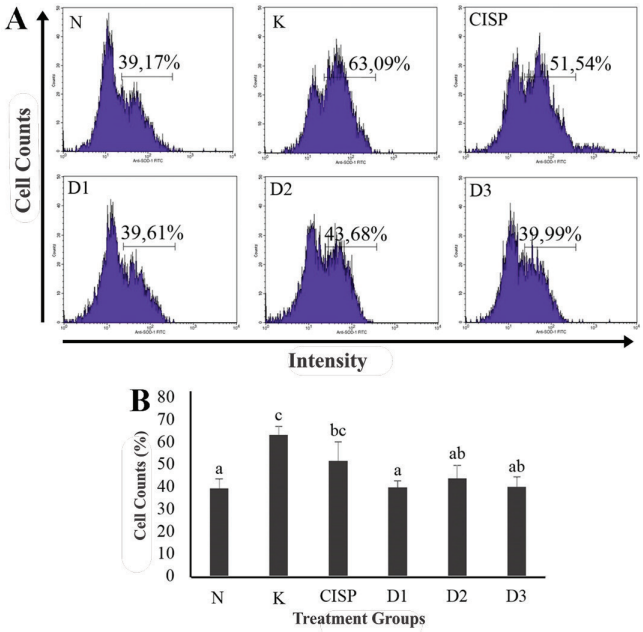

Figure 2. The effect of Cheral treatment on liver-derived Superoxide Dismutase (SOD) levels compared to normal healthy (N), cancer (K), and Cisplatin group (CISP). Cheral can modulate SOD levels better than cisplatin and it also can induce normal-like condition, especially for dose 1, shown by flow cytometric histogram (A) and statistical analysis (B). N: normal healthy mice, no-treatment; K: breast cancer mice, no-treatment; CISP: breast cancer mice, cisplatin treatment; D1: breast cancer mice, dose 2,466 mg/kg treatment; D2: breast cancer mice, dose 1,233 mg/kg treatment; D3: breast cancer mice, dose 4,932 mg/kg treatment. Results represent mean ± SD, n = 4. Different notation shows significant difference among groups based on Tukey HSD test ($P \leq 0.05$).

group (Figure 5). These results pointed out the possibility of the IFN-γ role in modulating oxidative stress. At the same time, this data also indicated the potential of Cheral potion in modulating oxidative stress due to breast cancer condition.

Discussion

There are conditions of oxidative stress in mice model of breast cancer (8) and it is well confirmed in this study by upregulation of HO and SOD in the cancer group compared with other groups (Figures 1 and 2). High levels of HO

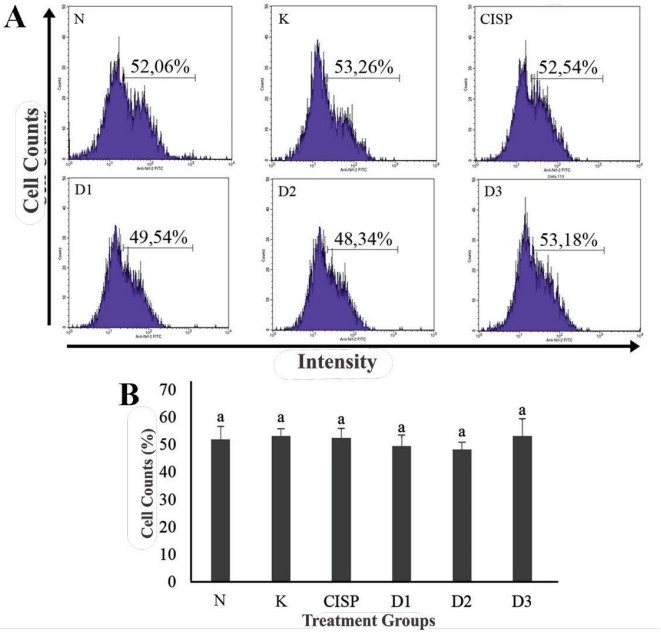

Figure 3. The effect of Cheral treatment on liver-derived Nuclear factor erythroid 2-related factor 2 (Nrf2) levels compared to normal healthy (N), cancer (K), and Cisplatin group (CISP). There is no significant difference for Nrf2 levels among all groups of treatment (A) and based on statistical analysis (B). N: normal healthy mice, no-treatment; K: breast cancer mice, no-treatment; CISP: breast cancer mice, cisplatin treatment; D1: breast cancer mice, dose 2,466 mg/kg treatment; D2: breast cancer mice, dose 1,233 mg/kg treatment; D3: breast cancer mice, dose 4,932 mg/kg treatment. Results represent mean ± SD, n = 4. Different notation shows significant difference among groups based on Tukey HSD test ($P \leq 0.05$).

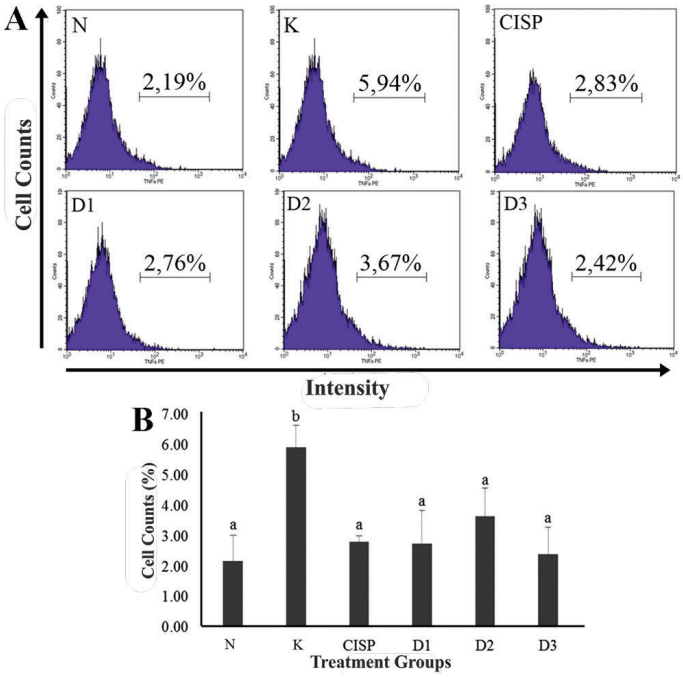

Figure 4. The effect of Cheral treatment on spleen-derived Tumor Necrosis Factor- α (TNF-α) levels compared to normal healthy (N), cancer (K), and Cisplatin group (CISP). Cheral can downregulate TNF-α to normal-like levels as well as cisplatin (A) and showed statistical difference with cancer group (B). N: normal healthy mice, no-treatment; K: breast cancer mice, no-treatment; CISP: breast cancer mice, cisplatin treatment; D1: breast cancer mice, dose 2,466 mg/kg treatment; D2: breast cancer mice, dose 1,233 mg/kg treatment; D3: breast cancer mice, dose 4,932 mg/kg treatment. Results represent mean ± SD, n = 4. Different notation shows significant difference among groups based on Tukey HSD test ($P \leq 0.05$).

and SOD are commonly used as indicators for oxidative stress condition in a tissue. Augmentation of HO level is a mechanism of cellular sensitivity to oxidative stress by producing HO in large quantities for oxidant elimination (28). In addition, high levels of SOD are also a form of cellular response under oxidative stress, particularly in liver disorders. Liver entering hepatocellular carcinoma pathogenesis also suggests an increase in SOD levels in the early stages (29).

The hepatoprotective effect of Cheral to oxidative stress in this study was shown by decreasing levels of HO and SOD as well as normal healthy group condition (Figures 1 and 2). Cheral potion has an advantage in modulating oxidative stress due to the combination of various antioxidant compounds in its composition. Antioxidants combinations have better efficacy than one type of antioxidant alone (30). *C. longa* and *P. niruri* as the main compositions of Cheral potion contain various types of antioxidants that have been widely evaluated to reduce oxidative stress and treat cancer. *C. longa* contains phenols, saponins, flavonoids, curcumin, and some polyphenol compounds which are widely proven as good antioxidants (18,19,22). *P. niruri* contains many phenols, flavonoid, and tannin that also widely proven their antioxidant activities (31).

The antioxidant ability of Cheral was performed through non-enzymatic reaction by alleviating HO and SOD levels, which can interact directly with oxidants (32). Flavonoid compounds are capable of forming a complex with copper or iron to prevent ROS formation (33,34). In addition, curcumin from *Curcuma* species can disrupt the structural stability of free radicals by transferring electrons to H atoms in OH and CH_2 groups that cause prevention of adverse cellular damage (35,36). When antioxidants from Cheral eliminate free radicals, the tissues will return to homeostasis conditions and the levels of the indigenous antioxidant proteins such as HO and SOD are reduced (Figures 1 and 2).

There was no significant difference for Nrf2 in all experimental groups (Figure 3) showing that HO and SOD regulation occurs without going through the Nrf2 pathway. In addition to the Nrf2 transcription factor, HO can be regulated by Bach-1 repressor transcription factor (37). Bach-1 protein is bound to the promoter region to prevent transcription of the HO gene (38). This process occurs when intracellular heme level is low (39). On the other hand, polyphenols and flavonoids can scavenge heme and make the intracellular heme reduced (40,41). Low level of heme generates Bach-1 interaction with the promoter region of the HO to suppress HO transcription. This mechanism will lead to HO reduction so that the HO level in the Cheral treatment becomes lower than the cancer group.

High level of SOD implies the presence of oxidative stress in tissue (29). The declining levels of SOD in this study (Figure 2) have a similar result with Schaffer et al

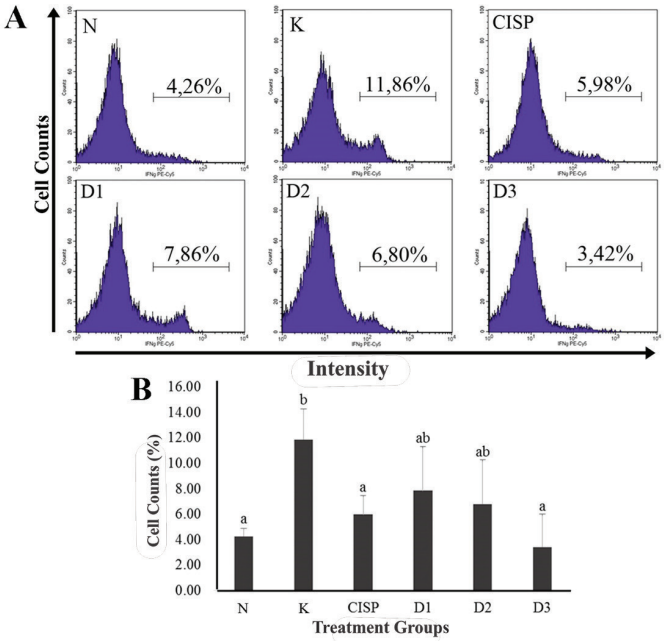

Figure 5. The effect of Cheral treatment on spleen-derived Interferon- γ (IFN-γ) levels compared to normal healthy (N), cancer (K), and Cisplatin group (CISP). IFN-γ levels decreased significantly in cisplatin group and dose 3, shown by flow cytometric histogram (A) and based on statistical analysis (B). N: normal healthy mice, no-treatment; K: breast cancer mice, no-treatment; CISP: breast cancer mice, cisplatin treatment; D1: breast cancer mice, dose 2,466 mg/kg treatment; D2: breast cancer mice, dose 1,233 mg/kg treatment; D3: breast cancer mice, dose 4,932 mg/kg treatment. Results represent mean ± SD, n = 4. Different notation shows significant difference among groups based on Tukey HSD test ($p \leq 0.05$). ($P \leq 0.05$).

(42) study which explains that polyphenols in the extract can act as a radical scavenger. Also, SOD provokes redox balance in the tissues (42). The downregulation of SOD levels in this study might involve the PI3k/Akt pathway and the transcription factor NF-κB since NF-κB controls the expression of SOD through the PI3K/Akt mechanism (43). In a previous research, Curcumenol from *C. longa* and extract from *P. niruri* were able to inhibit the PI3K/Akt pathway to decrease NF-κB (16,44) and downregulate SOD level.

Breast cancer causes the liver to undergo oxidative stress through the elevation of HO and SOD levels compared with other groups (Figures 1 and 2). Oxidative stress can be induced by inflammation through the elevation of TNF-α and IFN-γ as represented in lymphoid organ conditions such as spleen (9,10,45). Under cancer conditions, immune cells will produce large amounts of TNF-α and IFN-γ and spread through blood vessels, lymphatic vessels, and lymphoid organs (9,10). Augmentation of TNF-α and IFN-γ can increase immune system activity in the liver and stimulate inflammation (14,46). On the other hand, both *P. niruri* and curcumin from *C. longa* extract can suppress TNF-α and IFN-γ (24,47,48). Thus, the decreasing levels of spleen-derived TNF-α and IFN-γ occur as a result of physiological improvement after Cheral administration and reduction in oxidative damage take place under breast cancer condition.

Conclusion

This study confirmed that mice models for breast cancer undergo oxidative stress in liver organ based on high HO and SOD levels. On the other hand, increasing concentration of TNF-α and IFN-γ in the cancer group signifies the role of the cytokine in modulating oxidative stress under breast cancer condition. Cheral could improve physiological conditions by diminishing HO, SOD, TNF-α, and IFN-γ levels. Hence, it might be a good candidate as alternative therapeutic agent for breast cancer condition.

Authors' contributions

FEH performed study and wrote first draft of the manuscript; AS accomplished statistical analysis and literature search; HT carried out literature research and review; MI comprehended the research idea; MR designed the work and revised the manuscript. All authors read and approved publication of the final manuscript.

Conflict of interests

All authors declare that there is no conflict of interest.

Ethical considerations

All animals conditions and handling were approved by the Ethical Committee of Brawijaya University (Ethical Clearance No. 925-KEP-UB).

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