Mangosteen extract reduce apoptosis via inhibition of oxidative process in rat model of traumatic brain injury

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ABSTRACT

Background: Traumatic brain injury is one of the primary causes of mortality, morbidity, and economic burden, especially in the young population. Following initial primary injury, there is a secondary insult, resulting in neuro-inflammatory response and free radical generation. These oxidative stress is a powerful precursor of apoptosis. Mangosteen is a powerful natural antioxidant that also has neuroprotective property. This study was conducted to evaluate whether the Mangosteen extract could decrease the expression of MDA and apoptosis in traumatic brain injury.

Method: Thirty Sprague-Dawley rats were randomized into three treatments group, i.e., sham-operated control, closed head injury (CHI), and CHI with mangosteen extract (treatment group). In the treatment group, the mangosteen extract was administered once daily every day after CHI for 1, 3, and 7 days. MDA, SOD, AIF, caspase 8, and caspase 9 were evaluated using immunohistochemistry staining while apoptosis was assessed using TUNEL essay.

Result: MDA, AIF, caspase 8, and caspase 9 were up-regulated in CHI group compared to sham-operated group (p<0.05), meanwhile the expression of SOD decreased significantly. MDA, AIF, caspase 8, and caspase 9 were significantly down-regulated in the treatment group compared to CHI group (p<0.05), while significant elevation was observed in SOD expression.

Conclusion: Mangosteen extract decreased neuronal apoptosis in traumatic brain injury by promoting expression of SOD and down-regulating MDA, AIF, caspase 8, and caspase 9.

Keywords: Mangosteen extract, traumatic brain injury, apoptosis

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INTRODUCTION

Traumatic brain injury (TBI) is a primary cause of mortality, morbidity, and economic burden. Head trauma causes two kinds of damage in the brain tissue. First is the primary injury, which refers to the initial physical forces applied to the brain at the moment of impact. Then the secondary injury, which occurs over hours or days after the initial trauma resulting in a neuroinflammatory response and free radical production.

Reactive oxygen species (ROS), generated after TBI, are involved in the secondary brain injury. Under normal conditions, ROS level is maintained by the action of pro-oxidant and antioxidant in cerebral tissue, and they are continuously formed in small amounts as products of oxidative metabolism. TBI leads to increased ROS production and lipid peroxidation which subsequently makes the way to the formation of Malondialdehyde (MDA). MDA is released into extracellular space and finally into the blood, thus, making MDA an effective biomarker of lipid oxidation.

Oxidative stress is a potent precursor of apoptosis. Following oxidative stress, the mitochondria release apoptotic proteins such as cytochrome c (Cyt c) and apoptosis inducible factor (AIF) into the cytosol. The release of Cyt c and other pro-apoptotic proteins can induce caspase activation and apoptosis. Once freed into the cytosol from the mitochondrial intermembrane space, Cyt c binds with apoptotic protein-activating factor-1 (Apaf-1) and procaspase-9 to form an “apoptosome”, which activate caspase-9 and caspase-3 respectively. Activated caspase-3 cause a wide range of homeostatic disturbance, reparative, and cytoskeletal proteins disruption and leads to neuron cell death.

Alpha-mangostin, the first xanthone extracted from mangosteen fruit, is a yellow colored substance that can be obtained from bark and dried sap of *Garcinia mangostana*, a tropical fruit from the family of Guttiferae. *G. Mangostana* is cultivated in the tropical rainforest of Indonesia, Malaysia, Sri Lanka, Philippines, and Thailand. For many years, people in these countries have used the pericarp (peel, rind, hull or ripe) of *G. Mangostana* as a traditional herb for indigestion, infected wounds, and chronic wounds. Several researches had shown that the most well-known substance in mangosteen extract, α-mangostin, could inhibit the oxidation of low-density lipoprotein (LDL) in vitro and scavenge the singlet...
oxygen, superoxide anion, and peroxynitrite anion. The antioxidant properties of the α-mangostin act as a free radical scavenger that ameliorates the neuronal death induced by 3-nitropropionic acid (3-NP). Study have shown that the treatment by using α-mangostin provide a neuroprotective effect on cerebellar granule neurons (CGNs) which was associated with the induction of heme oxygenase-1 (HO-1).

Considering the wide variety of antioxidant and protective properties of α-mangostin, this study conducted to evaluate whether Mangosteen extract could reduce the expression of MDA and apoptosis.

MATERIAL AND METHOD

Mangosteen extract (ME) preparation
The Mangosteens were collected from Malang Regency in East Java, Indonesia. The fruit bodies of mangosteen were cleaned to remove any residual compost. The pericarps were then separated and dried. All dried pericarps were placed in 70% ethanol (100 gr in 500 ml) and then were shaken with a rotation speed of 50 rpm in 24 hours. The maceration filtrate was evaporated using rotary evaporator in 2 hours. From 100 gram dried mangosteen pericarp, 50 ml Mangosteen extract was obtained.

Mouse model of closed head injury
Unilateral focal brain injury was induced on the right frontal area using rat model of closed head injury (CHI), performed with the modified Feeney’s weight-drop protocol. This study had been approved by the local ethical committee. Thirty-three Sprague-Dawley rats weighing 250-400 gram were randomized into three groups, i.e., sham-operated controls, CHI only (positive control group), and CHI with mangosteen extract (treatment group). All animals were given ketamine HCl (intramuscular dosage 100 mg/kg) and xylazine base concentration 20 mg/ml (intramuscular dosage 0.15 ml/kg). The scalp was cleaned with povidone iodine, and aseptic techniques were used throughout surgery. The scalp was opened on the right frontal area. The rats were placed securely in stereotactic apparatus then impacted with 40 mg metal mass from 1.5 m height (Supplementary figure 1A). Mangosteen extract (100mg/kg per oral) was given once daily until the seventh day via nasogastric tube. Afterward, animals were sacrificed through cervical dislocation after giving ketamine HCl (100 mg/kg, intramuscular). In positive control group as well as the treatment group, subjects were divided into three groups based on sacrificial timing, i.e., first, third, and seventh day respectively. During the follow-up, three rats died directly after the trauma procedure. The brain was removed after craniocervical dislocation under anesthesia using ketamine HCl and xylazine base (Supplementary figure 1B). After removed (supplementary figure 2) the brains were fixed in 10% buffered formalin. The specimens were then processed in paraffin for immunohistochemistry staining. Sham-operated rats underwent anesthesia and surgery without prior trauma and treatment.

Immunohistochemistry staining
The MDA expression was served as an oxidative marker in this study. For apoptosis marker, we used the expression of AIF, caspase 8, and caspase 9. The expression of all markers was investigated on paraffin-embedded sections using the avidin-biotin-peroxidase complex method. Five-millimeter-thick paraffin sections were de-waxed, rehydrated, and put in the microwave for 10 minutes. The endogenous peroxidase activity of the investigated specimens was blocked with 3% H₂O₂ for 10 minutes, followed by 25 minutes of washing with phosphate-buffered saline (PBS). The tissue sections were incubated with normal rabbit serum for 10 minutes before incubated at room temperature with monoclonal mouse MDA, SOD, AIF, caspase-8, and caspase-9 (Santa Cruz). Sections were washed with PBS and incubated with a secondary antibody for 30 minutes. All sections were washed twice with PBS, developed with 0.05% 3,3-diamino-benzinetetrahydrochloride for five minutes, and slightly counterstained. Apoptotic cells were detected by TUNEL (terminal transferase-mediated dUTP nick end labeling) staining in (para)formaldehyde-fixed paraffin-embedded (FFPE) using the In Situ Cell Death Detection Kit from Roche’s protocols.

All samples were evaluated by the first author (not blinded to specimen). The positive signal for MDA, SOD, AIF, caspase 8, and caspase 9 and the number of apoptosis cells in brain tissue were quantitatively estimated based on the distribution of positively stained cells in the cortical brain. Cell counts were carried out using a light binocular microscope with 1000 times magnification in 20 high power fields.

Statistical Analysis
The total stained cells were presented as mean and standard deviation. When comparisons were made between groups, the significance of between-group variability was analyzed using the one-way ANOVA test with additional Tukey method as post hoc test. Differences were considered significant at the P < 0.05.
RESULT

α-mangostin inhibit the MDA expression

In our study, we found that the significant difference of MDA expression among three groups with the lowest MDA expression was found in the treatment group (Table 1, figure 1, p= 0.0001).

In the positive control group, MDA expression is shown to decrease in day 3 and day 7 compared to the first day with the value of 8.67 ± 1.53 in day 7 compared to 13.67 ± 1.53 in day 1. It is shown to be a significant difference of MDA expression in the positive control group (Table 2, figure 1, p= 0.041). In the treatment group, the lowest expression of MDA was shown on the third day, and a slight increase is shown on day 7 (5 ± 1.58 and 5.2 ± 1.92 respectively). We noted that there is no significant difference of MDA expression of the day 1, 3, and 7 in the treatment group (Table 3, figure 1).

α-mangostin promote the SOD expression

We found that the lowest SOD expression is found in the treatment group with the significant difference of SOD expression among the sacrificial timing (Table 1, supplementary figure 3, p= 0.0001). In the positive control group, SOD expression is shown to increase in day 7 compared to the first day and third day; with the value of 5.25±0.50 in day 7 compare to 4.25±1.708 in day 1, and 3.00±0.816 in day 3. It is shown to be no significant difference SOD expression in the positive control group (Table 2, figure 4, p= 0.532). In the treatment group, the highest expression of SOD is shown on the seventh day. We noted that there is a significant difference in SOD expression in the treatment group (Table 3, supplementary figure 3).

α-mangostin inhibit the caspase 8, caspase 9 and AIF expression

When compared to positive control group, treatment with mangosteen extract was shown to influence the expression of apoptotic pathways such as caspase 8, caspase 9 and AIF expression in the treatment group (p=0.0001 in all apoptotic pathway) in which shown similar expression in the negative control group (Table 1, supplementary figure 4-6). The p-value of all apoptotic pathway in the positive control group was below 0.05, which means that there is a significant correlation of inhibition of apoptotic pathway by α-mangostin (Table 2, supplementary figure 4-6).

Table 1  Profile of apoptosis and apoptotic pathway (Mean ± SD)

|          | MDA     | SOD     | Caspase 8 | Caspase 9 | AIF     | Apoptosis |
|----------|---------|---------|-----------|-----------|---------|-----------|
| Group 1  | 5.78±1.99 | 5.50±2.51 | 5.67±1.87 | 4.33±2.12 | 6.56±1.81 | 5.67±1.66 |
| Group 2  | 11.67±2.83 | 4.17±1.40 | 15±2.74   | 12.67±2.12 | 12.67±2.12 | 14.67±3.87 |
| Group 3  | 6±2.27   | 17.25±4.78 | 6±3.32   | 6.27±3.51 | 5.87±3.14 | 7.67±2.16 |
| p-value  | 0.0001  | 0.0001  | 0.0001    | 0.0001    | 0.0001  | 0.0001   |

Significant if p<0.05. Group 1: negative control, group 2: CHI, group 3: treatment. Post-hoc MDA: 1 vs 2 = 0.0001, 1 vs 3 = 0.0001; caspase 8: 1 vs 2 = 0.0001, 1 vs 3 = 0.0001; caspase 9: 1 vs 2 = 0.0001, 1 vs 3 = 0.0001; AIF: 1 vs 2 = 0.0001, 1 vs 3 = 0.0001; apoptosis: 1 vs 2 = 0.0001, 1 vs 3 = 0.0001.

CHI: closed head injury; MDA: malondialdehyde; AIF: apoptosis inducing factor; SOD: superoxide dismutase.

Table 2  Temporal profile of apoptosis and apoptotic pathway in CHI group (Mean ± SD)

|          | MDA     | SOD     | Caspase 8 | Caspase 9 | AIF     | Apoptosis |
|----------|---------|---------|-----------|-----------|---------|-----------|
| Day 1    | 13.67±1.53 | 4.25±1.70 | 17±1      | 13.67±1.53 | 13.33±2.08 | 10.33±1.53 |
| Day 3    | 12.67±2.52 | 3.00±0.816 | 16±2     | 14±1       | 13±1      | 15±2       |
| Day 7    | 8.67±1.53 | 5.25±0.50 | 12±2      | 18.33±1.53 | 18.67±2.08 | 18.67±1.15 |
| p-value  | 0.041   | 0.532   | 0.0271    | 0.0311    | 0.0141    | 0.0021      |

Significant if p<0.05. Post-hoc MDA: 1 vs 7 = 0.043; Caspase 8: 1 vs 7 = 0.028; caspase 9: 3 vs 7 = 0.039; AIF: 1 vs 7 = 0.025, 3 vs 7 = 0.019; apoptosis: 1 vs 7 p= 0.002, 1 vs 3 p= 0.027. CHI: closed head injury; MDA: malondialdehyde; AIF: apoptosis inducing factor; SOD: superoxide dismutase.

Table 3  Temporal profile of apoptosis and apoptotic pathway in the treatment group (Mean±SD)

|          | MDA     | SOD     | Caspase 8 | Caspase 9 | AIF     | Apoptosis |
|----------|---------|---------|-----------|-----------|---------|-----------|
| Group 3  | 7.8±2.39 | 11.75±2.36 | 9±3.54   | 9.4±3.85  | 8.4±2.89 | 9.4±1.14  |
| Day 3    | 5±1.58  | 18.25±2.21 | 4.6±2.7  | 6.2±1.64  | 6.4±2.07 | 14.76±1.52 |
| Day 7    | 5.2±1.92 | 21.75±2.21 | 4.4±1.34 | 3.2±1.3  | 2.8±1.3 | 6±2.45 |
| p-value  | 0.083   | 0.0027  | 0.0311    | 0.0081    | 0.0057  | 0.00341   |

Significant if p<0.05. Post-hoc caspase 8: 1 vs 7 p= 0.046; caspase 9: 1 vs 7 p= 0.006; AIF: 1 vs 7 p= 0.004; Apoptosis 1 vs 7 p= 0.027. MDA: malondialdehyde; AIF: apoptosis inducing factor; SOD: superoxide dismutase.
**Figure 1** MDA expression in sham control, CHI, and treatment group

**Figure 2** Apoptosis in sham control, CHI, and treatment group

**Supplementary figure 1** Animal model. A. Modified Feeney’s weight-drop model after CHI; B. cranial defect after CHI

**Supplementary figure 2** Mice brain after removal. A. Sham-operated group; B. CHI group; C. Treatment group

**Supplementary figure 3** SOD expression in sham control, CHI, and treatment group

**Supplementary figure 4** Caspase 8 expression in sham control, CHI, and treatment group
In the treatment group, the apoptotic pathway expression is shown to be the lowest in day 7. The \( p \)-value of 0.031 also supports it, 0.008 and 0.005 in caspase 8, caspase 9 and AIF respectively (Table 3, supplementary figure 4-6).

\( \alpha \)-mangostin lowers the apoptosis profile

The apoptosis profile was shown to have a significant difference among the three groups (\( p \)-value of 0.0001). This means that \( \alpha \)-mangostin decreases the apoptosis profile in the treatment group compared to positive control group (Table 1, figure 2). In the positive control group, apoptosis profile tends to be higher on day 7 compared to the first day (18.67 ± 1.15 vs. 10.33±1.53). There is a significant difference of apoptosis profile in the positive control group (\( p \)-value of 0.002) (Table 2, figure 2).

DISCUSSION

Since TBI is a multi-pathway process, medicinal plants have been evaluated as resources of agents for alternative treatments. Medicinal plants usually have multiple compounds and work via various pathways. Mangosteen has been used as traditional medicine for years. Many reports have been published regarding the pharmacological activities of \( \alpha \) and \( \beta \)-mangostin, the active substance in mangosteen pericarp.\(^{17}\) It has a strong effect in reducing the expression of inflammatory mediators, such as tumor necrosis factor alpha (TNF-\( \alpha \)) and interleukin 6 (IL-6).\(^{18,19}\) Xanthone from mangosteen, especially \( \alpha \)-mangostin, inhibits the NMDA and glutamate receptors.\(^{20}\)

In this study, we investigated the effect of mangosteen extract on the expression of NMDA as the marker of lipid peroxidation and apoptosis. We observed through immunohistochemistry that mangosteen extract inhibits oxidative process and apoptosis after traumatic brain injury. Based on these data, we investigated the molecular mechanism of mangosteen extract on the suppression of apoptosis. The result showed that mangosteen extract reduced the expression of caspase 8, caspase 9, and apoptosis-inducing factor. This result was interesting since many research in oncology showed \( \alpha \)-mangostin induces apoptosis. It proposed that \( \alpha \)-mangostin triggered the loss of mitochondrial membrane permeability and activation of caspase family.\(^{21,22}\)

Caspases are a family of genes essential in maintaining homeostasis through regulating cell death and inflammation. Caspases involved in apoptosis have been subclassified by their mechanism of action and are either initiator caspases (caspase-8 and -9) or executioner caspases (caspase-3, -6, and -7).\(^{23}\) Pathologically, insults such as trauma, at the appropriate severity that occurs below the threshold required for necrosis, may result in cellular apoptosis. Apoptosis may also assume an important role in the pathogenesis of many illnesses. “Too little” apoptosis may cause uncontrolled cell growth, as found in cancer. Conversely, “too much” apoptosis may contribute to several neurodegenerative processes, like Alzheimer's and Huntington's disease.\(^{24}\) In TBI, apoptosis is one of the long term consequences.\(^{25}\) Inhibiting apoptosis may be one alternative modality in traumatic brain injury.
In summary, we had demonstrated that the down-regulation of oxidative stress and apoptosis in traumatic brain injury after administration of mangosteen extract. Currently, this is the first study that demonstrates the effect of Mangosteen extract in apoptotic pathway after traumatic brain injury. The main limitation of this study was the nature of the injury since we only demonstrate focal injury in this research. Meanwhile, the diffuse injury is commonly found after traumatic brain injury and correlates significantly with the secondary injury. Inhibiting apoptosis after traumatic brain injury remains controversial because apoptosis is a vital mechanism for the biological system to eliminate abnormal cell. Therefore, the study about the effect of antiapoptosis should not be limited to the expression of molecular status only, but also to clinical in functional outcome.

CONCLUSION

Mangosteen extract decreased neuronal apoptosis in traumatic brain injury by promoting expression of SOD, and down-regulation MDA, AIF, caspase-8, and caspase-9. Further study is needed to evaluate the pharmacokinetic profile as well as to further deepening our understanding about the mangosteen pharmacodynamics as well as its other potential beneficial effects.

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