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Original research article

The Effect of Mangiferin Against Brain Damage Caused by Oxidative Stress and Inflammation Induced by Doxorubicin

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ABSTRACT

Doxorubicin (DOX) is an anthracycline antibiotic used for anticancer therapy. However, this agent can cause various systemic side effects including cognitive impairments in chronic use. Brain damage due to DOX is caused by an increase of tumor necrosis factor-alpha (TNF-α) level in the brain. Increased TNF-α can further lead to chronic inflammation which can lead to neuronal deaths or neurodegenerative diseases. Mangiferin (MAG), a compound extracted from *Mangifera indica*, has been found neuroprotective activities, but its effect on DOX-induced brain damage is unknown. This study aims to determine the effect of MAG on brain damage induced by DOX. Male Sprague-Dawley rats were induced by DOX intraperitoneally. MAG was given orally at the doses of 30 and 60 mg/kg bw for 7 consecutive weeks. The parameters measured were inflammatory and oxidative stress markers in brain tissue. Coadministration of MAG with DOX reduced inflammation which was marked by the reduction of TNF-α mRNA expression, decreased TNF-α level and reduction of oxidative stress marked by increase of superoxide dismutase level and decrease of malondialdehyde level. In conclusion, MAG was shown to have a neuroprotective effect on brain damage induced by DOX, partly due to inhibition of inflammation and oxidative stress.
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1. Introduction

Doxorubicin (DOX), an anthracycline antibiotic, is widely used for anticancer agent, including breast cancer, cancer in children such as *Wilms tumor*, soft tissue sarcomas, also *Hodgkin's* and *non-Hodgkin's* lymphoma (Hortobagyi 1997). Despite having a wide anticancer effect, the utilization of DOX can cause side effects manifested as heart cell damage (Hortobagyi 1997) and cognitive disorders (Tannock *et al.* 2004). Cognitive disorders may decrease daily activity performances, such as work performances, access to health service, and interaction and awareness to family members (Janelsins *et al.* 2011). Long-term research on DOX-based chemotherapy showed that 76% of patients experienced cognitive degradation on acute phase, and 61% of patients experienced cognitive degradation on a low phase (Wefel *et al.* 2010).

Although the biochemical basis for these cognitive problems is unknown, it has been demonstrated that cancer therapeutics agents such as DOX can modulate endogenous levels of cytokines such as tumor necrosis factor (TNF) alpha (Usta *et al.* 2004). In addition, enhanced circulating TNF-α can initiate local TNF production via activation of glial cells leading to production of reactive oxygen or nitrogen species (Szelényi 2001). DOX causes an increase of peripheral TNF-α (Tangpong *et al.* 2006; Aluise *et al.* 2010; Gilliam *et al.* 2011). The increase of peripheral TNF-α is caused by raised TNF-α production by heart muscles (Mukherjee *et al.* 2003) and immune cells (Ujhazy *et al.* 2003). The increase of TNF-α in brain tissue may most likely be caused by receptor uptake at the blood-brain barrier (Osburg *et al.* 2002) and activation of glia which causes increased production of local TNF-α through signal activation of nuclear factor–kappa B (NF-κB) (Mohamed *et al.* 2011; McCoy and Tansey 2008). Further escalation of TNF-α can induce mitochondrial damage (Tangpong *et al.* 2006; Joshi *et al.* 2005) and constant glia activation, which in turn play roles in chronic inflammation that can lead to neuronal deaths or neurodegenerative diseases (Gonzales-Scarano and Baltuch 1999). Thus, it is

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possible that an increase in TNF-α level may be a link between DOX-induced oxidative stress and central nervous system injury.

Mangiferin (MAG) is a xanton glycoside, initially isolated from *Mangifera indica* L, which is found in many types of mango trees. A particular species of mango tree that grows in Indonesia, *Mangifera foetida* Lour. (*Anacardiaceae*; locally called *bacang*), has been proved to contain higher levels of MAG compared with other mango varieties. MAG is a potent antioxidant (Pal *et al.* 2013), with hepatoprotective (Das *et al.* 2012) and neuroprotective (Liu *et al.* 2013) activities. Our recently published studies showed that MAG also has cardioprotective effects, especially on DOX-induced rats, by regulating the intracellular calcium homeostasis (Arozal *et al.* 2015; Agustini *et al.* 2015). MAG has also been found to decrease inflammation and cell damage in the brain through the decrease of TNF-α and negative regulation of NF-κB (Marquez *et al.* 2012). The neuroprotective effect of MAG has also been studied on diabetic rat model by delivering the mentioned MAG dosage for 8 weeks (Liu *et al.* 2013).

To the best of our knowledge, there has not been any research on the effect of MAG toward brain damage induced by DOX. The focus of the present study was to understand the potential protective effect of MAG to prevent brain damage induced by DOX as an effort to enrich the use of evidence-based Indonesian natural herbal medicine.

2. Materials and Methods

2.1. Materials

Unless otherwise stated, all reagents were of analytical grade and purchased from Sigma-Aldrich (Singapore) or Merck Millipore (Jakarta, Indonesia). DOX hydrochloride injection was obtained from Kalbe Pharma (Jakarta, Indonesia). MAG was of analytical grade and obtained from Plamed Science Technology Company (Xi-an, China). RNA isolation, cDNA synthesis and reverse transcription polymerase chain reaction (RT-PCR) kits were purchased from Roche (Jakarta, Indonesia).

2.2. Animals

Study animals used were male Sprague-Dawley rats aged 12–16 weeks weighing about 180–200 g obtained from Badan Pengawas Obat dan Makanan, Jakarta, Indonesia. Rats were kept in a room with constantly-controlled temperature (21°C) and humidity (55%) with a 12 hour light/ dark cycle. They were allowed free access to standard laboratory food and water. The protocol has been approved by Animal Care Committee from Ethics Committee, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.

2.3. DOX and MAG preparation

To prepare DOX, a certain volume of DOX (supplied as 2 mg/mL of DOX in saline 0.9%) was extracted from the original product vial using syringe in a fume hood. No dilution was needed. MAG 60 mg/kg bw was prepared by mixing 200 mg of MAG powder with 10 mL of 0.5% sodium carboxymethyl cellulose (CMC) using mortar and pestle until MAG was perfectly suspended (Arozal *et al.* 2015). MAG 30 mg/kg bw was prepared using the same procedure, except the amount of MAG powder used was 100 mg.

2.4. Experimental design

Dosing and schedule of the study were determined according to our previous study (Arozal *et al.* 2015) with some adjustments. Previously, higher dose of MAG (100 mg/kg bw/day) had been proven to give negative results. Hence, for this study the MAG doses were decreased to 30 and 60 mg/kg bw/day. Dosing and schedule of DOX treatment were selected according to the work of Ibrahim *et al.* (2009), where cumulative dose of15 mg/kg bw of DOX divided in six injections within 2 weeks was able to induce cardiotoxicity in rats.

After 2 weeks of acclimatization, rats were randomly divided into four groups consisting of five rats each. The groups were as follows: normal group (control) which only received CMC 0.5% and saline (vehicles), toxic control group (DOX) which only received DOX, and two MAG groups which received both DOX and MAG (both groups received DOX with the same dose as DOX group; DOX + MAG30 group was given MAG 30 mg/kg bw/day and DOX + MAG60 group was given MAG 60 mg/kg bw/day). MAG was given orally every day for 7 consecutive weeks in CMC 0.5% as vehicle. DOX was given intraperitoneally with a total dose of 15 mg/kg bw divided in six injections with saline 0.9% as vehicle, starting from the beginning of second week until the end of third week.

Throughout the experiment, rats were monitored closely for signs of toxicity and mortality. Rats were weighed every day. At the end of the seventh week, rats were sacrificed by cervical dislocation method. Brains (the whole cerebrum in both right and left hemisphere) were extracted then rinsed with cold 0.9% normal saline. The brains were subsequently froze at −80°C before undergoing biochemical and molecular examinations.

2.5. Total RNA isolation

Brain tissue was homogenized using Ultra Turrax electric homogenizer. Total RNA was isolated from brain homogenate using *Tripure Isolation Reagent* (Roche) according to the manufacturer's protocol. The isolated total RNA concentration and purity were measured spectrophotometrically at 260 nm using Nanodrop 2000 (Thermo Scientific, Wilmington, USA). Only the samples with sufficient purity ($A_{260/280} > 1.8$) were subjected to the next treatment.

2.6. cDNA synthesis

cDNA synthesis reaction was performed using *Transcriptor First Strand cDNA Synthesis Kit* (Roche). The resulted cDNA concentration and purity were measured spectrophotometrically at 260 nm using Nanodrop 2000 (Thermo Scientific).

2.7. Examination of TNF-α mRNA Expression

RT-PCR was conducted using the *FastStart Essential* DNA *Green Master* (Roche) kit on *LightCycler Nano* (Roche). Primers used for amplification are presented on Table 1. The relative quantification calculation of target mRNA was based on the expression of β-actin mRNA as a comparator. Amplification was performed in 45 cycles followed by melting curve analysis. Number of cDNA templates used were 250 ng and the primary concentration used was 0.4 μM. The amplification conditions for each gene were as follows: β-actin (predenaturation: 95°C for 10 minute, denaturation: 95°C for 10 seconds, annealing: 53°C for 10 seconds, elongation: 72°C for 23 seconds) and TNF-α (predenaturation: 95°C for 10 minute, denaturation: 95°C for 10 seconds, annealing: 60°C for 30 seconds, elongation: 72°C for 1 second).

After RT-PCR, the amplification product underwent electrophoresis on 2% agarose gel stained with SYBR Green for band analysis. Only the amplification products that showed one band

Table 1. Gene-specific primer sequences used in RT-PCR

Gene	Primer sequence	PCR product
TNF-α	F: 5' – TCT CAA GCC TCA AGT AAC AAG C – 3' R: 5' – ATG AGG TAA AGC CCG TCA GC – 3'	330 pb
β-actin	F: 5' – TGT TGT CCC TGT ATG CCT CT – 3' R: 5' – TAA TGT CAC GCA CGA TTT CC – 3'	222 pb

RT-PCR = reverse transcription polymerase chain reaction.
Primer sequences used were referred to previous publication by Mohamed *et al.* (2011).

with the intended product length were quantified. Levels of mRNA expression were quantified based on $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

2.8. Production of brain homogenates for determination of TNF-α level, lipid peroxides, and superoxide dismutase (SOD) Activity

The whole cerebrum tissues were cut and weighted 100 mg then put into a glass mortar. The tissues were suspended with PBS nine times volume of the brain tissue weight and 5 μL of protease inhibitor solution (Sigma) was added for every 100 mg of tissue. This mixture was then homogenized by using mortar and pestle. The homogenate was then centrifuged at ×10,000 *g* for 15 minutes at 4°C, then supernatant was taken. Supernatant was used for measuring protein level, TNF-α level, malondialdehyde (MDA) level and SOD activity.

2.9. Measurement of protein level

Protein level was measured using *Bradford* (Sigma-Aldrich) method and measured with spectrophotometer at wavelength of 595 nm.

2.10. Determination of TNF-α level

Measurement of TNF-α level was conducted using ELISA method using ELISA TNF-α kit for rat tissue (Sigma-Aldrich).

2.11. Determination of SOD activity

SOD (Cu, Zn and Mn) activity was measured based on epinefrin autooxidation inhibition rate (Misra and Fridovich 1972).

2.12. Determination of lipid peroxides

MDA, a measure of lipid peroxidation in serum and brain tissue was determined by reacting MDA with TBA in an acidic atmosphere and measured at wavelength of 532 nm (Abdel-Wahab *et al*. 2003). Results were expressed in nmol/mg protein.

2.13. Statistical analysis

The data obtained were analyzed using statistical program SPSS Version 19. One-way analysis of variance was used for comparison among groups, followed by post hoc test using Tukey. $p < 0.05$ was considered to be significant.

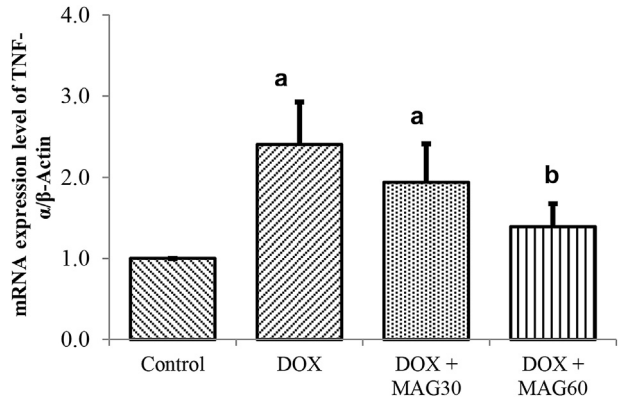

Figure 1. Effect of mangiferin (MAG) on doxorubicin (DOX)-induced alteration of brain tumor necrosis factor (TNF)-α mRNA expression levels. mRNA expression levels are normalized to β-actin as housekeeping gene. Values are presented as mean ± standard deviation, [a]$p < 0.05$ versus the normal group; [b]$p < 0.05$ versus the DOX group. Normal = normal group, DOX = doxorubicin 15 mg/kg bw, DOX + MAG30 = DOX 15 mg/kg bw and MAG 30 mg/kg bw, DOX + MAG60 = DOX 15 mg/kg bw and MAG 60 mg/kg bw.

3. Results

At the first week of experiment, stable increase of body weight was observed in all groups. However, in all groups receiving DOX (DOX, DOX + MAG30, DOX + MAG60), there was a considerable decrease of body weight which occurred when DOX treatment was given (data not shown). DOX, DOX + MGR30 and DOX + MGR60 also showed signs of general acute toxicity, including diarrhea, nose and mouth bleeding, and pallor. However, body weight improved and toxicity signs gradually subsided after DOX treatment ended. Mortality rates were 0% in all groups .

3.1. TNF-α mRNA expression level

TNF-α mRNA expression analysis results as depicted in Figure 1 show that DOX treatment increased TNF-α mRNA expression level significantly ($p < 0.05$) in the DOX-only treated group compared with the normal group (140%), whereas both groups receiving MAG (DOX + MAG30 and DOX + MAG60) showed decreased TNF-α mRNA expression level compared with the DOX only-treated group (19.17% for DOX + MAG30 and 42.08% for DOX + MAG60). However, the change was only statistically significant at group receiving MAG 60 mg/kg bw ($p < 0.05$).

3.2. TNF-α level in brain tissues

DOX caused an increase in brain TNF-α level by 19.29% compared to the normal group, as shown in Figure 2. It is of interest that only co-treatment with MAG at a dose of 60 mg/kg bw could significantly decrease brain TNF-α level compared to DOX-only treated group (26.49%).

3.3. Brain SOD activity

DOX caused a decrease in brain SOD activity level by 18.97% compared to the normal group, as provided in Table 2. It is of interest that only co-treatment with MAG at a dose of 30 mg/kg bw could significantly increase brain SOD activity compared to DOX-only treated group (92.20%).

3.4. Brain MDA level

Brain level of MDA was increased by 25.72% in DOX-only treated group compared with normal group, although not statistically significant. Table 2 also explains that coadministration of MAG at doses of 30 and 60 mg/kg bw could significantly guard against the increases of MDA level in brain tissue compared to DOX-only group (by 39.62% and 47.50% accordingly).

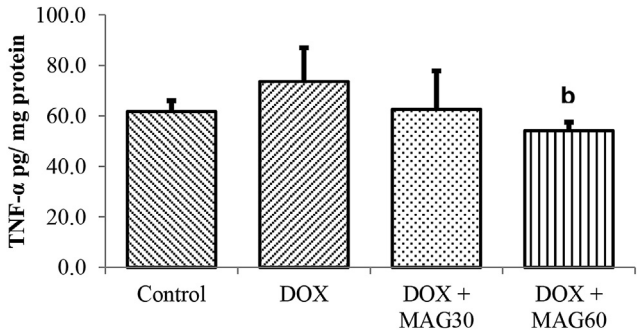

Figure 2. Effect of mangiferin (MAG) on doxorubicin (DOX)-induced alteration of brain tumor necrosis factor (TNF)-α levels. Values are presented as mean ± standard deviation, [a]$p < 0.05$ versus the normal group; [b]$p < 0.05$ versus the DOX group. Normal = normal group, DOX = doxorubicin 15 mg/kg bw, DOX + MAG30 = DOX 15 mg/kg bw and MAG 30 mg/kg bw, DOX + MAG60 = DOX 15 mg/kg bw and MAG 60 mg/kg bw.

Table 2. Brain levels of MDA and SOD activity

Group	MDA (nmol/mg prot)	SOD (U/mg prot)
Control	7.97 ± 1.314	2.53 ± 1.229
DOX	10.02 ± 3.057	2.05 ± 1.162
DOX + MAG30	6.05 ± 1.329*	3.94 ± 0.626*
DOX + MAG60	5.26 ± 0.535*	3.04 ± 0.325

DOX = doxorubicin; MAG = mangiferin; MDA = malondialdehyde; SOD = superoxide dismutase.
Effect of MAG on DOX-induced alteration of brain MDA levels and brain SOD activity. Values are presented as mean ± SD. Normal = normal group, DOX = doxorubicin 15 mg/kg bw, DOX + MAG30 = DOX 15 mg/kg bw and MAG 30 mg/kg bw, DOX + MAG60 = DOX 15 mg/kg bw and MAG 60 mg/kg bw.
*$p < 0.05$ versus the DOX group.

4. Discussion

This research showed that a total dose of 15 mg/kg bw of DOX was able to cause brain toxicity as evidenced by elevated brain TNF-α gene expression and elevated brain TNF-α levels. Furthermore, there was evidence of oxidative stress marked by increase of MDA level and decrease of SOD activity, although SOD is not the only important enzyme for antioxidant defense system. Treatment with MAG at both 30 and 60 mg/kg bw was able to improve inflammation and oxidative stress induced by DOX administration. To the best of our knowledge, this is the first study that revealed MAG potency as a neuroprotective agent in DOX-induced brain damage.

In this study, signs of toxicity and body weight decrease were observed in all groups receiving DOZ (DOX, DOX + MAG30, and DOX + MAG60) after DOX treatment. However, the gradual improvement of body weight that occurred after DOX treatment ended with no difference between groups receiving MAG (DOX + MAG30 and DOX + MAG60) and not receiving MAG (DOX). There were no deaths observed in all groups. Considering body weight and mortality rate data, it can be concluded that MAG seems to have effect on general acute toxicity.

Acute side effects observed as results of DOX administration were pallor, diarrhea and hemorrhage through mouth and nose. These side effects occurred in all DOX-treated groups (DOX, DOX + MAG30, and DOX + MAG60). The coadministration of MAG together with DOX did not prevent the occurrence of these acute side effects.

To understand the role of MAG in preventing brain damage induced by DOX administration, this research conducted analysis on brain TNF-α level, brain TNF-α mRNA expression, brain MDA level and brain SOD activity. TNF-α is a cytokine which has a role in congenital immune response (*innate*) as a response to several stress. This substance is hypothesized as the cause of cognitive damage on neurodegenerative diseases (McCoy and Tansey 2008; Medeiros *et al.* 2007; McAlpine *et al.* 2009; Rubio-Perez and Morillas-Ruiz 2012; González-Scarano and Baltuch 1999). TNF-α together with other cytokines has roles in acute and chronic inflammation (Feghali and Wright 1997). This research found that DOX group tended to experience an increase of TNF-α level in brain tissues. In addition, a relative increase of TNF-α mRNA expression occurred compared with the normal group. Statistically significant differences of the TNF-α level and TNF-α mRNA expression in brain tissue samples between DOX group and control group were not completely understood. The significant difference between TNF-α level and TNF-α mRNA expression might be influenced by eukaryotic elongation factor-2 which is an elongation control on TNF-α translation (González-Terán *et al.* 2013). The administration of MAG decreased both the TNF-α level and the TNF-α mRNA expression which were statistically significant at MAG 60 mg/kg bw of administration dosage compared with the DOX group.

This research conducted evaluations on oxidative stress by measuring SOD activity and MDA level on the brain tissues. SOD enzymes, which up to now are known to consist three different kinds, are among the antioxidant enzymes that transform O^- to become H_2O_2 (Misra and Fridovich 1972; McCord 2008; Blokhina *et al.* 2003; Zelko *et al.* 2002). It has been known before that the increase of TNF-α causes the increase of iNOS expression, which in turn causes the increase of O^-, leading to increased peroxynitrite, NF-κB activity regulation, and other protein activity regulation (Alderton *et al.* 2001; Bogdan 2001). SOD enzymes have a role in catching O^- radical, preventing the formation of OH^- radical and more dangerous peroxynitrite radical (Krishnamurthy and Wadhwani 2012). The measurements of SOD activity showed that on DOX group, there was a tendency of decreased SOD activity compared to normal group. Previous study conducted by Mohamed *et al.* (2011) discovered that the decrease in SOD activity in the group inducted by DOX had a significant difference compared to control group. The cause of this significant difference was not precisely known. There is a possibility that there are effects of NO to the decreasing SOD activity, but this still has to be proved. The coadministration of 30 mg/kg bw MAG increased SOD activity significantly compared to DOX group. Meanwhile, coadministration of 60 mg/kg bw MAG showed SOD level with no significant difference compared with DOX group. This increase in SOD might be influenced by cytokines which have roles in inflammation, as known from previous research that SOD activity could be increased by TNF-α, IL-1α, IL-1β, and interferon-γ (Zelko *et al.* 2002).

MDA is a marker for lipid peroxidation (Krishnamurthy and Wadhwani 2012). Reaction of lipid peroxidation is defined as a reaction between polyunsaturated fatty acids. Lipid peroxide is then decomposed into several molecules, including MDA (Alessio 2000). In this research, DOX group tended to experience an increase in MDA. In previous study conducted by Mohamed *et al.* (2011), it was known that the increase of MDA and decrease of SOD activity in group inducted with DOX only was significant compared to the control group. The difference between this research and that previous research in particular may be caused by the difference of sacrifice time of study animals: in this research, study animals were sacrificed 4 weeks after DOX induction; in the previous research, study animals were sacrificed 2 weeks after DOX induction. The coadministration of MAG decreased MDA level significantly compared to DOX-only group. Based on the SOD and MDA analysis, it can be concluded that MAG has a role in decreasing DOX-induced oxidative stress which occurs in brain tissue.

Based on evaluation of all parameters, this research shows that there were no significant differences between coadministration of DOX with 30 mg/kg MAG or 60 mg/kg MAG; although coadministration of 60 mg/kg MAG showed slightly better results. Based on another previous research, 30 days administration of 500 mg/kg MAG did not show morphological changes nor death; hence, in the next study, administration of MAG with higher dosage is very possible (Sellamuthu *et al.* 2009). The increase in MAG dosing should still consider the fact that dosage increase from 30 mg/kgBB to 100 mg/kgBB will cause area under curve increase, which is not linear, and also lengthening of half time ($t_{1/2}$) (Lai *et al.* 2003).

5. Conclusion

Based on the overall results, it can be concluded that MAG can be a potential compound which can be used to prevent brain damage caused by inflammation and oxidative stress on DOX-based anticancer therapy.

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Conflict of interest

The authors report that they have no conflict of interest.

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