

## ORIGINAL ARTICLE

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## Propolis inhibited Bax expression and increased neuronal count of hippocampal area CA1 in rats receiving sodium nitrite

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## ABSTRACT

**BACKGROUND**

Sodium nitrite induces hypoxia and oxidative stress in the hippocampus, decreasing the number of neurons in the hippocampus and cognitive function. Propolis contains chrysin that has antioxidant effects that are expected to inhibit neuronal damage in the hippocampus. This study aims to determine the effects of propolis on the expression Bcl-2-associated X protein (Bax) and the number of neurons in the rat hippocampus receiving sodium nitrite.

**METHODS**

This study of laboratory experimental design was conducted on 18 male Wistar strain rats (*Rattus norvegicus*), they were randomized into 3 groups: one control group (K) received sodium nitrite and two intervention groups (P1 and P2) received sodium nitrite and propolis at doses of 100 and 200 mg/kgBW. Treatment with sodium nitrite and propolis were given for 60 days, followed by termination. The number of neurons and Bax expression in the hippocampal CA1 area were measured. One-way ANOVA was used to analyze the data.

**RESULTS**

There were significant differences in Bax expression between group K and groups P1 and P2 ( $p < 0.001$ ). The lowest number of neurons in the hippocampal CA1 area was in the K group. There were significant differences in the number of neurons between control (K) group and groups P1 and P2 ( $p < 0.001$ ).

**CONCLUSION**

Propolis inhibited the expression of Bax and decreased the number of neurons in the hippocampal CA1 area of rats receiving sodium nitrite. This study provides information about the benefits of propolis as an antioxidant in the brain.

**Keywords:** Propolis, Bax, hippocampus, sodium nitrite

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## INTRODUCTION

Exposure to sodium nitrite results in structural and functional changes in various organs of the body. In the brain, the exposure results in congestion of the blood vessels and bleeding in the meninges, ventricles, brainstem and cerebellum. Exposure to sodium nitrite also results in perineuronal edema, vacuolation, neuronal degeneration, neurophagy and necrosis of the Purkinje cells in the cerebellum.<sup>(1)</sup> The study conducted by Jebasingh et al.<sup>(2)</sup> showed that intraperitoneal administration of sodium nitrite at a dose of 60 mg/kgBW in male Wistar rats for 30 days decreased the capacity for learning and memory and increased anxiety. In addition, administration of sodium nitrite resulted in changes in the morphology of the cortex, hippocampus, thalamus, and cerebellum. These changes are among others pyknotic nuclei and fragmented and vacuolated neurons resulting from apoptosis and neuronal degeneration.

Sodium nitrite is a compound that converts hemoglobin into methemoglobin, which causes hypoxia, thus leading to ischemia and inflammation of the brain.<sup>(3)</sup> According to Halim and Ibrahim<sup>(4)</sup> the hypoxia increases reactive oxygen species (ROS), resulting in the degradation of lipid membranes and enzymes, and in DNA damage. Oxidative stress disturbs metabolic activity, inhibits expression of the factors of neuronal regeneration and proliferation in the hippocampus and decreases the number of receptors in the hippocampus.<sup>(5)</sup> The hippocampus is one of the brain areas that are susceptible to oxidative stress. Exposure to stress in the hippocampus results in a decreased number of neurons in the hippocampus and amygdala and decreased memory and cognitive function.<sup>(6)</sup>

To protect the neurons in the hippocampus from hypoxia and oxidative stress as a result of exposure to sodium nitrite, a compound is needed, such as propolis, that possesses neuroprotective effects and prevents damage and death to the neurons. Propolis contains chrysin and has

antioxidant and neuroprotective effects. Administration of propolis at a dose of 200 mg/kgBW in rats exposed to stress and showing increases in H<sub>2</sub>O<sub>2</sub> has the effect of decreasing the H<sub>2</sub>O<sub>2</sub> concentration.<sup>(7)</sup> Administration of propolis to rats with cerebral ischemia reduces apoptosis with increased expression of Bcl-2 and decreased expression of Bax and caspase-3.<sup>(8)</sup> Propolis-containing polyphenols may prevent the death of dopaminergic neurons in the substantia nigra as a result of exposure to 6-OHDA (6-hydroxydopamine), may increase the expression of brain-derived neurotrophic factor (BDNF), increase the antioxidant activity of enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GSR), and decrease the concentration of malondialdehyde that may cause oxidative stress in the neurons.<sup>(9)</sup> In neuronal cell culture, administration of propolis at a dose of 25 µg/mL may prevent oxidative stress, inhibit release of LDH and increase the viability of the neurons as a result of exposure to H<sub>2</sub>O<sub>2</sub>.<sup>(10)</sup> In rats with neurotoxicity induced by lead (Pb) for four weeks, administration of propolis may inhibit the neurotoxic effect of Pb. This is shown in the normal activity of acetylcholinesterase, decreased MDA concentrations in the brain, and increased activity of NADH, succinate dehydrogenase (SDH) and cytochrome C oxidase in mitochondria.<sup>(11)</sup> A previous study had evaluated the effect of propolis on the neuronal count in the hippocampus.<sup>(12)</sup> Caffeic acid phenethyl ester (CAPE) is a phenolic chemical compound present in numerous plants and is extracted from honeybee hive propolis.<sup>(13)</sup>

The study of Wang et al.<sup>(14)</sup> states that CAPE may inhibit the apoptosis of neurons as a result of sevoflurane exposure. This is shown by the decreased expression of the pro-apoptotic proteins Bax and Bcl 2 associated agonist of cell death (Bad) and the increased expression of the antiapoptotic proteins B cell CCL/lymphoma 2 (Bcl 2) and Bcl 2 like 1 (Bcl xL). CAPE inhibits neuronal apoptosis in the hippocampus that is marked by decreased numbers of terminal

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells and decreased expression of activated caspase-3.<sup>(14)</sup>

In contrast with all of the above-mentioned studies, the present study aimed to evaluate the neuroprotective effect of propolis in inhibiting Bax expression and the decrease in hippocampal neuronal count of rats exposed to sodium nitrite.

## METHODS

### Research design

This was an experimental laboratory study of posttest only control group design, conducted at the Research Laboratory, Faculty of Medicine, Universitas Islam Indonesia (FK UII) from July to December 2017.

### Research subjects

The subjects of this study were four-month-old male Wistar strain rats (*Rattus norvegicus*) weighing 200-300 grams. The required number of subjects were calculated from the formula of Charan and Biswas<sup>(15)</sup> as follows:

$E = \text{Total number of animals} - \text{Total number of groups}$

$E = 10 \text{ to } 20$

$\text{Total number of animals} = 20 - \text{total number of groups}$

$\text{Total number of animals} = 20 - 3$

From above calculation the required number of subjects was 17 rats. To obtain 6 subjects per group, the total number of subjects used was 18 rats. All experiments were performed according to the institutional guidelines of the Experimental Animal Center and in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals.

### Preparation of propolis extract

The propolis used in this study was a product available in the open market, in which 1 ml of the material contains 600 mg propolis. From this propolis preparation 0.1 ml was taken and 0.9 ml distilled water was added, so as to

obtain a concentration of 60 mg/ml. The diluted propolis solution was then administered to the rats in the appropriate doses, i.e. 100 mg/kgBW and 200 mg/kgBW, respectively, in accordance with the study by Lee et al.<sup>(16)</sup>

### Exposure to sodium nitrite and administration of propolis

In the sodium nitrite only group (group K) sodium nitrite at a dose of 50 mg/kgBW/day was administered orally for 60 days by means of a gastric tube. In the group receiving sodium nitrite and propolis (groups P1 and P2), sodium nitrite at a dose of 50 mg/kgBW/day and propolis at doses of 100 and 200 mg/kgBW/day, respectively, were administered by the oral route for 60 days by means of a gastric tube.

### Sample preparation

On day 61, when the rats were terminated and underwent transcardial perfusion, the rats were anesthetized by intramuscular injection of ketamine (100 mg/kg). The transcardial perfusion was done with a solution of NaCl at a volume of 100-200 ml until the emerging perfusion fluid was clear. After perfusion, the brain was carefully excised, the part containing the hippocampus was taken and fixed in phosphate-buffered formalin for 24 hours. It was subsequently made into paraffin blocks and sectioned at 3  $\mu\text{m}$  thickness. From each rat brain one slice was taken for immunohistochemical staining and one slice for hematoxylin-eosin staining.

Immunohistochemical staining was initiated by deparaffinizing with xylol and alcohol in decreasing concentrations. The tissue was then incubated with 3%  $\text{H}_2\text{O}_2$  in 10% methanol for 20 minutes and washed three times with distilled water followed with three times phosphate-buffered solution (PBS). Subsequently antigen retrieval was carried out in citrate buffer at pH 6 in a microwave oven. For about 10 minutes, the slices were heated to a high temperature (100°C), followed by med-low temperature for 20 minutes. After cooling, the slices were again

washed three times with PBS. Subsequently the slices were blocked with background sniper for 10 minutes. Without washing again, the tissue was dripped with Bax primary antibody (Ab) and incubated overnight at 4°C, then washed three times in PBS and incubated with Trekkie Universal Link for 10 minutes. After washing three times with PBS, the tissue was incubated with horseradish peroxidase-conjugated streptavidin (SA-HRP complex) for 10 minutes, then washed again three times with PBS. Bax-labeled pyramidal cells were visualized with 3,3'-diaminobenzidine (DAB) (1 : 100) for 5 minutes. The tissue was then washed five times with distilled water, followed by counterstaining with Mayer's hematoxylin for one minute. After washing in running water for 2 minutes, the slices were dehydrated in ethanol 70%, 80%, 90%, 95% and 100%, for one minute, respectively, cleared in xylol and covered with Canada balsam and coverslip.

Hematoxylin-eosin staining was performed according to the protocol of the Research Laboratory of the Faculty of Medicine, UII. Briefly, the protocol was as follows: The slides with the tissue sections were placed in the staining jar, deparaffinized, then washed twice with distilled water. Then the slides were placed in hematoxylin solution for 10 minutes, washed three times with distilled water, and checked under the microscope. If the hematoxylin staining was adequate, the slides were washed in running water. Subsequently the slides were placed in eosin and washed twice with distilled water. The slides were then placed in 70%, 80%, 90%, 95% and 100% alcohol, and dried with tissue paper. The dried slides were placed in xylol I, II and III for 5 minutes, respectively, followed by mounting and were then observed under the microscope.

### Observation of Bax-expressing cells

Observation of Bax-expressing cells was performed in the hippocampal area CA1. The slide preparations were observed under the light microscope at 400 x magnification. The whole

hippocampal area CA1 was observed and the Bax expression interpreted using the Allred score, which evaluates the proportions and staining intensities of cells with positive Bax expression.

The Allred score is semi-quantitative, using the proportion of positive cells (scores 0-5) and staining intensity (scores 0-3), which are summed to yield the total score (0-8).<sup>(17)</sup>

As per the Allred score for Bax-expression, the proportion score (PS) and the % positive tumor cells are respectively, (-) (0%), (+) (<1%), (++) (1-10%), (+++) (11-33%), (++++) (34-66%), and (+++++) (67-100%). The intensity of staining (IS) for the nuclear positivity of the cells graded as 0, 1, 2, and 3 was as negative, weak, mild, intermediate, and strong, respectively. Allred score is obtained by adding up the proportion score and intensity score.

### Hippocampal neuronal count

The neuronal count was performed in area CA1 of the hippocampus. The slides were observed under the light microscope at 400 x magnification. The cells were counted in the whole hippocampal area CA1. Then the mean number of neurons per field of view for each slide was calculated with the formula: mean number of cells = number of cells in area under observation/number of fields of view.

### Data analysis

To compare the mean Allred score for Bax expression and neuronal count between groups in the hippocampus, one-way Anova was used, followed by Tukey's multiple comparison post hoc test to determine the differing groups. A p value of less than 0.5 shows a significant difference.

### Ethical clearance

This study obtained ethical clearance from the Committee on Research Ethics, Faculty of Medicine, Universitas Islam Indonesia, under no. 03/Ka.Kom.Et/70/KE/VII/2017.

Table 1. Mean Bax expression and pyramidal neuronal count in the hippocampus

	Intervention			p value
	K	P1	P2	
Bax expression	7.27 ± 0.43	4.07 ± 0.65	4.06 ± 0.72	0.000
Pyramidal neuronal count	26.0 ± 2.53	46.0 ± 4.27	49.0 ± 3.05	0.000

K: sodium nitrite; P1: sodium nitrite + propolis at 100 mg/kgBW; P2: sodium nitrite + propolis at 200 mg/kgBW

## RESULTS

The results of the evaluation of Bax expression in area CA1 of the hippocampus using the Allred score are presented in Table 1. Group K receiving treatment with sodium nitrite had the highest mean Allred score. Analysis using one-way ANOVA showed significant results ( $p=0.000$ ). Post-hoc analysis demonstrated significant differences between group K and groups P1 and P2. There was no significant difference between groups P1 and P2 (Table 2).

The results of the pyramidal neuronal count in hippocampal area CA1 of the control group (K), treatment group 1 (P1), and treatment group 2 (P2) are presented in Table 1. Analysis with one-way ANOVA showed significant results ( $p=0.000$ ). The post-hoc analysis also showed significant differences between group K and groups P1 and P2. However, there were no significant differences between group P1 and group P2 (Table 2).

## DISCUSSION

In this study, exposure to sodium nitrite increased Bax expression in area CA1 of the hippocampus, which agrees with the study of Zaidi<sup>(18)</sup> stating that exposure to sodium nitrite causes degeneration in the hippocampus in areas CA1-CA4 and the gyrus dentatus, showing the presence of pyknotic nuclei, darkly stained cells, cells with condensed nuclei, and vacuolation. The neuronal count in the hippocampus also underwent a decrease as a result of the exposure to sodium nitrite. In another study Zaidi<sup>(19)</sup> found that sodium nitrite causes damage to the Purkinje cells in the cerebellum. Morphological changes in the Purkinje cells comprised swollen, autolyzed dark and

shrunken nuclei. This shows that Purkinje cells of the cerebellum are susceptible to the hypoxia that results from the exposure to sodium nitrite so that the cells degenerate. Amin et al.<sup>(1)</sup> report that exposure to sodium nitrite decreases the number of red blood cells and the hemoglobin concentration. Hypoxia may cause leakage of the mitochondrial membrane so that cytochrome-C will enter the cytosol and activate pro-apoptotic proteins followed by apoptosis of the cell.<sup>(20)</sup>

In the present study the pyramidal neuronal count in area CA1 of the hippocampus underwent a greater reduction in group K as compared with groups P1 and P2. This result is similar to that of the study by Amin et al.<sup>(1)</sup> who found congestion of blood vessels and bleeding in meninges, cerebrum and cerebellum. In addition, apparently the Purkinje cells of the cerebellum undergo degeneration and necrosis. Exposure to sodium nitrite in the brain results in neurotoxicity that is marked by an increase in serum biochemical parameters, such as nitrates, thiobarbituric acid-reactive substances (TBARS) and a decrease in glutathione and dopamine concentrations.<sup>(21)</sup> The study conducted by Jebasingh et al.<sup>(2)</sup> shows that intraperitoneal administration of sodium nitrite at 60 mg/kgBW in male Wistar rats for 30 days

Table 2. Results of Tukey's multiple comparison test

		Mean difference	p value
Bax expression			
K	P1	3.20	0.001
	P2	3.21	0.000
P1	P2	0.01	0.989
Pyramidal neuronal count			
K	P1	20	0.000
	P2	23	0.000
P1	P2	3	0.309

K: sodium nitrite; P1: sodium nitrite + propolis at 100 mg/kgBW; P2: sodium nitrite + propolis at 200 mg/kgBW

decreases learning ability and memory and increases the anxiety level in tests with the elevated plus maze. In addition, induction of hypoxia with subcutaneous administration of sodium nitrite at a dose of 50 mg/kgBW decreases locomotor activity and motor coordination in male Wistar rats.<sup>(22)</sup>

According to Jebasingh et al.,<sup>(2)</sup> morphological examination of the cortex, hippocampus, thalamus, and cerebellum shows changes resulting from exposure to sodium nitrite. The changes comprise pyknotic and rounded nuclei, with fragmented dead neurons and vacuolation. The afore-mentioned brain areas play an important role in the regulation and coordination of movement and behavior. The morphological changes are caused by apoptosis and neuronal degeneration. Mohammed<sup>(23)</sup> reports that administration of sodium nitrite at a dose of 30 mg/kgBW causes histopathological damage in various brain areas, including area CA1 of the hippocampus that is marked by loss of pyramidal cells and increase in vascular endothelial growth factor (VEGF).

The results of the present study show that administration of propolis at doses of 100 and 200 mg/kgBW may inhibit the decrease in pyramidal neuronal count in area CA1 of the hippocampus. This condition is consistent with previous studies showing that propolis inhibits the decrease in neuronal count in area CA1 of the hippocampus and the cortex prefrontalis in rats with induced stress.<sup>(12)</sup> Propolis may inhibit oxidative stress with increased activity of antioxidant enzymes in rats that were induced with 6-hydroxydopamin to trigger Parkinson's disease.<sup>(9)</sup> Propolis contains caffeic acid phenyl ester (CAPE) as antioxidant, that may inhibit neurotoxicity and neuronal degeneration in the cerebrum and cerebellum as a result of exposure to ethanol.<sup>(24)</sup>

In this study, administration of propolis in groups P1 and P2 decreased Bax expression in area CA1 of the hippocampus. The results of this study are in agreement with the study by Basarslan et al.<sup>(24)</sup> who reported that administration of CAPE may inhibit the apoptosis

of neurons in the cerebrum and cerebellum. In rats receiving ethanol and CAPE, expression of Bax and caspase-3 was lower than in rats exposed to ethanol only. Expression of Bcl-2 was higher in rats exposed to ethanol and then given CAPE. Propolis contains flavonoids with strong antioxidant and anti-inflammatory effects. Administration of propolis at doses of 100 and 200 mg/kgBW decreases apoptosis in rat brain tissue examined by TUNEL immunohistochemistry, decreases expression of TNF- $\alpha$  in brain tissues of a rat model of traumatic brain injury (TBI) and decreases the area of necrosis.<sup>(25)</sup> Annis et al.<sup>(26)</sup> report that administration of propolis at doses of 50, 100 and 200 mg/kgBW for 7 days may decrease apoptosis and increase expression of BDNF in the brain of a *Rattus norvegicus* traumatic model. Decrease in apoptosis and increase in BDNF expression is proportional to dose increases. The most effective dose of propolis is 200 mg/kgBW/day. The dose of 50 mg/kgBW is less effective in increasing BDNF expression. The study of Khalil et al.<sup>(27)</sup> shows that propolis may reduce the toxic effect of methotrexate in the liver and brain. Propolis increases the glutathione (GSH) concentration, increases the antioxidant activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GSR) in the liver and brain tissues and decreases the malondialdehyde (MDA) concentration. Propolis is capable of modulating enzyme antioxidant activity and reduce the oxidative damage caused by methotrexate. Administration of propolis in a rat model of spinal cord injury was able to decrease the concentrations of caspase 3 and cathepsin B.<sup>(28)</sup>

The results of the present study differ from those of the study by Hemieda et al.<sup>(29)</sup> who tested the effect of the administration of propolis in a rat model of diabetes. Diabetic rats were given propolis orally in two separate doses, 50 or 100 mg/kg BW/day for 6 weeks. The obtained results show that administration of propolis induced apoptosis and cell cycle arrest in the pancreas of diabetic rats, that are marked by

increased p53 and caspase-3 in the G0/G1 phases; and decreased Bcl-2, as compared with the untreated diabetic rats. In spite of this, the administration of propolis markedly reduced oxidative stress, as seen in the decreased concentrations of malondialdehyde (MDA) and carbonyl proteins in the liver and pancreas. Administration of propolis increased the capacity of the antioxidant defense system by increasing the content of glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and total antioxidants (TAC) in the liver and pancreas. Administration of propolis significantly improved inflammation as shown in the decrease in serum markers of inflammation such as C-reactive protein (CRP), interleukin 10 (IL-10) and transforming growth factor  $\beta$  (TGF- $\beta$ ), in comparison with untreated diabetic rats.

One of the clinical implications of the results of this study is the use of propolis to inhibit the neurodegeneration resulting from exposure to sodium nitrite. One of the limitations of this study is that only one piece of the brain was taken as the sample. Further studies should take a larger number of samples using stereology. This study only determined the expression of Bax as one of proapoptotic proteins. Further studies may be performed by determining all markers of apoptosis, such as caspases (caspase 3, 8 and 9), Bad, Bcl 2 and Bcl-XL and count the number of cells undergoing apoptosis with the TUNEL assay.

## CONCLUSION

Administration of propolis is capable of inhibiting Bax expression and the decrease in neuronal count in area CA1 of the hippocampus in rats receiving sodium nitrite. This study provides information on the benefits of propolis as a brain antioxidant.


## CONFLICT OF INTEREST

There was no conflict of interest in this study.

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## CONTRIBUTORS

K designed the study, wrote the protocol and the first draft of the manuscript. ZSN revised the manuscript. ESH revised the protocol and manuscript. FAR, ZLW, MZ carried out the study. All authors have read and approved the final manuscript. 

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