Anti-inflammatory effects of functional beverage from a mixture of moringa leaves, pandanus leaves, and red ginger in mice induced with monosodium urate crystal

T D Widyaningsih¹, M Rachmawati¹, E Prabawati¹, and S Winarsih²

¹Department of Food Science and Technology, Universitas Brawijaya, Jl. Veteran, Malang, ZIP 65145, Indonesia
²Faculty of Medicine, Universitas Brawijaya, Jl. Veteran, Malang, ZIP 65145, Indonesia
E-mail: tridewantiw@ub.ac.id

Abstract. Production of uric acid that exceeds normal limits in the blood (hyperuricemia) can cause the formation of urate crystals and inflammatory reactions. In various studies, moringa leaves, pandanus leaves, and red ginger are known to contain phytochemical components (phenols and flavonoids) that are useful as antioxidants, anti-inflammatory, inhibiting xanthine oxidase enzyme activity, overcoming rheumatism, and antidiabetic. Functional drinks from the mixture of moringa leaves, pandanus leaves, and red ginger were optimised using the response surface method (RSM) in previous studies. The effect of this functional beverage product was tested for its effect as an anti-inflammatory in vivo. The testing process of the anti-inflammatory effect was using intradermally monosodium urate (MSU)-induced mice. Mice were measured for changes in edema, and after two days of treatment, the spleen was taken for flow cytometry testing of inflammatory cytokine expression of CD11b⁺ TNFa⁺, CD11b⁺ IL6⁺, CD11b⁺ IL10⁺, and blood was taken to test the expression of cytokine CD11b⁺ TNFa⁺. The results showed functional beverage products could potentially be used as an alternative anti-inflammatory agent in gout because it significantly inhibited edema, significantly inhibited the expression of proinflammatory cytokines CD11b⁺ TNFa⁺ and CD11b⁺ IL6⁺ and increased the expression of anti-inflammatory cytokines CD11b⁺ IL10⁺, where the anti-inflammatory effect was not significantly different from the control of indomethacin drugs.

1. Introduction
The incidence of hyperuricemia has been associated with gout, rheumatism, and other metabolic disorders such as heart disease, insulin resistance, diabetes, obesity, and others [1, 2]. Increased uric acid levels correlate with increased oxidative stress in cells through the mechanism of activating NADPH [2]. Excessive production of uric acid can lead to the formation of urate crystals that accumulate and cause inflammatory reactions in the joints, commonly called gout or arthritis [3-5]. In patients with arthritis or gout, there are elevated levels of proinflammatory cytokines [6]. Chemical treatment therapy for hyperuricemia and arthritis has side effects in the body, especially digestion and kidney [7]. Natural antioxidants can be used as an alternative combination treatment to reduce the harmful effects of chemical drugs.
Moringa leaves (*Moringa oleifera*), pandanus leaves (*Pandanus amaryllifolius* Roxb.), and red ginger (*Zingiber officinale* Roscoe) are plants that are often found and utilised, including in Indonesia. Moringa leaves contain vitamins and minerals, mainly flavonoids quercetin and kaempferol, β sitosterol, and caffeoylquinic acid [8][9]. Red ginger and pandanus leaves have been studied to have positive effects on arthritis and gout [10]. Components contained in moringa leaves, pandanus leaves, or red ginger indicate antioxidant activity associated with inhibition of increased free radical compounds that occur in the mechanism of increased levels of uric acid and gout inflammation [11].

The previous studies carried out an integrated food therapy product formulation from moringa leaves, Pandanus leaves, and red ginger in the form of functional drinks. The results of the analysis of these functional beverage products indicate the presence of antioxidant activity and phytochemical compounds of the phenol and flavonoid are thought to be able to act as an anti-inflammatory. This study aims to determine the anti-inflammatory activity if integrated food therapy products in the form of functional drinks through specific mechanisms in animal experiment (*in vivo*).

2. Materials and Methods
Materials used in this study are, i.e. experimental animals of male mice, the spleen of male mice, MSU crystals, functional drinks of moringa leaves, pandanus leaves, and red ginger, indomethacin, EDTA, PBS solution, anti-TNF-α antibodies, anti-IL-6, and anti-IL-10.

In this study, experimental male mice were used. Experimental animals were divided into six groups, feeding and drinking ad libitum. Experimental mice were adapted before treatment. Checking the conditions and weighing the mass of experimental animals was done at the end of the adaptation period to ensure the animals were in a healthy condition. The division of experimental animals treatment groups were as follows: 1) Group P0: normal mice with normal diet and pure water treatment; 2) Group P1: positive control, mice induced inflammation with intradermal injection of MSU crystals by 50 µl and pure water treatment; 3) Group P2: drug control, mice induced inflammation with intradermal injection of MSU crystals of 50 µl and treatment of indomethacin drugs 19.5 mg/kg BW; 4) Group P3: inflammatory induced mice with intradermal injection of MSU crystals of 50 µl and treatment of traditional functional drinks mixed with moringa leaves, pandanus leaves, and red ginger at 26 ml/kg BW; 5) Group P4: mice induced inflammation with intradermal injection of MSU crystals by 50 µl and treatment of functional powder I drink at a dose of 0.91 g/kg BW; 6) Group P5: mice were induced inflammation by intestinal injection of MSU of 50 µl and treatment of functional powder II drinks at 1.82 g/kg BW.

One hour before MSU crystal injection, treatment was carried out in each group. Induction of inflammatory states in experimental animals by 50 µl intradermal injection of monosodium urate crystals (MSU) in groups P1, P2, P3, P4, and P5. Changes in foot volume were observed at 0, 4, 24, and 48 hours after MSU crystal injection. On the final day of treatment, mice were surgically removed for blood to be tested for CD11b+ TNFα+ cytokines, and the spleen was taken to test the expression of cytokines CD11b+ TNFα+, CD11b+ IL-6+, and CD11b+ IL-10+ by flow cytometry method.

Data on changes in foot volume, CD11b+ TNFα+, CD11b+ IL-6+, and CD11b+ IL-10+ cytokine levels were analysed using the Minitab 16 program with various ANOVA analyses.

3. Results and Discussion
Hyperuricemia can be a cause of gout or arthritis. Hyperuricemia can cause urate crystal deposition and formation. Gout is one of the inflammatory diseases that can occur when tissue in the body is supersaturated with urate, causing the formation and deposition of MSU in the articular and periarticular tissues [12].

In various studies testing anti-inflammatory effects, it is known that MSU crystal injection can cause an increase in leg thickness in experimental animals. Changes in the volume of mouse feet can be seen in Figure 1.
Figure 1. Changes in the volume of mouse feet during the experiment.

Changes in the foot volume of the positive control group were used as a reference for maximum inflammation and then compared with other treatments to evaluate the anti-inflammatory effect [13]. After the injection of MSU crystals, the volume of mice's foot increased significantly compared to the negative control group; this indicates the occurrence of edema due to MSU crystals. Changes in leg volume continue to increase at the 4th hour to the 24th hour after MSU crystal injection. The average foot volume after being treated with functional drinks or indomethacin drugs decreased better than positive controls at 24 and 48 hours. This indicated that the functional drink a mixture of moringa leaves, pandanus leaves and red ginger has an inhibitory effect in the final phase of the development of inflammation that caused edema.

Functional drinks made of moringa leaves, pandanus leaves, and red ginger are indicated as potential anti-inflammatory agents because they can inhibit the formation of edema after MSU crystal injection. Phytochemical components of moringa leaves, pandanus leaves, and red ginger in functional beverage products have antioxidant activity that can inhibit the formation of free radical compounds, where the increase in free radical compounds plays a role in inflammatory events such as gout [14].

Ferrari et al. [13] reported that phagocytosis of urate crystals initiates chemotaxis and changes in cell permeability, flavonoid components, especially quercetin are thought to reduce edema due to MSU crystal formation. Research by Tsala et al. [15] shows that water extract of moringa leaves can significantly reduce edema in animals induced by xylene and egg white (acute inflammation) and cotton pellet granulomas (chronic inflammation).

Inflammatory induction by MSU crystals has the characteristic of neutrophil infiltration which results in tissue damage, which is due to the release of free radicals, lysosomal enzymes, prostaglandin E2 (PGE2), leukotrienes, and interleukin 1 [16, 17]. Acute inflammation that occurs due to MSU crystal invasion results in the release of cell mediators such as cytokines and prostaglandins and free radicals. Free radicals produce nitric oxide (NO), hydroxyl radicals (OH\(^-\)), and superoxide anions (O\(_2^\cdot\)\(^-\)). Strong antioxidants such as phenolic components are reported to neutralise free radicals by donating an electron or hydrogen atom to reactive oxygen compounds [18].

MSU crystals trigger the activation of inflammatory mediators. MSU crystals stimulate synovial cells, monocytes, macrophages, and neutrophils for TNF-\(\alpha\) and IL-6 secretion. MSU crystals can stimulate the expression of inflammatory cytokines and chemokines as a quick attempt to eliminate dangerous agents [19]. The results of the analysis of CD11b + TNF-\(\alpha\) expression in the spleen of mice are presented in Figure 2. The expression of TNF-\(\alpha\) produced by CD11b + in the spleen of mice was
confirmed further by testing the same parameters in the blood of mice. The results of TNF-α testing by CD11b + on the blood of mice are presented in Figure 3.

**Figure 2.** Results of TNF-α expression by CD11b on the spleen samples of mice.

**Figure 3.** The results of TNF-α expression by CD11b on the blood samples of mice.

**Table 1.** Relative values of TNF-α in spleen and blood of mice.

| Treatment                | relative% TNF-α spleen | relative% TNF-α blood |
|--------------------------|------------------------|-----------------------|
| Negative control         | 11.59 ± 4.76<sup>b</sup> | 5.41 ± 5.02<sup>b</sup> |
| Positive control         | 24.04 ± 3.58<sup>a</sup> | 19.03 ± 9.51<sup>a</sup> |
| Indomethacin             | 15.38 ± 4.49<sup>b</sup> | 10.89 ± 5.96<sup>b</sup> |
| Traditional drink        | 12.64 ± 6.40<sup>b</sup> | 5.55 ± 3.66<sup>b</sup> |
| Powder Drink I           | 13.54 ± 8.17<sup>b</sup> | 6.03 ± 5.19<sup>b</sup> |
| Powder Drink II          | 13.90 ± 7.84<sup>b</sup> | 6.90 ± 5.19<sup>b</sup> |
| **BNT value α 5%**       | 7.98                   | 7.87                  |

Information:
- The average value is ± SD from 5 replicates data.
- Indomethacin drug control dose 19.5 mg/kg body weight; traditional drinks 26 ml/kg body weight; powder drink 10.91 g/kg BW; Powder drink II 1.82 g/kg body weight.
- The same letter notation is not significantly different from BNT test α 5%.

Based on the flow cytometry plot results in Figure 2 and Figure 3, an increase in TNF-α cytokine expression was found in the positive control group. Results of analysis and further tests of the relative percentage of flow cytometry plot graphs Figure 2 and Figure 3 are presented in Table 1. For both TNF-α testings in the spleen and blood of mice, TNF-α proinflammatory cytokines in the positive control group experienced a significant increase (p < 0.05) compared with negative or healthy control. The data in Table 1 shows that the group treated with indomethacin drugs as well as traditional functional drinks and powdered drinks, both doses had TNF-α percentage values that were significantly different from the positive control group.
Tumour Necrosis Factor Alpha (TNF-α) is a proinflammatory cytokine that plays the most role in the inflammatory process and is used as an indicator of cells experiencing oxidative stress, apoptosis, or even necrosis. TNF-α levels produced by macrophages or lymphocytes are known to increase during inflammation [20].

This study indicates that treatment using herbal functional drinks from a mixture of moringa leaves, pandanus leaves, and red ginger can inhibit TNF-α in mice exposed to free radicals produced by MSU crystals. Excessive production of free radicals can stimulate the synthesis of proinflammatory cytokines through the activation of one of them NLRP3 inflammation (Nucleotide-binding oligomerisation domain, leucine-rich repeat-containing gene family, and pyrin domain-containing 3), where NLRP3 is the main key to crosslink pathways signal between redox and inflammatory responses. Inflamasome stimulates the production of cytokines IL-1β from the cytoplasm into the extracellular environment and activates Toll-like receptors (TLR-1). Activated TLR-1 triggers the activation of NF-κB and MAPK to induce the transduction of proinflammatory signals and produce cytokines such as IL-6 and TNF-α. [21].

In addition to TNF-α cytokines, interleukin 6 (IL-6) cytokines also play an important role in the inflammatory state. Increasing the concentration of IL-6 is positively correlated with various inflammatory diseases, so IL-6 is one of the markers of inflammation. IL-6 increases neutrophil and macrophage withdrawal [22]. The results of IL-6 cytokine testing by CD11b+ on the spleen of mice are presented in Figure 4. From the flow cytometry plot graph, the relative values of IL-6 cytokines produced by CD11b are presented in Table 2.

![Flow Cytometry Plot](image)

**Figure 4.** The results of IL-6 expression by CD11b on spleen samples of mice.

**Table 2.** Relative values of IL-6 spleen of mice.

| Treatment              | % relative IL-6  |
|------------------------|------------------|
| Negative control       | 14.89 ± 6.05b    |
| Positive control       | 33.42 ± 6.91a    |
| Indomethacin           | 21.21 ± 5.56b    |
| Traditional drink      | 18.00 ± 6.19b    |
| Powder Drink I         | 18.53 ± 5.33b    |
| Powder Drink II        | 19.36 ± 7.56b    |

BNT value α 5% 8.25

Information:
- The average value is ± SD from 5 replications data
- Indomethacin drug control dose 19.5 mg/kg body weight; traditional drinks 26 ml/kg body weight; powder drink I 0.91 g/kg BW; powder drink II 1.82 g/kg body weight.
- The same letter notation is not significantly different from the BNT test α 5% CD11b+ IL10b.

Figure 4 and Table 2 show the lowest expression and relative value of IL-6 cytokines by CD11b+ cells in the negative control group, where the experimental animals were not induced by MSU crystals.
The administration of NSAID-indomethacin drugs to MSU-induced crystalline animals, the administration of a functional drink mixed with moringa leaves, pandanus leaves, and red ginger in mice induced inflammation by MSU crystals can significantly reduce IL-6 compared with positive control. Known trends in changes in expression of CD11b+ IL-6+ are in line with changes in expression of CD11b+ TNF-α+. IL-6, and TNF-α are important cytokines in inflammation, especially in gout. Decreased levels of TNF-α and IL-6 contribute to the improvement of the inflammatory response due to the induction of monosodium urate crystals [23].

The results of this study indicate that both indomethacin drugs, traditional functional drinks and powdered drinks of all types of doses can significantly counteract the production of proinflammatory cytokines TNF alpha and IL-6 by CD11b. This indicates functional drinks from a mixture of moringa leaves, pandanus leaves, and red ginger is thought to play an important role in suppressing the expression of proinflammatory cytokines in inflammatory conditions due to MSU crystals. The phenolic component has a protective effect against inflammation through various mechanisms. First, as an exogenous antioxidant, Second, it prevents local and systemic inflammation by increasing the activity of antioxidant enzymes. Third, polyphenols modulate NLRP3 activation so that they can inhibit TLR-1 (Toll-like receptor-1) and ultimately suppress the production of proinflammatory cytokines [24].

Both the raw materials of moringa leaves, pandanus leaves, and red ginger, as well as functional beverage products, are known to contain a lot of flavonoid components. Flavonoids are known as phytochemical components with the best antioxidant activity. Routine flavonoids and quercetin are known to play a role in anti-inflammatory caused by MSU crystals.

**Figure 5.** The results of IL-10 expression by CD11b on spleen samples of mice.

IL-10 is an anti-inflammatory cytokine centre that plays a role in the inflammatory state. IL-10 controls the inflammatory process by suppressing the expression of proinflammatory cytokines, chemokines, and adhesion molecules found in macrophages, monocytes, neutrophils, and T cells. Macrophages that take MSU crystals can produce anti-inflammatory cytokines such as TGFβ and IL-10 [25]. The results of the analysis of CD11b+ IL10 expression in the spleen of mice are presented in Figure 5 and Table 3. The smallest expression of cytokine IL-10 was shown in groups of experimental animals that were injected with MSU crystals but without any drug treatment or functional drinks. The administration of indomethacin drugs and functional drinks can provide an increased expression of IL-10 anti-inflammatory cytokines in experimental animals that have been injected with MSU crystal. The administration of traditional drinks and powder drinks with a dose of 0.91 g/kg BW for mice was able to show a significant increase in the expression of cytokine IL-10 compared to positive controls. IL-10 is a potent anti-inflammatory status promoter. IL 10 works by decreasing or inhibiting the expression of several proinflammatory cytokines and other mediators [26]. In vitro research shows that IL 10 suppresses proinflammatory cytokines produced by macrophages and monocytes such as TNF-α, IL-1,
IL-6, IL-8, and IL-12. In this case, IL-10 as an immunoregulator can help reduce the inflammatory response. IL-10 mediates anti-inflammatory effects by inhibiting the transcription factor NF-κB.

**Table 3.** Relative values of IL-10 spleen of mice.

| Treatment                | % relative IL-10 |
|--------------------------|------------------|
| Negative control         | 35.72 ± 7.53a    |
| Positive control         | 23.50 ± 6.67b    |
| Indomethacin             | 37.04 ± 5.58a    |
| Traditional drink        | 36.62 ± 4.23a    |
| Powder Drink I           | 35.02 ± 5.94a    |
| Powder Drink II          | 32.22 ± 9.92ab   |

BNT value α 5% 8.98

Information:
- The average value is ± SD from 5 replications data
- Indomethacin drug control dose 19.5 mg/kg body weight; traditional drinks 26 ml/kg body weight; powder drink I 0.91 g/kg BW; powder drink II 1.82 g/kg body weight.
- The same letter notation is not significantly different from BNT test α 5%

4. Conclusions

The functional beverage product mix of moringa leaves, pandanus leaves, and red ginger significantly inhibit edema and the expression of proinflammatory cytokines namely CD11b+ TNFα and CD11b+ IL6+, and increases the expression of anti-inflammatory cytokines CD11b+ IL10+, where the anti-inflammatory effect is not significantly different from the control of indomethacin drugs.

5. References

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