

## Pomegranate (*Punica granatum L*) powder reduced malondialdehyde (MDA) level in cigarette smoke exposed rats

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### Abstrak

**Latar belakang:** Mengetahui efek pemberian bubuk “pomegranate” selama 14 hari terhadap peroksidasi lipid berdasarkan pengukuran kadar malondialdehida (MDA) pada tikus yang dipaparkan asap rokok.

**Metode:** Rancangan acak lengkap diterapkan pada penelitian ini. Tiga puluh tikus Sprague-Dawley dibagi menjadi tiga kelompok, yaitu: kelompok tanpa penambahan bubuk pomegranate (kontrol), kelompok R1 dengan penambahan 5% (kandungan flavonoid 0,351%/100g) dan kelompok R2 dengan penambahan 10% (kandungan flavonoid 0,566%/100g) bubuk pomegranat ke dalam ransum. Ransum diberikan ‘ad libitum’ selama 14 hari. Tikus dipaparkan pada asap rokok selama tiga kali sehari. Kadar MDA diukur sebelum pemaparan, hari ke-8 dan -15 pemaparan. Data dianalisis menggunakan uji ANOVA setelah pengujian normalitas data.

**Hasil:** Kadar MDA sebelum pemaparan adalah  $0.35 \pm 0.06$  nmol/mL,  $0.38 \pm 0.06$  nmol/mL dan  $0.38 \pm 0.06$  nmol/mL berturut-turut untuk kelompok kontrol, R1 dan R2 ( $P = 0.65$ ). Pada hari ke-8, kadar MDA adalah  $0.70 \pm 0.06$  nmol/mL,  $0.57 \pm 0.06$  nmol/mL dan  $0.56 \pm 0.06$  nmol/mL berturut-turut untuk kelompok kontrol, R1 dan R2. Kadar MDA pada hari ke-15 berturut-turut untuk kelompok kontrol, R1 dan R2 adalah  $1.02 \pm 0.06$  nmol/mL,  $0.89 \pm 0.06$  nmol/mL dan  $0.80 \pm 0.06$  nmol/mL. Terdapat perbedaan bermakna ( $P = 0,001$ ) rerata kadar MDA hari ke-8 dan hari ke-15 antar kelompok. Rerata kadar MDA pada kelompok kontrol paling tinggi dibandingkan kelompok R1 dan R2 baik pada hari ke-8 maupun hari ke-15. Rerata kadar MDA pada kelompok R2 paling rendah dibandingkan kelompok R0 dan R1 pada hari ke 8 maupun hari ke 15. Peningkatan kadar MDA pada hari ke delapan dibandingkan sebelum pemaparan pada kelompok R0, R1 dan R2 berturut-turut adalah 97%, 52% dan 48%, sedangkan peningkatan MDA pada hari ke 15 dibandingkan sebelum pemaparan pada kelompok R0, R1 dan R2 berturut-turut adalah 187%, 137% dan 113%. Peningkatan kadar MDA terbesar adalah pada kelompok R0.

**Kesimpulan:** Pemberian bubuk pomegranat pada kadar 5% dan 10% dapat menekan terjadinya peroksidasi lipid yang ditunjukkan dengan kadar MDA dibandingkan dengan kelompok kontrol. (*Med J Indones* 2011; 20:34-9)

### Abstract

**Background:** To analyze the effect of pomegranate (*P. granatum*) powder consumption for 14 days on lipid peroxidation as shown by malondialdehyde (MDA) level in cigarette smoke exposed rats.

**Methods:** Thirty Sprague-Dawley male rats were randomly divided into three groups, i.e.: a control group and two treatment groups. The treatment groups either received 5% (R1: 0.351% flavonoids/100g) or 10% (R2: 0.566% flavonoids/100g) pomegranate extract powder, respectively. The diets in the form of pellets were freely consumed (*ad libitum*) and were given for 14 days. Rats were exposed to cigarette smoke three times per day. Blood samples were taken on day 0, day 8th and 15th for MDA analyses. Comparison of MDA levels was done by ANOVA's test on normal data.

**Results:** On day 0, the MDA levels were  $0.35 \pm 0.06$  nmol/mL,  $0.38 \pm 0.06$  nmol/mL and  $0.38 \pm 0.06$  nmol/mL for control, 5% and 10% pomegranate powder group, respectively ( $P = 0.65$ ). On day 8th, the MDA levels were  $0.70 \pm 0.06$  nmol/mL,  $0.57 \pm 0.06$  nmol/mL and  $0.56 \pm 0.06$  nmol/mL, and on day 15th, the MDA levels were  $1.02 \pm 0.06$  nmol/mL,  $0.89 \pm 0.06$  nmol/mL and  $0.80 \pm 0.06$  nmol/mL in control, 5% and 10% pomegranate powder group, respectively. There was a significant difference ( $P < 0.001$ ) in MDA levels on day 8th and 15th between groups. The average MDA level for rats consuming control diet was the highest on day 8th and 15th. On the other hand, the lowest average MDA level on day 8th and 15th was observed in rats given 10% pomegranate extract powder. In comparison to MDA level before cigarette smoke exposure, the increases in MDA levels for rats consuming control diet, 5% and 10% pomegranate extract powder were 97%, 52% and 48% on day 8th, and 187%, 137% and 113% on day 15th, respectively. The highest increase in MDA level was observed in control group.

**Conclusion:** The use of pomegranate powder at 5% and 10% concentration was able to prevent the occurrence of lipid peroxidation as shown by the MDA levels and the effect was dose dependent. (*Med J Indones* 2011; 20:34-9)

**Key words:** antioxidant, flavonoids, lipid peroxidation

Cigarette smoke contains more than 4000 elements and at least 200 of them are harmful to health. The main toxins in cigarettes are tar, nicotine, and carbon monoxide. In addition, cigarette smoke also contains other chemicals that are not less toxic such as ammonia, formic acid, formaldehyde, hydrogen cyanide, etc.<sup>1,2</sup>

The combustion of cigarettes can lead to the production of reactive oxygen species (ROS). Free radicals, components of ROS are found in cigarette mainstream and side stream smoke. Side stream cigarette smoke contains more toxic gases and free radicals than the mainstream cigarette smoke.<sup>3,4</sup> The adverse effects of smoking may result from the accumulation of oxidative damage brought about by ROS, which is called oxidative stress.

Oxidative stress is a condition that occurs due to imbalance between free radical and antioxidant productions. This will cause serious damage to biological macromolecules and disregulation of normal metabolism and physiological functions.<sup>5</sup> Free radicals can cause lipid peroxidation in cell membranes, which in turn produces compounds that are toxic to cells, such as malondialdehyde (MDA). Elevated levels of MDA show the increased activity of lipid peroxidation.<sup>6</sup>

As a response to oxidative damage, antioxidants are produced. Antioxidants are molecules that slow or prevent the oxidation of other chemicals.<sup>7</sup> Antioxidants can be derived from the body or from outside the body. Antioxidants from outside the body are natural food ingredients from fruits. One of the antioxidant containing fruits is pomegranate (*Punica granatum L.*). Pomegranate contains high concentration of antioxidants (11.33 mmol/100 g). The main natural antioxidants in pomegranate are polyphenols;<sup>8</sup> and one of the polyphenols is the flavonoid.

Rat is one of the animals, which are widely used as laboratory animals due to their similar anatomy to mammals, and some other advantages such as easy handling, their short life cycle, and they are readily available. Moreover, rats resemble humans in most conditions, and their reproduction also resemble those of large mammals.<sup>9</sup>

Currently, there is no study that has determined the effect pomegranate powder consumption against cigarette smoke induced oxidative stress. Therefore, the objective of this study was to evaluate the possible effects of pomegranate in reducing the blood MDA content in cigarette smoke exposed rats (*Rattus norvegicus*).

## METHODS

This study was conducted in a completely randomized design that compared three groups of rats. The treatments were R0 (control diet), R1 (95% of R0 + 5% of pomegranate powder), and R2 (90% of R0 + 10% of pomegranate powder). The experiment was carried out for two months in an animal house at the Laboratory of Animal Genetic and Breeding, Department of Animal Production and Product Technology, Faculty of Animal Science, Bogor Agricultural University, and at the Laboratory of Biochemistry, Faculty of Medicine and Health Sciences Syarif Hidayatullah State Islamic University, Jakarta.

### Subjects

Thirty male white rats (*Rattus norvegicus*) of Sprague-Dawley strain (3 weeks of age) with average body weight of 80 g were used in this experiment. These rats were divided into 3 groups (R0, R1 and R2) that consisted of 10 rats each. These rats were individually kept in a plastic cage.

### Pomegranate powder

Pomegranate powder was commercial pomegranate powder (Taify®). Nutrient composition of the pomegranate powder is shown in Table 1.

### Diet and drink

A standard diet of commercial feed for rats in powder form was used. For the control rats no pomegranate powder was added. For the treatment groups, pomegranate powders were added at the final levels of 5 and 10%. All diets were made into -pellets. The pellets were freely consumed (*ad libitum*) through oral (without gavage) and were given weekly, for two weeks (14 days). Food intake was measured by subtracting the amount of food that was given with the amount of food that was left. The result was divided by 7 days to obtain food intake per day. Food intake was then multiplied with dry matter (DM) content to obtain DM intake. Flavonoid intake was calculated by multiplying DM intake with flavonoid content of each treatment diet.

### Cigarette exposure

Rats were exposed to cigarette smoke 3 times per day (in the morning, at noon and in the evening) using 1 cigarette per rat. The cigarette used was a commercial filter cigarette. A burning cigarette was placed in a glass

cup and was put into each of the plastic cage. The top of each of plastic cage was then fully covered with a paper cartoon to expose the rats with cigarette smoke. This treatment was applied until the cigarette was burned, and the paper cartoon was then replaced.

**Blood sampling**

Blood sampling was taken at day-0, -8, and -15 by cutting the tip of the tail ( $\pm 0.5$  cm from the tail tip); the tail was then massaged from its base up to the tip of the tail to collect the blood (2 ml). Blood was collected using a vacutainer and stored in an ice box to be brought to the laboratory for MDA measurement.

**Malondialdehyde measurement**

Measurement of MDA level was done according to the method of soewoto et al,<sup>10</sup> in brief: the blood sample was added to 0.50 mL of 10% cold TCA solution, which was then centrifuged for 15 minutes. The supernatant formed was added to 0.75 mL of 0.67% TCA solution, and the mixture was then placed into a boiling-water containing water bath for 10 minutes. After it was cold, it was read using a spectrophotometer at a wavelength of 532 nm to determine the MDA concentration. The MDA concentration was obtained by dividing the absorption with  $\epsilon$  ( $\epsilon=153.000 \text{ M}^{-1}\text{cm}^{-1}$ ).<sup>10</sup>

**Data analysis**

The data obtained were noted and tabulated to be entered and processed using Microsoft Excel 2007 software. All results were expressed as mean $\pm$ standard deviation. Statistical test to compare the three groups was done using two ways repeated ANOVA's test on normal data. Differences were considered significant if  $P < 0.05$ .

**RESULTS**

All the rats were in healthy conditions throughout the study. The diet was controlled every day and added when necessary. Cages were cleaned once a week and the replacement of husk as bedding was also carried out at the same time. Every time after cigarette smoke exposure the rats looked weak.

The composition of nutrients in the diet <sup>11</sup> is presented in Table 1. Addition of pomegranate powder into the diet changed the composition. Generally, the amount of almost all nutrients was increased; except for crude protein, it decreased on the addition of pomegranate powder at 5%, and a similar result was found in ash and

Beta-N contents when pomegranate powder was added at 10%. The control diet already contained flavonoids. With the addition of pomegranate powder, the flavonoid content was increased nearly one-fold in R1, and the increase was doubled in R2.

Table 1. The content of nutrients in the diet

Nutrients	Pomegranate powder	Diet		
		R0	R1	R2
Dry matter (DM - %)	81.50	89.35	91.50*	91.46*
Ash (%DM)	5.06	8.66	8.81*	8.28*
Crude Protein (%DM)	4.86	18.18	17.4*	18.47*
Fiber crude (%DM)	16.82	10.44	11.47*	15.22*
Fat crude (%DM)	1.31	3.67	4.14*	4.20*
Beta N (%DM)	71.95	48.40	56.67*	45.29*
Energy (kcal/gram)	3855	3.88	4.04*	4.07*
Flavonoids (%/100g)	4.22	0.253	0.351*	0.566*

R0= control diet, R1= 95%R0+5% pomegranate powder, R2= 90%R0+10% pomegranate powder, \*= ANOVA test result:  $P < 0.01$  compared to R0

Food intake in all three groups can be seen in Table 2. There was no significant difference in food intake between the three groups ( $P = 0.65$ ). Flavonoid intakes are presented in Figure 1. In R0 group, flavonoid intake was lower than in that of R1 ( $P= 0.000$ ) and R2 group ( $P= 0.000$ ). In R1 group, flavonoid intake was lower than in the R2 group ( $P = 0.000$ ). The addition of pomegranate powder in the diet will increase the content of flavonoids. The higher the addition of pomegranate extract powder, the higher the flavonoid content will be.

Table 2. Average of food intake in each group (g/head/day)

Groups	First week <sup>2</sup>	Second week <sup>2</sup>	<i>P-value</i> <sup>3</sup>
R0	10.40 (0.97)	10.50 (0.53)	0.65
R1	10.45 (0.52)	11.27 (1.62)	
R2	10.30 (0.48)	10.70 (0.48)	

R0= control diet, R1= 95%R0+5% pomegranate powder, R2= 90%R0+10% pomegranate powder, <sup>2</sup>= Mean (standard deviation), <sup>3</sup>= ANOVA test result

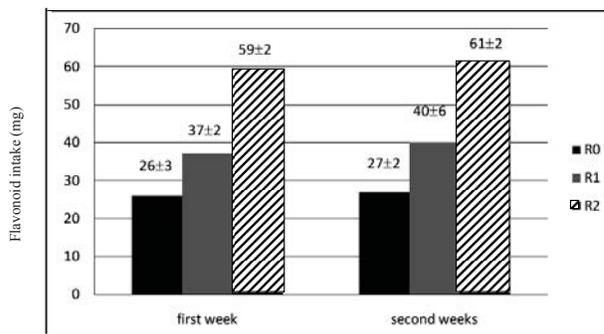


Figure 1. Average flavonoid intake in the first and second week (mg).

The mean MDA levels before treatment in all three groups were not significantly different ( $P = 0.65$ ) (Table 3). On day 8<sup>th</sup>, the MDA levels were  $0.70 \pm 0.06$  nmol/mL,  $0.57 \pm 0.06$  nmol/mL and  $0.56 \pm 0.06$  nmol/mL, and on day 15<sup>th</sup>, the MDA levels were  $1.02 \pm 0.06$  nmol/mL,  $0.89 \pm 0.06$  nmol/mL and  $0.80 \pm 0.06$  nmol/mL in R0, R1 and R2 group respectively. Results of ANOVA's test showed that there were significant differences ( $P = 0.001$ ) in mean concentrations of MDA at day-8 and -15 between groups. The MDA levels in R0 group were the highest compared to both R1 and R2 groups on day-8 and -15. On the other hand, the lowest MDA levels were found in R2 group both on day-8 and -15 (Figure 2). A significant increase in MDA level was shown in each group by day-8 and -15 compared to MDA levels before. Elevated levels of MDA on day-8 compared to before treatment in group R0, R1 and R2 were 97%, 52% and 48% respectively, while the increase in MDA level at day-15 compared to before treatment in group R0, R1 and R2 were 187%, 137% and 113% respectively. Elevated level of MDA was the largest in the R0 group.

Table 3. Average of MDA levels before treatments (nmol/mL)

Groups	MDA level before treatments <sup>2</sup>	<i>P</i> -value <sup>3</sup>
R0	0.35 (0.06)	0.65
R1	0.38 (0.06)	
R2	0.38 (0.06)	

R0= control diet, R1= 95%R0+5% pomegranate powder, R2= 90%R0+10% pomegranate powder, <sup>2</sup>= Mean (standard deviation), <sup>3</sup>= ANOVA test result

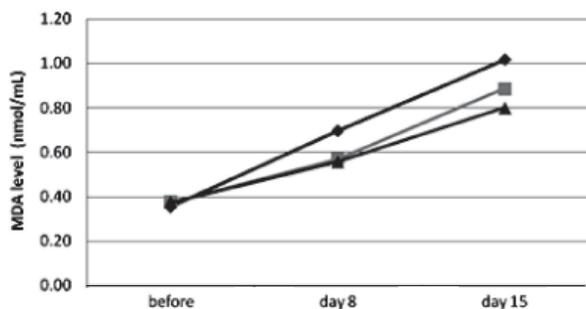


Figure 2. Average of malonaldehyde (MDA) levels before treatment, day-8 and -15 after treatments.  $p = 0.001$ , ANOVA'S test

◆= R0, ■= R1, ▲= R2

## DISCUSSION

Table 1 shows that almost all nutrient contents increased by the addition of pomegranate powder at levels of 5 and 10%, but the increase did not follow a linear trend. The decrease in crude protein content in diet

after the addition of 5% pomegranate powder, and the reduction in ash and Beta-N contents after the addition of 10% pomegranate powder were probably due to a less homogeneous mixing at the time of sampling for composition analysis.

Since there were no significant differences in food intakes among the treatment groups ( $P = 0.65$ ), this fact indicates that pomegranate powder can be included into the diets at 5 to 10% without affecting the rat's appetite, and food intake. Pomegranate was given in the diet and not by gavage, so the intake depended on the rat's appetite, and became the limitation of this study.

According to Parakkasi,<sup>12</sup> the level of food consumption can describe the diet palatability. Our result indicated that the degree of preference was the same for the diet with and without added pomegranate powder. However, the intake was less than those stated by Smith and Mangkoewidjojo,<sup>13</sup> who stated that food intake of adult rats were between 12 to 20 g/day. On the other hand, the National Research Council (NRC) indicated that the consumption of white rat at growth period was about 10-15 g food/head/day.<sup>14</sup> The lower food intake in the present study could be due to the cigarette smoke; however, these effects were not improved by the addition of 5 and 10 % pomegranate into the diet for 14 days.

The mean MDA levels before treatment in all three groups were not significantly different ( $P = 0.65$ ) as shown in Table 3. The mean MDA levels were much lower than the levels of MDA in other studies although the same test methods were carried out, i.e. 3.63 mmol/L,<sup>15</sup> 2.70 mmol/L,<sup>16</sup> 2.0 mmol/L,<sup>17</sup> and 3.5 mmol/L.<sup>18</sup> Erichsen conducted an examination of MDA level using high performance liquid chromatographic (HPLC) and the result was 1.29 mol/L.<sup>19</sup> By using the same methods of examination as applied in this study, Ogawa found that the level of MDA was 0.6 mmol/L.<sup>20</sup>

There are several methods of examination to measure MDA, such as spectrophotometer and HPLC. Research conducted by Lovri showed no difference in the results by using the two methods.<sup>17</sup> A very low level of MDA was likely due to the age of the rats used that were very young i. e. about three weeks, while some studies used mice at the aged of 6-9 weeks on average.

Elevated levels of MDA that showed the lipid peroxidation process in the three groups were due to the exposure of various chemical substances containing cigarette smoke that included free radicals.<sup>3, 21</sup> In this study, rats were exposed to passive cigarette smoke.

Cigarette smoke directly inhaled by active smokers is called mainstream smoke. Side stream smoke is the smoke generated from burning cigarettes. The side stream smoke contains more toxic gases and free radicals compared to the main smoke. This cigarette smoke can cause lipid peroxidation.<sup>3</sup>

Giving pomegranate powder could suppress the increase in MDA level (Figure 2). A similar result to that obtained in this study was also found by Kaplan *et al*,<sup>22</sup> who gave pomegranate in the form of juice for two months to rats.

The study used rats with atherosclerosis that showed low levels of plasma lipid peroxidation at weeks 3, 6 and 8 in treatment groups compared to control. However, these effects depended on the concentration of pomegranate juice. The higher the concentration of pomegranate juice, the lower was the lipid peroxidation level.<sup>22</sup>

The ability of pomegranate powder in suppressing the increase in MDA level showed the antioxidant capacity in pomegranate powder to counteract free radicals. Pomegranate is rich in antioxidants of the polyphenol group including flavonoids, which can protect tissues from free radicals and pathological processes such as cancer, cardiovascular and inflammatory diseases.<sup>23</sup>

In this study, the flavonoid consumption as much as 26-27 mg in R0 was not able to suppress the increase in lipid peroxidation due to cigarette smoke. The suppression of lipid peroxidation just occurred when the flavonoid intake was increased to 37-40 mg (R1), and a more noticeable effect could be obtained when the flavonoid consumption was increased to 59-61 mg (R2). This is different from the study by Kaplan *et al*. in which pomegranate juice containing 0.175 mg flavonoids /day given to atherosclerotic mice was able to decrease macrophage lipid peroxidation.<sup>22</sup> In another study, consumption of pomegranate juice that contained 0.035 mg flavonoids could reduce oxidative stress in atherosclerotic rats.<sup>24</sup> This difference is probably due to the exposure to cigarette smoke that led to higher oxidative stress conditions; so the intake of flavonoids have to be higher to be able to suppress the occurrence of lipid peroxidation.

Flavonoids contribute to counteract the free radicals in several ways. Some flavonoids work by inhibiting an enzyme that is responsible for the production of superoxide anions such as xantin oxidase. Moreover, it can also acts as a scavenger of free radicals by donating electrons to superoxide radicals or lipid radicals to be stable.<sup>25</sup>

In addition to the content of polyphenols in pomegranate, there are other natural antioxidants, i.e. vitamin C, vitamin E, beta-carotene and zinc. Antioxidant concentrations in pomegranate are the highest compared to those present in grapes, oranges and other fruits.<sup>22</sup> This research showed that the content of natural antioxidants in pomegranate powder can counteract oxidative stress due to exposure to cigarette smoke. However, a more in depth examination of all antioxidant contents that are present in pomegranate powder and their capacity needs to be conducted in future studies. These studies are necessary to determine the type of antioxidants that have significant roles in pomegranate powder. This research did not show which antioxidant in pomegranate were the most responsible for this results, and this fact was the limitation of this study.

In conclusion, giving pomegranate powder at the levels of 5% and 10% could suppress the occurrence of lipid peroxidation as indicated by MDA levels compared to control group.

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