

ANTIOXIDANT ACTIVITY OF GINGER (*Zingiber officinale*) OLEORESIN ON THE PROFILE OF SUPEROXIDE DISMUTASE (SOD) IN THE KIDNEY OF RATS UNDER STRESS CONDITIONS

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Diterima 11 Juli 2007/ Disetujui 18 Februari 2008

ABSTRACT

Stress condition has been reported to decrease intracellular antioxidant-superoxide dismutase (SOD) in the liver and kidney of rats. This study was conducted to evaluate the antioxidant activities of ginger oleoresin on the profile of superoxide dismutase (SOD) in the kidney of rats under stress condition. The stress condition was achieved by five days of fasting together with swimming for 5 min/day. Ginger oleoresin was orally administrated in a dose of 60 mg/KgBW/day for seven days. Drinking water was provided ad libitum to all groups. The treatment of ginger oleoresin significantly decreased malondialdehyde (MDA) levels and increased SOD activity, as well as immunohistochemical, increased the content of copper, zinc-SOD (Cu,Zn-SOD) in the kidney tissues compared to that of untreated group. The antioxidant content in ginger oleoresin such as shogaol, zingeron, and gingerol, etc. were shown to have activities in the kidney tissues of rats under stress condition that is increasing the profile of SOD. Ginger oleoresin treatment in combination both before and after stress gave the best results.

Key words : antioxidant, ginger oleoresin, kidney, stress, superoxide dismutase (SOD)

INTRODUCTION

Wresdiyati and Makita (1995) reported that stressful conditions morphologically altered the kidney of Japanese monkeys, and increased the number of peroxisomes in the organ. It was also reported that stress produced inflammation and decreased the intracellular antioxidant-Cu,Zn-SOD in the liver and kidney of rats (Wresdiyati et al., 2002; Wresdiyati 2003a; Wresdiyati 2003b). These alterations may account for the fasting stress condition, inducing production of reactive oxygen species-free radical and thereby creating the situation known as oxidative stress. Oxidative stress can lead to a variety of biochemical and physiological lesions often resulting in metabolic impairment and cell death. These highly reactive oxygen radicals can readily react with various biological macromolecules such as DNA, proteins, lipids, and cause protein destruction. The lesions in turn lead to various diseases and degenerative processes such as aging and carcinogenesis in human and animals (Halliwell & Gutteridge 1995).

Ginger (*Zingiber officinale* Roscoe) has been used in many ways since long time ago. It is known to be effective as an appetite enhancer, an improver of digestive system, and an anti-cold remedy. Oleoresin is a non volatile compound in most of rhizomes. Oleoresin can be obtained by extracted the rhizomes using methanol or ethanol. Rhizomes oleoresin is well known to contain phenolic compounds which play a role as antioxidant. Wresdiyati et al., (2003) and Wresdiyati

(2003a) reported that *in vivo* methanol extract of ginger oleoresin has anti inflammatory effects on the livers and kidneys of rats under stress conditions. Ginger oleoresin has also been reported to have more antioxidative effect than α -tocopherol *in vitro* (Kikuzaki & Nakatani 1993). However, there are few reports of the antioxidant activities of ginger oleoresin on the profile of intracellular antioxidant-superoxide dismutase (SOD) in kidney tissue, *in vivo*, especially under condition of stress.

The present study was conducted to examine the role of antioxidant activity of ginger extract-oleoresin on the profile of SOD activity in kidney tissue *in vivo* especially under stress condition.

METHODS

Materials and equipments

Ten month old ginger (*Zingiber officinale*) was used for this study. It was obtained from Balitro Cimanggu, Deptan Bogor. A total of 60 male Wistar rats (250 ± 5 g) were used as experimental animals.

Some chemicals used for this study were ethanol, methanol, GF-254, hexane, diethyleter, linoleic acid, ammonium thiocyanate 30%, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ 20mM, HCl 3,5%, Bouin solution, xylol, paraffin, hematoxylin, eosin, monoclonal antibody Cu,Zn-SOD from Sigma (S2147), secondary labelled antibody from Dako (K1491), diaminobenzidine, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, NaH_2PO_4 , H_2O , NaCl, Trisma HCl, and Trisma Base.

Spectrofotometer, spectrofluorometer Shimadzu RF 540, incubator, oven blower, rotary vacuum-evaporator, centrifuge, tissue embedding console, microtome, humid chamber for immunohistochemical analysis, vortex, and micropipet were used for this study.

Extraction of ginger oleoresin

The ginger was cleaned and dried in an oven (40-60°C) for 30-36 hr to obtain dried ginger with 8-11% water content. The dried ginger was ground and then sieved to obtain ginger powder of 30 mesh. A total of 250 g of ginger powder was extracted four times using two kinds of solvents (500 ml), methanol and ethanol. The resulting extract then was sieved under vacuum condition, and distilled using a rotary vacuum-evaporator. The resulting oleoresin was a light to dark brown solid materials. The oleoresin was weighed.

Total phenol of ginger oleoresin analysis

Total phenol was analysed in both methanol and ethanol extracts of ginger oleoresin by AOAC 1984 method. 0.1 ml oleoresin was added to 75 ml of distilled water, 10 ml Folin-ciocalteu and 10 ml Na₂CO₃. The solution was then made up to 100 ml with distilled water, mixed and left at room temperature for 30 minutes. Absorbance of λ 760 nm of the solution was then read by a spectrophotometer. The negative control was made by replacing the 0.1 ml oleoresin with 0.1 ml distilled water. The standard of phenol is tannic acid.

Antioxidant activity analysis of ginger oleoresin

A 200 ppm ginger oleoresin was added to 2 ml linoleic acid (50 mM) in 99.8% ethanol, 2 ml phosphate buffer (0.1 M pH 7.0), and 1 ml dionized water. The solution then was incubated at 37°C. Every two days during 14 days, 50 μ l of the solution added 2.35 ml ethanol (75%), 50 μ l ammonium thiocyanate (30%), and 50 μ l FeCl₂·4H₂O (20 mM in HCl 3.5%). After 3 minutes, the solution was then observed using spectrophotometer at 500 nm (Chen et al., 1996). Distilled water and α -tocopherol were also observed for comparison to the ginger oleoresin.

Identification of ginger oleoresin

GF-254 plate was heated at 110°C for 4 hours. Ginger oleoresin was put on the start line of the plate, then stored at development media that contain eluent hexane and diethyleter in ratio 3:7. Then let eluent run to the final line. The plate then was taken from the media and shown some separate fractions which have different *Retardation Factor* (Rf) from each others. In order to clean and detailed seen of the fractions, reagent *Folin-ciocalteu* was sprayed to the fractions. Rf is ratio distance of oleoresin (start spot to final spot) to the distance of mobile phase.

Treatment of animals and tissue sampling

A total of 25 male Wistar rats (250 \pm 5 gr BW) were used for this study. The animals were adapted to the situation and conditions of the animal laboratory for 2 weeks, and then divided into 5 treatment groups (Table 1); (1) control group, without treatment of either stress or oleoresin, (2) stress group, treated by fasting and swimming only, without oleoresin, (3) treatment with oleoresin and followed by fasting and swimming, (4) treatment by fasting and swimming followed by treatment with oleoresin, and (5) treatment with oleoresin, followed by the stress regime, and followed again by treatment with oleoresin. Stress condition was created by 5 days of fasting while the animals were given access to only drinking water and making them to swim for 5 min per day. The oleoresin was orally administrated by using a sonde for 7 days. The oleoresin-treated animals were given standard laboratory diet *ad libitum*.

Table 1. Animal groups assigned to stress and ginger oleoresin treatments

Group	Treatment		
	Oleoresin*	Stress**	Oleoresin*
C	-	-	-
S	-	+	-
S+O	-	+	+
O+S	+	+	-
O+S+O	+	+	+

C = control, S = stress, S+O = stress+oleoresin, O+S = oleoresin+stress

O+S+O = oleoresin+stress+oleoresin

+ : treated

- : no treatment

* : 60 mg/KgBW/day for 7 days

** : fasting stress with only drinking distilled water for 5 days and swimming 5 minutes per day

Tissue sampling was carried out at the end of each treatment. Following cervical dislocation kidney tissues were collected from each animal in all groups. Tissues were then divided into three part and subjected to analysis for malondialdehyde (MDA), SOD activity and immunohistochemical analysis of Cu, Zn-SOD.

MDA analysis

A total of 50 μ l of sample or standard (1,1,3,3-tetraethoxypropane) was added to 1 ml thiobarbituric acid (10 mmol TBA in 75 mmol/L phosphate buffer at pH 3.0), then mixed for 5 sec. The solution was incubated for 60 min at 95 °C. Following cooling for 5 min, added 5 ml 1-butanol, then mixed again for 1 min. The solution was then centrifuged at 4000 rpm for 10 minutes, and the supernatant was then observed by using spectrofluorometer at 515 nm (excitation) and 553 nm (emission) (Conti et al., 1991; Raharjo & Sofos 1993).

SOD activity analysis

The kidney tissue was homogenized in phosphate buffer PH 7.4 (1:0.5) added and centrifuged at 10.000 rpm for 20 min. The lysate was collected and stored at -20°C. A total of 400 µl cold chloroform/ethanol 37.5/62.5 (v/v) was added to 150 µl lysate, and mixed for 3 sec. The solution was then centrifuged at 4400 rpm (4°C) for 10 min. The resulted supernatant was collected and stored at -2-8°C. A total of 2.9 ml xanthine and cytochrome c solution was added to the 50 µl supernatant sample, then mixed slowly using vortex. The reaction begin at the time 50 µl xanthine oxidase was added to the solution and mixed again. Observation was carried out using spectrophotometer. For phosphate buffer blanks were used to substitute for the sample. Distilled water was used as control.

Immunohistochemical analysis for Cu,Zn-SOD

Kidney tissues obtained from the animals of all groups were fixed in Bouin's solution for 24 hr. After fixation, the tissues were then dehydrated in a series of alcohols and cleared in xylol. The tissues were then embedded in paraffin before microtome sectioning (4 µm thickness). Tissue sections were immunohistochemically stained for Cu,Zn-SOD as described previously (Dobashi et al., 1989) with slight modifications. The tissue sections were washed for 15 min with 3 changes of PBS between each step. After deparaffinization and rehydration, the tissue sections were exposed to 3% H₂O₂ for 10 min to inactivate endogenous peroxidase activity and then to 10% normal goat serum to block nonspecific binding. Following rinsing with PBS, the tissue sections were incubated with the primary antibody of Cu,Zn-SOD (Sigma S2147) at 4°C. The tissues were then incubated with enhanced labelled polymer peroxidase (Dako K1491). The reaction product of antigen-antibody was visualized using diaminobenzidine (DAB). The tissue sections were then counterstained with haematoxylin, followed by dehydrated through a series of alcohols, and cleared with xylol. Finally, the sections were mounted on glass slides with entelan. For the control of staining, tissue sections were incubated with PBS instead of Cu,Zn-SOD antibody. The tissue sections of control staining showed a negative reaction with minimal background staining.

The distribution and frequency of positive reaction product on the tissues of control group were compared qualitatively with those of the treatment groups. The Cu,Zn-SOD reaction products were observed in the glomeruli, tubuli proximalis, tubuli distalis of the kidney. The observation of Cu, Zn-SOD in the tissues was based on the brown colour intensity in the cells and the distribution of the reaction product.

Data analysis

The amount of oleoresin and total phenol of ginger extract using methanol and ethanol were

statistically analysis using T-test. The MDA level and SOD activities of kidney tissues of all groups of treated animals were statistically analyzed using ANOVA and Duncan test. The immunoreaction products of the Cu,Zn-SOD were observed qualitatively by means of light microscopy.

RESULTS AND DISCUSSION

Oleoresin and total phenol of ginger extract

Methanol appeared to extract more oleoresin than ethanol, but the results were not significantly different. However, the phenolic compound in oleoresin following methanol extraction differed from that extracted by ethanol. Methanol extraction yielded total phenols of 647.22 ± 28.66 mg/mL, whereas ethanol extraction resulted in total phenols 522.22 ± 36.33 mg/mL which was significantly different ($P < 0.05$).

Based on the results obtained using methanol and ethanol extraction, oleoresin extracted with methanol was used in the following study on antioxidative activity of ginger oleoresin in animal treatment.

Antioxidative activity of ginger oleoresin

Antioxidative activity of ginger oleoresin that was compared to control (aquadest) and α -tocopherol were showed in Figure 1. Ginger oleoresin and α -tocopherol showed have antioxidative activities better than control, and antioxidative activity of ginger oleoresin showed the best from others. Antioxidative activity was decided from how can they stop linoleic acid oxidation to resulting peroxide radical. The radical then oxidize Fe²⁺ to Fe³⁺ and give red colour. Intensity of red colour showed the score of absorbancy. The figure of antioxidative activity of ginger oleoresin showed lower than α -tocopherol (Figure 1).

Identification of phenolic compound of ginger oleoresin

The identification was done using GF-254 plate. The plate showed several spots of certain active compound such as gingerol at R_f 0.24, zingeron at R_f 0.32, and shogaol at R_f 0.38 (Figure 2). It showed that ginger which used in this study contain active compounds; gingerol, shogaol, and zingeron. In this study, all of active compounds contain in ginger oleoresin (crude extract) were used to treat the experimental animals. We did not use active compound separately. It was reported that antioxidative activity of oleoresin is bigger than that of each phenolic compound in the oleoresin (Kikuzaki and Nakatani, 1993).

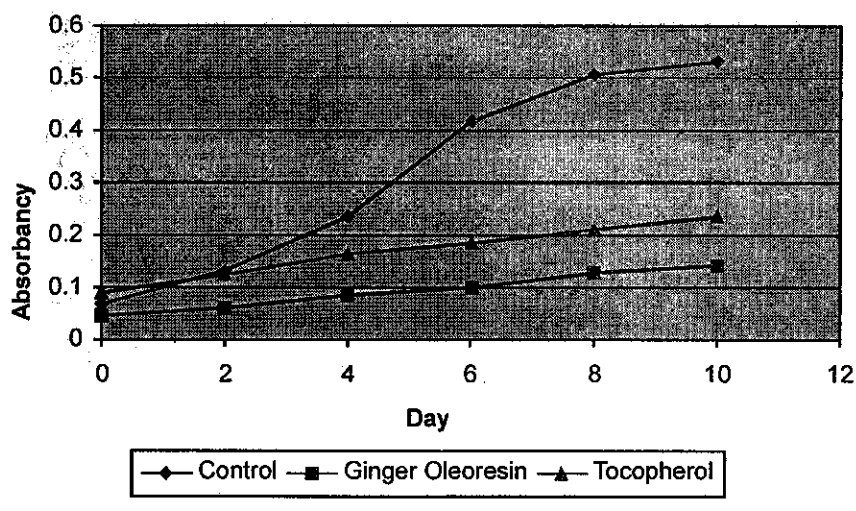


Figure 1. Antioxidative activity of ginger oleoresin, α -tocopherol and control (aquadest)

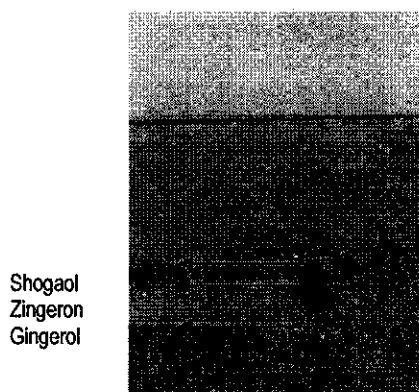


Figure 2. Thin Layer Chromatogram fractionation of phenolic compounds of ginger oleoresin. Ginger oleoresin contain active compounds such as gingerol at (Rf 0.24), zingeron (Rf 0.32), and shogaol (Rf 0.38)

MDA level of kidney tissues of treated rats

MDA is one of the final products of lipid peroxidation by free radicals. Thus MDA is used as an indicator of the presence of free radicals and has been widely used for detection of oxidative impairment by free radicals. The high level of MDA indicates a large number of free radicals.

The MDA level ($\mu\text{mol/g}$ protein) of the control group was the lowest, $947.47 \pm 29.41 \mu\text{mol/g}$, and statistically different ($P < 0.05$) to the MDA levels of the other groups. The highest MDA level was $2290.61 \pm 161.99 \mu\text{mol/g}$ which was found in the stress group (Figure 3). This result showed that stress condition could induce the production of free radicals that reactively oxidized lipids. The MDA level in the stress condition group that was treated with ginger oleoresin was significantly lower ($P < 0.05$) compared to that of the

stress group. The MDA level of stress+oleoresin, oleoresin+stress, and oleoresin+stress+oleoresin groups were $1378.84 \pm 44.93 \mu\text{mol/g}$, $1163.16 \pm 61.23 \mu\text{mol/g}$, and $1002.92 \pm 38.32 \mu\text{mol/g}$ respectively. These results showed that ginger oleoresin has antioxidative activity. The antioxidative activity scavenges free radicals resulting from condition of stress. It showed that the treatment of oleoresin before stress (O+S) more effective than oleoresin treatment after stress condition (S+O). However, a combination of both times of treatment, both before and after stress (O+S+O), gave the best result that was not statistically different compared to that of the control group. This means that ginger oleoresin had decreased the number of free radicals to level found in the normal-control group.

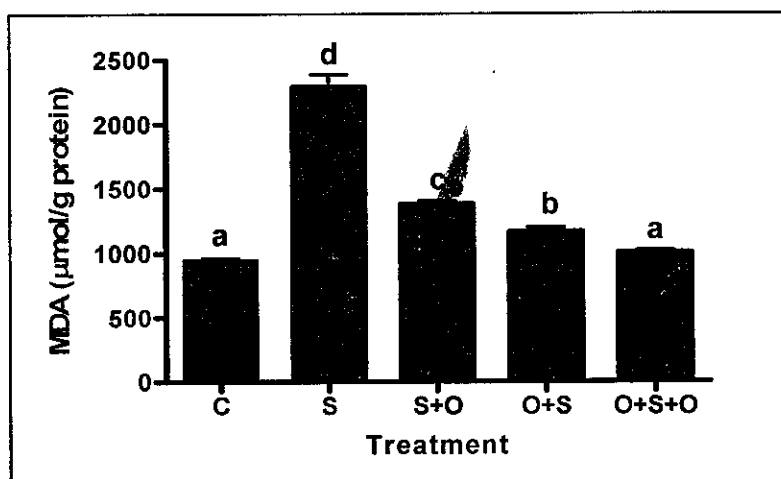


Figure 3. MDA level in the kidney tissues of treated rats. The control (C) group showed the lowest level, while Stress (S) group showed the highest level of MDA. Ginger oleoresin treatment after stress (S+O), before stress (O+S), and combination of both times of treatment (O+S+O) showed decreased MDA level. Different small letters are significantly different ($P < 0.05$).

SOD activity in the kidney tissues of treated rats

SOD activity (U/g wet weight) analysis using spectrophotometer showed that the average SOD activity of the stress group was 1175 ± 265 U/g compared to 4480 ± 370 U/g for the control group ($P < 0.05$). The average SOD activity of oleoresin treated groups was higher than that of the stress group, and was 3195 ± 185 U/g in the group which received oleoresin after stress (S+O). This SOD activity was significantly higher ($P < 0.05$) than that of the stress group and not significantly different to that of the control group. This was interpreted to mean that ginger oleoresin treatment

after stress condition (S+O) could increase activity of SOD in the kidney tissue of rats. However, the level remained statistically below the SOD activity in the control group. The average SOD activity of the groups that received oleoresin before stress (O+S) and combination of before and after stress (O+S+O) were 3745 ± 385 U/g and 3925 ± 189 U/g respectively. They were not significantly different to that of the control group. This was interpreted to mean that ginger oleoresin treatment in these latter two groups increased the SOD activity up to the levels under control condition (Figure 4).

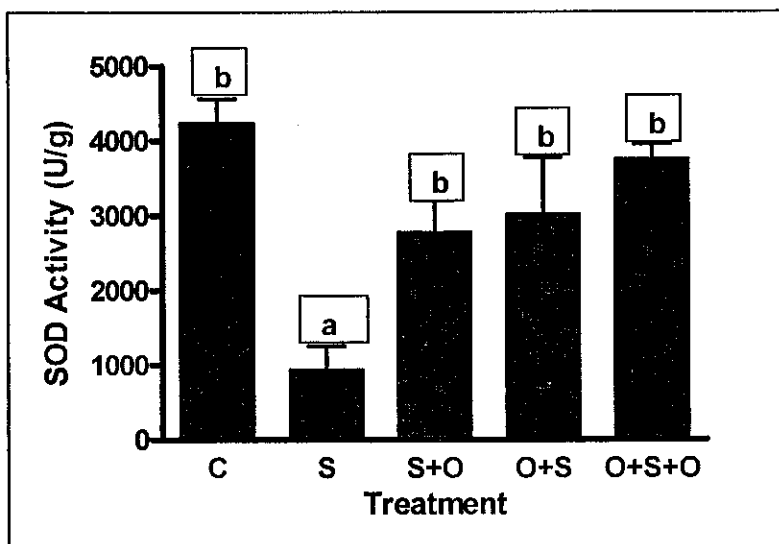


Figure 4. SOD activity in the kidney tissues of treated rats. SOD activity in the control (C) group showed the highest than that of other groups. While the lowest level of SOD activity showed in the stress (S) group. The SOD activity in the ginger oleoresin treated groups; after stress (S+O), before stress (O+S), and combination of both both times of treatment (O+S+O) was higher than that of the stress group and slightly lower than that of the control group. Different small letters are significantly different ($P < 0.05$).

Immunohistochemically-Cu,Zn-SOD content in the kidneys of treated rats

The positive reaction products of immunohistochemically localization to the Cu,Zn-SOD showed brown colour in the kidney tissues (Figure 5). The content of the enzyme based on the intensity of brown colour and their distribution in the tissues. The largest amount of Cu,Zn-SOD content was qualitatively found in the control group, which gave the highest intensity of brown colour reaction product and it was distributed in all of part of the tissue. That in the stress group was qualitatively lower compared to that of control group (Table 2 and Figure 5). The oleoresin treated groups showed more Cu,Zn-SOD content compared to that of stress group. The groups that treated ginger oleoresin before stress and combination on both way before and after stress gave higher Cu,Zn-SOD content in the kidney than the group that treated before stress. These results were comparable to the results of SOD activity, where there was an increase of SOD activity in the oleoresin treated groups compared to the stress group.

Table 2. Immunohistochemically-Cu,Zn-SOD content in the kidney tissues of treated rats

Group	Treatment	Content of Cu,Zn-SOD in the kidney
1	C	+++
2	S	+/-
3	S+O	+
4	O+S	++
5	O+S+O	++

C = control, S = stress, S+O = stress+oleoresin,

O+S = oleoresin+stress

O+S+O = oleoresin+stress+oleoresin

MDA level in the kidney tissues of the stress group was significantly higher compared to that of control group, whereas SOD activity and the Cu,Zn-SOD content in the tissues of the stress group was lower compared to that of control group. These results showed that free radicals were produced under the condition of stress imposed.

It was reported for Japanese monkeys that stress condition resulted in an alteration of the morphological peroxisomes and a remarkable almost three-fold increase in their number in kidney tissues (Wresdiyati & Makita 1995). Increasing the number of peroxisomes may also increase the number of oxidations that take place in the peroxisomes, subsequently more free radicals are produced as a side effect of stress condition. It has also been reported that fasting stress condition increased β -oxidation and cytochrome P-450 oxidase levels (Ishii et al., 1980; Orellana et al., 1992). These conditions result in a remarkable production of free radicals, such as anion superoxide. Anion superoxide can be dismutated by SOD and change to singlet oxygen molecules that able to oxidize unsaturated

fatty acid. The results of this oxidation may alter the cellular membrane as indicated by MDA production.

The remarkable increase in free radicals production results in more intracellular antioxidant, such as SOD, being needed to catalize these free radicals. Thus the activities and the cellular content of the antioxidant, SOD, is decreased, as is shown by the SOD activity and the content of Cu,Zn-SOD in the kidney tissues of rats under stress condition. They also relate to the elevated MDA level found under stress condition.

Oral administration of ginger oleoresin decreased MDA level and increased both SOD activity and Cu,Zn-SOD content in the kidney tissues of rats under stress condition. These results showed that antioxidant activities of ginger oleoresin could maintain the profile of SOD in the kidney under stress condition. The ginger used in this study contains some phenolic compounds such as gingerol, zingeron, and shogaol (Figure 2). These phenolic compounds, in ginger extract-oleoresin, have antioxidant activity (Figure 1). The phenolic compounds of ginger oleoresin would appear to neutralized free radicals, such as lipid radicals, to more stable products by transferring their hydrogen molecules to the free radicals. Subsequently, alteration of the cellular membrane is lower and MDA levels are also lower, in this study.

The increased activity of SOD and increased content of Cu,Zn-SOD in the kidney tissues of rats treated with ginger oleoresin under stress condition were both caused by the antioxidant activity of phenolic compounds in the ginger oleoresin. The phenolic compounds neutralized the free radicals, anion superoxide, which would normally be done by SOD. Consequently, intracellular antioxidant-SOD can be saved in both number and activity to neutralize the increase in anion superoxide that results from stress condition. Finally, the SOD activity and Cu,Zn-SOD content in the kidney tissues was higher in the ginger oleoresin treated group compared to that of the stress group without treatment of ginger oleoresin.

Oleoresin treatment, before and after stress condition, had the best effect on kidney tissues of rat by increased SOD activity and Cu,Zn-SOD content, as well as decreased MDA levels. It has been reported that 88 to 95% of phenolic compounds can be absorbed into the body, and that retention in the body is from 62% to 95% (Desminarti 2001). In the current experiments absorption of the phenolic compounds took place in the small intestine. Extending the timespan of oleoresin treatment (before and after stress) in this experiments appeared to have caused more phenolic compounds to be absorbed and retained in the tissues, resulting in a stronger and more positive effect of these compound.

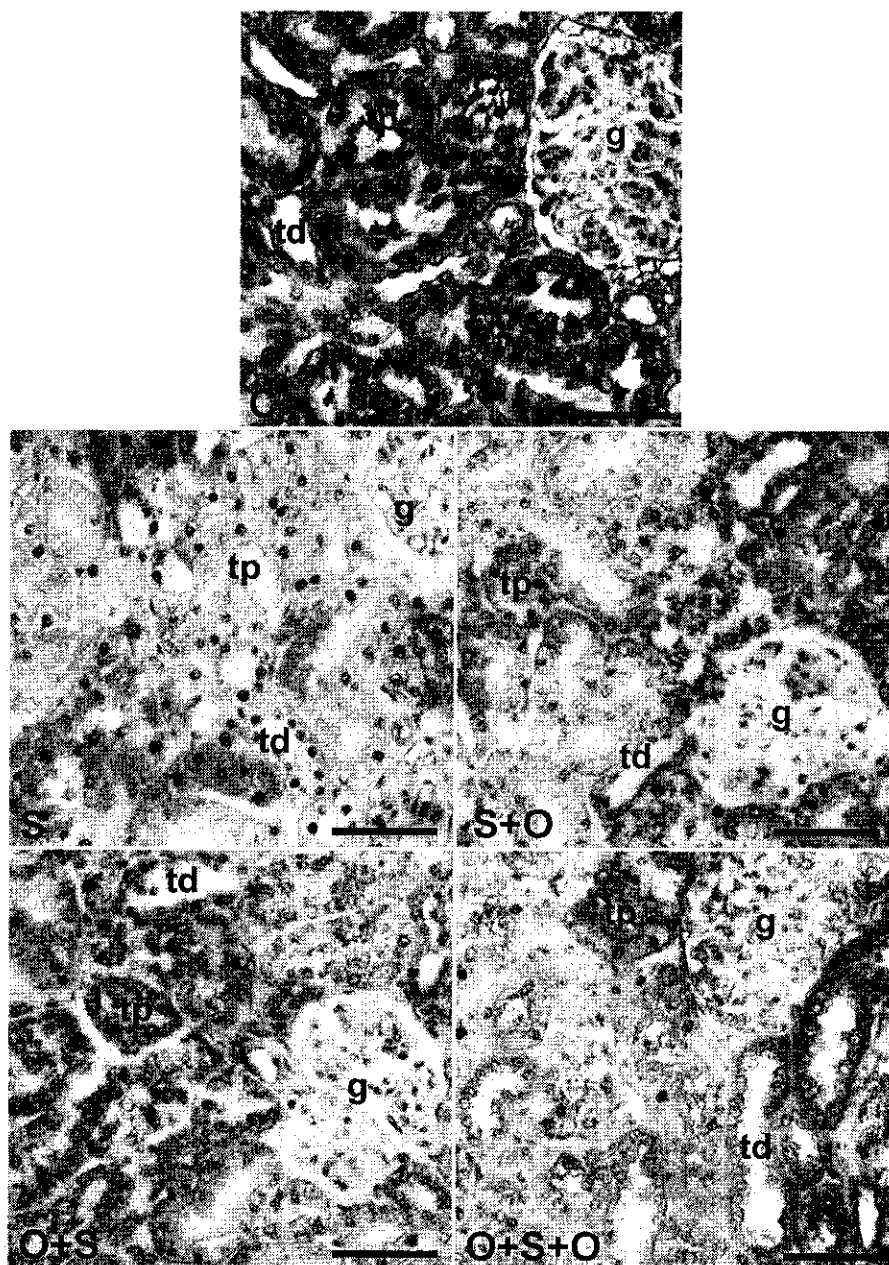


Figure 5. Photomicrograph of immunohistochemical localization of Cu,Zn-SOD in the kidney tissues of treated rats. The content of Cu,Zn-SOD in control (C) group qualitatively showed the highest than that of others group. While the lowest level of Cu,Zn-SOD content showed in the stress (S) group. The Cu,Zn-SOD in the ginger oleoresin treated groups ; after stress (S+O), before stress (O+S), and combination of both times of treatment (O+S+O) qualitatively showed increased compared to that of stress group. g=glomeruli, td=tubuli distalis, tp=tubuli proximalis, — = 50 μ m (400X magnification).

CONCLUSION

The study concluded that the antioxidant activity of ginger (*Zingiber officinale*) oleoresin increased SOD activity and the content of Cu,Zn-SOD in the kidney of the rat under stress condition. The antioxidant activity was also shown by the reduced level of MDA in the tissues of rats under stress condition. These results were shown in the groups of rats that were given by ginger oleoresin whether before or after stress condition. However, the group of rats that were given by ginger oleoresin both before and after stress showed the best results.

ACKNOWLEDGMENT

This study was part of a research project funded by a grant (*Hibah Bersaing X*) from the Ministry of Education of Indonesia No. 103/LII/BPPK-SDM/IV/2002 for Tutik Wresdiyati in 2003.

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