Effect of mangiferin on mRNA expression of transforming growth factor beta in rats with liver fibrosis induced by thioacetamide

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Abstract. The bioactive compound mangiferin is known for its inhibition of fibrosis, a process that is reversible as a result of a liver injury. In this study, we aimed to explore the possible antifibrotic effect of mangiferin through the inhibition of mRNA expression of transforming growth factor beta (TGF-β), which is the primary profibrogenic cytokine. We induced liver fibrosis in rats by intraperitoneally injecting them with thioacetamide 200 mg/kg three times per week for 5 weeks. We gave mangiferin orally at a dose of 50 mg/kg/day and 100 mg/kg/day for 5 weeks. We had the following four treatment groups: the control group, the thioacetamide only group, the thioacetamide + mangiferin 50 mg/kg/day group, and the thioacetamide + mangiferin 100 mg/kg/day group. We measured the expression of TGF-β mRNA with qRT-PCR and calculated it using the Livak method. We found an increased expression of TGF-β mRNA in the group that was administered only thioacetamide compared with that of the control group. In the treatment groups, we found a lower TGF-β mRNA expression than that in the thioacetamide-only group, but the only treatment achieving a statistically significant result compared with that of the thioacetamide only group was the group with mangiferin at a dose of 50 mg/kg BW. Mangiferin may, therefore, have a beneficial effect in inhibiting thioacetamide-induced fibrogenesis by lowering the mRNA expression of TGF-β.

1. Introduction
One of the most important health problems in Indonesia is liver cirrhosis, a disease with many etiologies but, in Indonesia, most commonly, infection by the hepatitis B virus. In Indonesia the prevalence of this virus infection had, by [date], reached 23 million or about 9.4% of the population. Meanwhile, in Asia and Africa about 500,000 to 1.2 million deaths every year are caused by liver cirrhosis, 2%–8% of which are from Southeast Asia. Liver cirrhosis itself is a condition preceded by a reversible process known as fibrosis, and characterized by the excessive accumulation of extracellular matrix (ECM), including collagen, caused by an imbalance between the synthesis and degradation of ECM [1–3].
Liver injury activates hepatic stellate cells (HSCs) into myofibroblast cells, which are not present in a normal liver, so initiating liver fibrosis. The myofibroblast cells then migrate to the injury location, where they produce an excessive amount of ECM—a process known as fibrogenesis. During liver injury fibrogenic cytokines, such as TGF-β, trigger the activation of HSCs. In laboratory animal studies, the induction of thioacetamide has been demonstrated to trigger liver fibrosis. The active metabolite of thioacetamide—thioacetamide sulfodioxide (TASO₂)—indirectly activates type M2 macrophages, which, in turn release TGF-β₁, thereby initiating the fibrogenesis that eventually becomes liver fibrosis. Untreated liver fibrosis—although reversible—can develop into cirrhosis or even hepatocellular carcinoma. The very reversibility of liver fibrosis makes the development of antifibrotic agents that can inhibit fibrogenesis or induce fibrolysis, a realistic goal. Animal studies have demonstrated that potential antifibrotic agents generally have anti-inflammatory and antioxidant mechanisms of action to inhibit the activation of HSCs and the apoptosis of hepatocytes, respectively. However, to the best of our knowledge, no potential antifibrotic agent effective for humans has to date been isolated [2,4,5].

Mangiferin, a natural xanthone found in many parts of the mango tree, is a common plant found in Indonesia and other tropical areas [6,7]. The many potential therapeutic effects of mangiferin currently being studied include their antioxidant, anti-inflammatory, antiulcerogenic, neuroprotective, hepatoprotective, and even antibacterial properties [6,7]. Studies using a rat model show that mangiferin extracted from the mango stem can, by decreasing the production of reactive oxygen species (ROS), suppress oxidative stress in the liver [6]. The anti-inflammatory effect of mangiferin has also been shown to limit the release of TGF-β, thereby lessening the activation of HSCs following liver injury [7]. The encouraging results of these studies suggest the need for more extensive studies of mangiferin to confirm these results in humans and to reveal any other potential therapeutic effects.

Based on these results, we aimed to investigate the effect of mangiferin in rats with thioacetamide-induced liver fibrosis acting through the expression of TGF-β, which is one of HSCs activators. We focused in this study on the mRNA expression of TGF-β in rats with thioacetamide-induced liver fibrosis and to compare the mRNA expression of TGF-β in rats with liver fibrosis and given mangiferin at doses of either 50 mg/kg/day or 100 mg/kg/day.

2. Materials and Methods
2.1 Materials
We purchased RNA isolation, cDNA synthesis, and reverse transcription polymerase chain reaction (RT-PCR) kits from Roche™.

2.2 Animals
In this experimental study we used liver tissues that were restored at a temperature of -80°C from the previous study. We derived the liver tissues from 20 male Sprague-Dawley (SD) rats aged 5 weeks with body weights of around 220 g–260 g. We acquired the rats used in this study from BPOM, Jakarta, Indonesia. We housed the rats in the animal laboratory of the Department of Pharmacology and Therapeutic Medicine, Faculty of Medicine, Universitas Indonesia, at a constantly controlled temperature of 21 °C and humidity of 55% with a 12 hour light/dark cycle. We gave the rats standard laboratory food and water ad libitum. The Animal Care Committee from Ethics Committee, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia approved the protocol.

2.3 Fibrosis induction
We divided 20 rats into four groups. We designated the first group as the normal group (n = 3) and gave them no treatment at all; we gave the second group thioacetamide only (n = 7); we gave the third group mangiferin at a dose of 50 mg/kg/day alongside thioacetamide (n = 5); and we gave the fourth group mangiferin at a dose of 100 mg/kg/day alongside thioacetamide (n = 5). We diluted thioacetamide (Merck, Darmstadt, Germany; catalogue number 108170) at a dose of 200 mg/kg in
physiological NaCl and delivered it three times per week for 5 weeks via intraperitoneal injection. We administered mangiferin (Plamed, Xian, China; catalogue number 4773-96-0) orally at the dosage of its group name. We weighed the rats every day.

2.4 Sample collection and preparation
At the end of week 5, we euthanized the rats to collect liver samples. We performed this sample collection and preparation in a previous study and then froze them at -80 °C before undertaking molecular examination. However, due to the limitations of study, we were able to examine only three samples of sufficient quality from each group.

2.5 Sample collection
We isolated total RNA from the liver tissue using TriPure® Isolation Reagent (Roche) according to the manufacturer’s protocol. We measured the concentration and purity of the isolated total RNA spectrophotometrically using Nanodrop 2000 (Thermo Scientific, Wilmington, USA) at 260 nm.

2.6 cDNA synthesis
We performed the cDNA synthesis reaction using the Transcriptor First Strand cDNA Synthesis Kit (Roche). We also measured the concentration and purity of cDNA spectrophotometrically using Nanodrop 2000 at 260 nm.

2.7 Examination of mRNA expression of TGF-β
We conducted RT-PCR using the FastStart Essential DNA Green Master (Roche) kit on LightCycler® 490 II (Roche). We present the primers used for amplification in Table 1. We based the relative quantification of TGF-β mRNA on the expression of β-actin as a housekeeping gene. We performed amplification in 45 cycles, followed by melting curve analysis. We quantified the mRNA expression levels based on the 2-ΔΔCT method (Livak and Schmittgen 2001).

| Gene     | Primer sequence                                      | Tm(C)     |
|----------|------------------------------------------------------|-----------|
| TGF-β    | F: 5'-TGAACCCGGCCTTTCTGCTTCTCATG-3’                  | 67.21 °C  |
|          | R: 5'-GCGGAAGTCAATGTACAGCTGCCG-3’                    | 68.59 °C  |
| B-actin  | F: 5'-CGTCATCCATGGCGAACT-3’                          | 57.48 °C  |
|          | R: 5'-CCGCGAGTACAACCTTCT-3’                          | 59.41 °C  |

Statistical analysis

We analyzed the data obtained using the statistical program SPSS Version 20. For the normality test of each group we used analysis with one-way ANOVA. We followed this with the post hoc test using LSD. We considered p < 0.05 to be statistically significant.
3. Results and Discussion

3.1 Results

Figure 1. Levels of mRNA expression of TGF-β in all four groups. Data are presented as mean ± SD. *p = 0.007, **p = 0.035 after analysis with one-way ANOVA followed by post hoc test using LSD with α 0.05. TCA only = thioacetamide, MGF 50 mg = thioacetamide + mangiferin dosage 50 mg/kg/day, MGF 100 mg = thioacetamide + mangiferin dosage 100 mg/kg/day.

We present the expression of the mRNA targets in Fig. 1, where we noted an increase in mRNA expression in the group that was given thioacetamide only (the TCA only group) compared with that in the control group. We found a lower mRNA expression in the group given mangiferin at the dose of 50 mg/kg/day (the MGF 50 mg group) alongside thioacetamide than that in the TCA only group, with a statistically significant difference in mRNA expression between the two groups (p < 0.05). We also found a lower expression in the mRNA level in the group given mangiferin at a dose of 100 mg/kg/day (MGF 100 mg group) alongside thioacetamide compared with that of the TCA only group. However, we found a higher level of mRNA expression in this group than that in the MGF 50 mg group. The difference in mRNA expression between the MGF 50 mg group and the MGF 100 mg group was considered statistically significant (p < 0.05).

3.2 Discussion

Liver fibrosis, a reversible condition, is characterized—due to the imbalance in its synthesis and degradation of ECM—by the accumulation of excessive ECM. It is initiated by repeated liver injury caused by chronic liver disease, such as infection with the hepatitis B or hepatitis C virus. Left untreated, liver fibrosis, although reversible, may develop into cirrhosis and hepatocellular carcinoma—conditions which, unlike fibrosis, are irreversible and mark the end-stage of chronic liver diseases [1–3].

An increase in the expression of TGF-β, one of the main profibrogenic cytokines, is the principal factor in the pathogenesis of liver fibrosis. Under normal condition, TGF-β, through its regulation of tissue remodeling and apoptosis, is important for maintaining cellular homeostasis in the liver [8]. However, injury to the liver triggers an inflammatory response, activating Kupffer cells and macrophages, thereby increasing the TGF-β production [8,9]. An increase in TGF-β, one of the main profibrogenic cytokines, triggers fibrogenesis [4,8,10,11].

In fibrogenesis, the role of TGF-β is to activate target cells, its main target being HSCs, where TGF-β triggers the proliferation and activation of HSCs into myofibroblast cells [9,11]. HSCs themselves are important in fibrogenesis because of their role as one of the main cell types producing ECM [9,11]. The production of ECM increases drastically in the presence of TGF-β and accumulates
in the hepatocytes. Along with the activation of HSCs, TGF-β also activates other cells such as hepatocytes and hepatic progenitor cells, and acts as the main regulator of fibrillar collagen, TIMP, and plasminogen activator inhibitor-1 (PAI-1), all important components in fibrogenesis [11].

In Figure 1, we show that the mRNA expression of TGF-β in the TCA only group was higher than that in the control group, demonstrating the pathological thioacetamide-induced condition in the liver. Because TGF-β is a profibrogenic cytokine, the elevation of TGF-β mRNA expression marks the possibility of fibrogenesis. In liver fibrosis, the expression of TGF-β—and especially in the sinusoid cells such as HSCs—is much higher than that in normal liver tissue [8,12] and endothelial cells [12]. This statement is further supported by another study in which the elimination of TGF-β receptors in rat liver tissue also decreased the severity of liver fibrosis. Thus, in the development of fibrosis, TGF-β plays a dominant role.

In the groups given mangiferin alongside thioacetamide, we demonstrated that the TGF-β mRNA expression in both groups was lower than that in the TCA only group. However, in the group given mangiferin at a dose of 50 mg/kg/day, the TGF-β mRNA expression was lower than that in the group given mangiferin at a dose of 100 mg/kg/day. Our data demonstrated that the antifibrotic effect of mangiferin is not dose-related. Theoretically, the effects of bioactive compound use for pharmacological treatments are expected to be enhanced along with the increasing dosage [14]. However, the use of a combination of several bioactive compounds at low concentrations is now known to be more beneficial than the use of a single bioactive compound at a high concentration [14]. An increase in dosage that exceeds the optimal dose may even have adverse effects [14,15]. The dosage may be increased to levels below the ED₃₀ level. Therefore, further studies are necessary to establish the optimum dosage of mangiferin in delivering the maximum therapeutic effect.

Several pharmacological effects of mangiferin—especially its anti-inflammatory and antioxidant effects—are currently being studied. The anti-inflammatory mechanism of mangiferin is expected to be through the inhibition of the expression of iNOS, COX-2, PGE₂, and NOS, in processes where they are important in the inflammation process [7]. Other mechanisms under investigation are the balancing of anti-inflammatory cytokines and proinflammatory mediators, the inhibition of the activation of inflammatory cells, the regulation of the expression of inflammatory genes, and the enhancement of cellular resistance against inflammatory injury [16]. The main mechanism of mangiferin as an antifibrotic agent is expected to be its anti-inflammatory activity. In a study of myocardial infarction in rats, cardiac fibrosis was not found in the sample groups that were given mangiferin [17]. In those groups, the administration of mangiferin alongside ramipril resulted in the inhibition of collagen aggregation, also decreasing the volume of collagen fractions [17]. An expression of TNF-α lower by 42% than that of the control group also demonstrated the anti-inflammatory activity of mangiferin in a context where TNF-α is known to trigger inflammation [7,17]. It can be concluded from these data that mangiferin is a potential antifibrotic agent because it has anti-inflammatory activity.

Another beneficial effect of mangiferin is its antioxidant action, in which mangiferin inhibits ROS and free radicals production [7]. Mangiferin can also trigger the enzymatic process involved in the production of natural antioxidants and modulate the membrane potential of mitochondria [7]. It can also downregulate COX-2 and NF-κB, thereby inhibiting and protecting against fibrogenesis [18]. The mechanism of the hepatoprotective effect of mangiferin is expected to lie in these antioxidant activities. In another study, mangiferin modulated the cell-growth regulator, thereby inhibiting the negative effect of oxidative stress induced by injury to the rat liver tissue [16]. In this study, we also demonstrated that mangiferin can have a hepatoprotective effect against liver disease associated with iron overload [6,7,19], through the mechanism of enhancing the excretion of iron from the liver by inactivating the iron in the Fenton reaction [7]. A study of vimang, an extract of mango bark frequently used in Cuba, demonstrated that the extract can lower the serum iron concentration, transferrin saturation, and iron in the liver [19]. However, an increase in the dosage may have adverse effects, such as increasing the serum iron concentration [19]. The extract can also give higher protective action against hepatotoxicity induced by CCl₄ than that of the antioxidant activity of...
vitamin C [6,7,19]. This beneficial antioxidant activity also supports the antifibrotic effect of mangiferin, especially in liver fibrosis induced by the drugs or other free radicals in the liver.

Other bioactive compounds, such as green tea extract, citrus extract, grape seeds extract, Rosmarinus officinalis, glycoside plants, triterpene plants, polyphenols, flavonoids, and alkaloids, also produce similar antifibrotic effects [18]. Most of those bioactive compounds are consider to be potential antifibrotic agents because they have anti-inflammatory and antioxidant activities [18], which are also found in mangiferin. Other bioactive compounds with antifibrotic effects—such as Liriodendron tulipifera—have a different mechanism, which is the inhibition of the proliferation and activation of HSCs and macrophages [20].

In addition to its antifibrotic effect, mangiferin is also hypothesized to have other therapeutic effects currently under investigation, such as antibacterial, antiviral, antiparasitic, antidiabetic, and even anticancer properties. Therefore, further studies to prove the pharmacological effects of mangiferin in humans, together with the optimum dosage that will give maximum beneficial effects, are now necessary.

4. Conclusion
Based on our study results, we can conclude that the mRNA expression of TGF-β in the group given thioacetamide was higher than that in the control group; their pathological condition was most probably fibrosis, because TGF-β is one of the main profibrogenic cytokines. The TGF-β mRNA expression in the groups given mangiferin alongside thioacetamide was lower than that in the group given thioacetamide only, although the decrease differed between these groups. This demonstrates the absence of a linear relationship between dose and response in the potential antifibrotic effect of mangiferin. However, mangiferin is still considered to be a bioactive compound potentially inhibiting the development of liver fibrosis.

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