

ANTIOXIDANT ACTIVITIES OF *ALLIUM SATIVUM* TOWARDS FORMALIN-INDUCED
OXIDATIVE STRESS IN RAT LIVER

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ABSTRACT

A. Sativum has been reported to be effective against diseases, in the pathophysiology of which free radicals have been implicated. In the present study, the antioxidant effect of *A. Sativum* towards formalin-induced oxidative stress in rat was investigated.

Wistar rats were divided into 4 groups: (1) control (2) exposure of 10% formalin by inhalation for 4 hrs/day, 5 days/wk in 8 wks (3) exposure of 10% formalin by inhalation for 4 hrs/day, 5 days/wk in 8 wks and later fed with 250 mg/kg aqueous extract of *A. sativum* for 14 days (4) control group fed with 250 mg/kg aqueous extract of *A. sativum* for 14 days. After the treatment, the liver was harvested for determination of malondialdehyde activities (MDA) and activities of anti oxidant enzymes superoxide dismutase (SOD) and catalase (CAT).

Inhalation of 10% formalin increased the concentration of MDA and also in group fed with *A. sativum*. However, increase level of SOD was seen in group 3 and group 4, suggesting the occurrence of antioxidant activity in group fed with *A. sativum*.

Inhalation of 10% formalin induced oxidative stress in rat liver. Supplementation of *A. sativum* has the potential to enhance the endogenous SOD antioxidant status thus the potential capabilities of preventing liver damage.

1.0 Introduction

Antioxidant is defined as a molecule that inhibits the generation of reactive oxygen species, origin from unstable molecule. Various type of antioxidant has been studied such as anthocyanins, beta-carotene, catechins, coenzyme Q10, vitamin C, vitamin E, vitamin A, and selenium [28]. Interested in antioxidant study is develop as they believed that the antioxidant is able to protect the body against damage by reactive oxygen species [17]. Natural antioxidants, especially from plant origin, has notably increased in recent years but so far no attempt was done in evaluating therapeutic intervention with the natural antioxidant like *Allium Sativum* (*A. sativum*).

Previous study has shown that *A. sativum* or garlic express higher free radical scavenging effect as compared to red onion [22]. This is due to the profusion of sulfhydryl which was reported as an excellent antioxidant [8] and capable to reduce oxidative stress induces by drugs including acetaminophen [2], ions, minerals, and other dissolved toxic gases.

Formaldehyde (FA) is dissolved, colorless and powerful-smelling gases that are found in aqueous solution are a known chemical to cause death in human [27]. Common route of toxic exposure to FA are through inhalation, oral and skin. Short-term exposure of this substance will lead to oxidative stress to the organ. While, there is no report showing toxicity

effect to human in an acute exposure [27]. Long-term inhalation exposure have a generalized systemic damaging effect i.e, immunological, lymphoreticular, neurological, reproductive, developmental, genotoxic, and carcinogenic. Most importantly, metabolites of FA will induce oxidative stress in human organs and biological systems.

At present, limited research has been done in investigating the protective effect of *A. sativum* towards formalin. It is well known that *A. sativum* posses' antioxidant properties and it is believed that the *A. sativum* will reduce the oxidative stress by increasing the level of antioxidant enzyme in rat liver. Previous studies have shown that antioxidant S-allylcysteine, one of an important compound in *A. sativum* acts as protection against oxidative stress induced by gentamicin [18] and 1-methyl-4-phenylpyridinium-induced Parkinsonism in mice [21]. This compound helps to reduced oxidative damage by increasing the level of endogenous antioxidant enzyme and scavenged excess free radical.

The objective of this study is to investigate the protective effects of *A. sativum* toward formalin-induced oxidative stress in rat liver by determine the level of malondealdehyde activities (MDA) and antioxidant enzyme (superoxide dismutase and catalase).

2.0 Materials and Methods

2.1 Sample Collection, Preparation and Extraction

Samples of *A. sativum* were obtained from fresh local produce. The aqueous *A. sativum* extract was prepared by base on the method by Thomson *et al.*, (2007) [26] and Hfaiedh *et al.*, (2011) [11]. Thirty grams (30 g) of *A. sativum* bulbs were peeled off and cleaned. The samples were minced into smaller pieces and homogenized in 70 ml of ice-cold saline (300 mM NaCl) for 15 min in a blender at high speed using 30 seconds burst spaced by cooling intervals to avoid heating of the mixture. The homogenized mixture was then filtered 3 times using cheesecloth. The filtrate was centrifuged at 2000 g for 10 minutes and the product, which is the clear supernatant, was diluted to 100 ml with normal saline. The aqueous *A. sativum* extract was stored in small aliquots at -20 °C until further use. One milliliter (1 ml) of this extract contains 300 mg of pure *A. sativum*.

2.2 Preparation of formalin

A commercially available (R & M Marketing, Essex, UK) 37 % formaldehyde solution was further diluted in isotonic saline. In this study, 4 % formaldehyde or 10 % buffered formalin is prepared by adding 100 ml of 40 % formaldehyde to 900 ml distilled water with 4 g sodium phosphatase, monobasic and 6.5 g sodium phosphate, dibasic (anhydrous).

2.3 Animal Preparation and Exposure

We obtained approval from our Institution's Research and Ethics Committee. The usage of animals for this project has been approved by the Institution's Animal Ethics Committee. Male Wistar rats, weighted approximately 200 - 250 g and averaging at 12 weeks old were used in this study. They were housed in polypropylene cages in an air-conditioned room at 25 ± 3 °C, relative humidity of 50 ± 5 °C and 12 hours alternating light and dark cycles. The rats were provided with chow diet and drinking water *ad libitum*. All animals were received human care according to the criteria outline in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Twenty male Wistar rats were divided into 4 groups. Group 1: control (received neither 10 % formalin exposure nor 250 mg/kg aqueous extract of *A. sativum*), Group 2: exposed to 10 % formalin only, Group 3: exposed to 10 % formalin and received 250 mg/kg aqueous extract of *A. sativum* orally, and Group 4: received orally 250 mg/kg aqueous extract of *A. sativum* only. Exposure to 10 % formalin was conducted for a period of 8 weeks, 4 hours/day, 5 days a week in order to induce oxidative stress in the rats [30], while oral supplementation of 250 mg/kg aqueous extract of *A. sativum* was conducted for 4 weeks [19].

The room temperature was maintained at 25 °C \pm 5 °C, relative humidity at 47 % - 55 % and airflow was adjusted at 1.2 m³/ min. The animals were routinely checked for visible sign of toxicity, such as behavior alteration, physical appearance, breathing pattern, and locomotors activity. Animals were weighted every day, at which time they will also be given complete physical examination. After exposure or oral supplementation, rats were sacrificed via diethyl ether overdose and liver was harvested for biochemical analysis.

2.4 Biochemical Analysis

The harvested liver of each rat were weighted and divided equally for biochemical analysis. Each small pieces of liver was immediately frozen in liquid nitrogen to stop any biochemical reaction in the organ and subsequently was subjected for malondialdehyde (MDA) assay [16], superoxide dismutase (SOD) assay [5], catalase (CAT) assay [1] and protein estimation [12].

2.4.1 Lipid Peroxidation of Malondialdehyde (MDA)

Lipid peroxidation in the liver upon FA exposure was determined according to the method of Ledwozyw *et al.*, (1986) [16]. This method determines the MDA, which is a stable peroxidation product by measuring the reaction

products with thiobarbituric acid (TBA). This reaction of MDA-TBA complex produced a pink color pigment and extracted with n-butanol, estimated by the absorbance at 532 nm using spectrophotometer.

A total of 0.2 g liver tissue sample was weighted and homogenized by using homogenizer. This process was done in low temperature to reduce in vitro oxidation. The sample was placed in a test tube and 0.8 ml distilled water was added (4 times the weight of sample). The homogenates was centrifuged for 10 minutes at 3000 rpm in 4 °C. The supernatant was collected for MDA and protein determination.

MDA standard curve was prepared every time the MDA assay for sample was determined. Standard was prepared using 1,1,3,3-tetraethoxypropane (malondialdehyde tetraethyl acetate). A total of seven MDA concentrations were used including 0, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 nmol/ ml.

A total of 0.1 ml standard or sample was diluted by adding 0.4 ml distilled water. A total of 2.5 ml 1.22 M trichloroacetic acid in 37 % HCl (TCA/ HCl) was added and vortexed in both standard and sample. For the sample, the test tube was incubated for 15 minutes in room temperature after the addition of TCA/ HCl. A total of 1.5 ml of thiobarbituric acid solution (0.67 %) in 0.05 M NaOH (TBA/ NaOH) was added into the standard or sample, vortexed, and thereafter was heated for 30 minutes in a boiling water bath (100 °C). After cooled to room temperature, 4 ml of n-butanol was added. Next, the mixture was shaken vigorously for 3 min and further subjected for centrifugation at 3000 rpm and 10 minutes. The organic layer was collected and its absorbance was measured at 532 nm. The n-butanol was used as a blank.

2.4.2 Superoxide Dismutase (SOD)

SOD activity was assayed according to the method by Beyer and Fridovich (1987) [5]. In this assay, the sensitizing dye (riboflavin) is activated by a photon, producing an excited state which oxidizes some electron donor such as L-methionine. The dye is reduced to a semiquinone which reduced oxygen (O_2) to superoxide anion ($O_2^{\cdot-}$), and turns nitro blue tetrazolium (NBT) to an insoluble purple formazan with the absorbance at 560 nm.

A total of 1.0 ml substrate mixture (a mixture of L-methionine (9.9×10^{-3} M), NBT (5.7×10^{-5} M) and triton X-100 (2.5×10^{-2} %) in the phosphate buffer solution (50 mM, pH 7.8)) was pipetted into a test tube and 0.02 ml sample was added. For the blank, the sample was replaced with phosphate buffer. Next, 10 μ l riboflavin was added into the mixture and mixed evenly. The mixture was placed in the aluminium box illuminated by 18-watt fluorescent lamps (Sylvania arolux)

for seven minutes. After that, sample absorption was determined using spectrophotometer at 560 nm.

2.4.3 Catalase (CAT)

CAT activity was estimated by following the method by Aebi (1984) [1]. The principle of CAT activity is based on the UV absorption of hydrogen peroxide at 240 nm, where the absorption decreases with time. The enzyme activity was calculated from the decrease in absorbance.

A total of 0.2 ml of sample was weighted and 0.8 ml of potassium chloride (KCl) was added before homogenization process. The sample was centrifuged at 2000 rpm, for 20 minutes, in well maintain temperature, 4 °C. The supernatant was taken and further centrifuged using microcentrifuge at 14 000 rpm, 20 minutes, in 4 °C. About 0.025 ml of the resultant supernatant was diluted with 10.0 ml phosphate buffer (PBS). The solution was mixed gently with a vortex. A total of 2 ml of the solution was taken and 1 ml of distilled water was added. The solution was mixed gently using a plastic pipette. For the blank, 1.0 ml hydrogen peroxide (H_2O_2) was replaced with PBS. The decrease in the absorption of the mixture at the temperature of 37 °C towards the blank containing 2.0 ml of sample solution and 1.0 ml phosphate buffer for 30 seconds at 240 nm wavelength was recorded.

2.4.4 Protein Assay

The estimation of protein was estimated following the method by Janairo *et al.*, (2011) [12]. The principle of protein estimation is based on the blue color resulted from the chemical interaction, indicating the presence of proteins. The chemical interaction was analyzed by spectrophotometer at 540 nm.

Protein standard curve was prepared following different bovine serum albumin (BSA) concentration. Distilled water and BSA was pipette out into a series of test tubes. The solutions were mixed well by vortex and incubated for 20 minutes. Changes of the color in the solutions were observed and the absorbance of each tube was read against the blank at 545 nm using spectrophotometer. Standard graph was drawn and the amount of protein in the sample was determined.

2.5 Statistical Analysis

All data were expressed as mean \pm standard error of mean (SEM) and was analyzed by using the Statistical Package for the Social Sciences software (SPSS, version 13.0). The statistical method of two-way analysis of variance (ANOVA) was used to compare the mean values among all 4 different groups followed by Dunnett's T3 for multiple comparisons.

All differences were considered as significant when the p-value is $p < 0.05$.

3.0 Results

3.1 Malondialdehyde Concentration

Significant increased of MDA level (Figure 1) was seen in Group 2 (Exposed to 10 % formalin only) (0.57 ± 0.03 nmol/mg protein) as compared to Group 1 (Control) (0.15 ± 0.04 nmol/mg protein). However, significant increased in MDA level was also noted in Group 3 (Exposed to 10 % formalin and fed with 250 mg/kg aqueous extract of *A. sativum*) (0.54 ± 0.02 nmol/mg protein) and Group 4 (fed with 250 mg/kg aqueous extract of *A. sativum* only) (0.33 ± 0.02 nmol/mg protein).

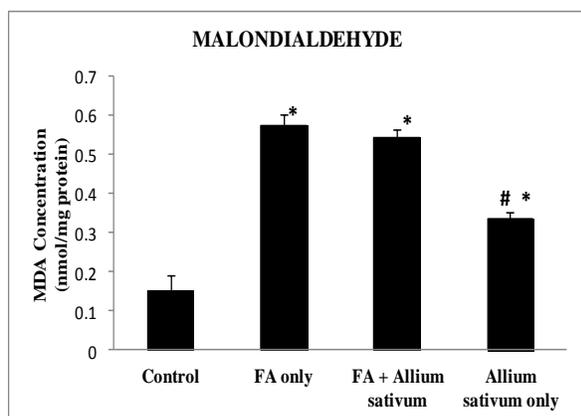


Fig.1 Level of MDA in all experimental groups. There are significant increased of MDA level was seen in Group 2 (Exposed to 10 % Formalin only) (0.57 ± 0.03 nmol/mg protein) as compared to Group 1 (Control) (0.15 ± 0.04 nmol/mg protein). Significant increased was also noted in Group 3 (Exposed to 10 % Formalin and Received 250 mg/kg aqueous extract of *A. sativum*) (0.54 ± 0.02 nmol/mg protein) and Group 4 (Received 250 mg/kg aqueous extract of *A. sativum* only) (0.33 ± 0.02 nmol/mg protein) comparable to control group.

3.2 Antioxidant Activity

After being fed with aqueous extract of *A. sativum* for 4 weeks, significant increased in SOD activity (Figure 2) was observed in Group 3 (4.92 ± 0.16 U/mg protein) and Group 4 (6.30 ± 0.35 U/mg protein) when compared to control group (2.59 ± 0.13 U/mg protein). SOD activity in Group 2 was also increased (3.76 ± 0.37 U/mg protein) when compared to control (2.59 ± 0.13 U/mg protein) with $p > 0.05$.

In contrast to SOD activity, there is no significant increase or decrease in CAT activity seen in all experimental groups (Figure 3). Level of CAT was maintain at 111.07 ± 5.36 μ mol decomposition of H_2O_2 /min/mg protein for Group 2, 107.50 ± 5.54 μ mol decomposition of H_2O_2 /min/mg protein for Group 3 and 124.47 ± 4.69 μ mol decomposition of

H_2O_2 /min/mg protein for Group 4 when compare to Group 1 (130.22 ± 6.10 μ mol decomposition of H_2O_2 /min/mg protein) with $p > 0.05$.

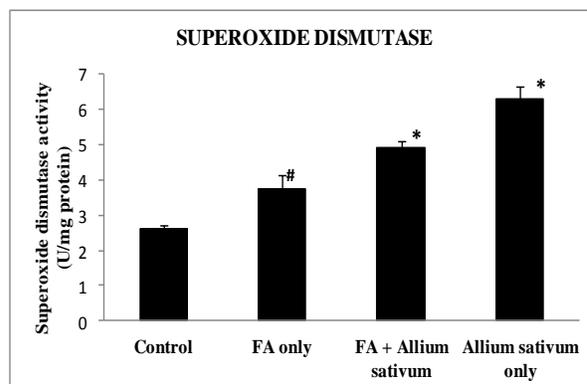


Fig.2 SOD activities in all experimental groups. Supplementation of aqueous extract of *A. sativum* significantly increased SOD activity in Group 3 (4.92 ± 0.16 U/mg protein) and Group 4 (6.30 ± 0.35 U/mg protein) when compared to control group (2.59 ± 0.13 U/mg protein). High level of SOD activity was also noted in Group 2 (3.76 ± 0.37 U/mg protein) when compared to Group 1 (2.59 ± 0.13 U/mg protein) with $p < 0.05$.

4.0 Discussion

This present study was conducted to determine the antioxidant activities of *A. sativum* towards formalin-induced oxidative stress in rat liver. Study by Gopalipour *et al.*, (2007) [7] concluded that the mean vapors concentration of dissection room is 1.5 ppm of formaldehyde, which is equal to 10 % formalin are able to induced oxidative stress in rat testis and that concentration was used in this study. Recommended dose of *A. sativum* extract (250 mg/kg body weight) was used to treat oxidative stress in rat liver [4].

Physical examination on rats' fur showed changes in color from its natural color, white to yellow as early as day 3 of 10 % formalin exposure (results not shown). Kerns *et al.*, (1983) [14] have proved that long-term inhalation exposure of formaldehyde produced a concentration-dependent increase in yellow discoloration of the rat hair coat. However, the hair discoloration is reversible when the exposure is discontinued. This study revealed an increase of MDA level after exposure to 10 % formalin for 8 weeks, indicating the occurrence of lipid peroxidation in the liver. Teng *et al.*, (2001) [25] reported that the addition of 4mM formaldehyde to hepatocytes resulted in generation of reactive oxygen species (ROS) and induced lipid peroxidation in dose- and time-dependent manner. This also proved that formaldehyde disturbs the oxidant-antioxidant balance in various tissue and cause oxidative stress in parallel with tissue damage [20]. Sogut *et al.*, (2004) [24] also found that MDA level of group exposed to formaldehyde (10 ppm and 20 ppm) were higher

compared to control group. Kum et al., (2007) [15] stated that the increased level of MDA might dependent on the increased production of enzymes utilized in the detoxification of formaldehyde.

MDA level was also noted higher in Group 4 which only received 250 mg/kg aqueous extract of *A. sativum* for 4 weeks. This observation showed that supplementation of *A. sativum* increased lipid peroxidation. According to Joseph *et al.*, in 1989 [13], aqueous extract of *A. sativum* at concentration of 200 gm/l in drinking water consumed for 10 days exhibited significantly higher levels of aspartate aminotransferase (AST) due to liver injury. Feeding of allicin (100 mg/kg/day) for 15 days in rats increased the activity of liver lipase and alpha glucal phosphorylase and decreased glucose-6-phosphatase activity [3]. Moreover, Chen *et al.*, (1999) [6] has reported that treatment of rats with fresh *A. sativum* homogenate for 7 days caused a significant decrease in liver catalase activity in doses of 2 and 4 gm/kg. Additionally, supplementation with diallyl sulfide (DAS), a flavor compound derived from *A. sativum*, at dose 200 mg/kg for 8 days, causing the hepatic catalase activity to decrease by 95 %.

Significant increased in MDA level was also seen in Group 3 where the rats were supplemented with 250 mg/kg aqueous extract of *A. sativum* after exposed to 10 % formalin when compare to control group. This finding showed that supplementation of *A. sativum* are unable to reduce oxidative stress resulted from inhalation of 10 % formalin, although there is study claimed that *A. sativum* possess antioxidant properties which helps to reduce oxidative stress in rat liver [23].

High SOD values in formaldehyde exposed rats may be due a response of increase oxidative stress in the liver tissue [20]. The similar finding was seen in the present study whereby the SOD level was increased after exposure to 10 % formalin when compare to control group, although it is not statistically significant. This may be due to liver's SOD activities in adult rats remain unchanged as the rat have compensated with the formaldehyde exposure [20]. Previously, Banerjee *et al.*, (2001) [4] showed that *A. sativum* intake at 250 mg/kg/day for 30 days caused an increased in endogenous antioxidant of SOD in liver and kidney, but higher doses (1000 mg/kg) have been shown to be toxic to the heart, liver and kidney. Thus, administration of *A. sativum* at dose of 250 mg/kg will restore the altered levels of SOD antioxidant molecules, which confirm that *A. sativum* contains antioxidant compounds and protects tissues against formaldehyde-induced oxidative stress in rat liver, but not in higher dose. Comparable with this result is in a group of rats which only received *A. sativum* for 4 weeks. SOD activity was seen higher as compared to control group. Similarly, Young-Min *et al.*, (2009) [29] showed that

SOD activity in mice liver is elevated upon receiving diet containing garlic and aged black garlic for 7 weeks. However this is shown in *A. sativum* that was administered orally only, whereas intraperitoneal injection of *A. sativum* showed just an opposite effect [10].

Increase in catalase activity may be indicative of the high degree oxidative stress due to elevated endogenous hydrogen peroxide (H_2O_2) or an adaptive response to oxidative stress induced by formaldehyde [20]. In the present study, exposure to 10% formalin did not significantly increase the CAT activity. This contrast result obtained may be due to formalin did not reduce the antioxidant level of the liver cells that cause the oxidative damage to occur but further increase the CAT activity in order to balance the toxic effect. Kum *et al.*, (2007) [15] reported that liver catalase activities in adult rats remain unchanged because the rats have compensated with the formaldehyde exposure. Gulec *et al.*, (2006) [9] also showed that CAT activity was significantly decreased in the liver tissues of formaldehyde-injected rats after 10 days of intoxication. Moreover, there is no significant increase was seen in CAT activity in group of rats supplemented with *A. sativum* only. There might be two possibilities, one, the CAT activity in control group might have reached the maximum limit. Thus, supplementation of *A. sativum* will no longer exceeded the level as shown in control group as it is the maximum limit for CAT activity in rat. Two, in different study, male Sprague-Dawley rats treated with *A. sativum* at daily doses of 50 or 200 mg/kg for 8 days and 29 days caused the hepatic catalase activity to decrease [6]. The dose used in this study might cause a toxic effect towards the liver cell, subsequently, leads to alteration in hepatocytes structure and function.

No significant increase in CAT activity was noted in group of rats supplemented with *A. sativum* upon exposure to 10 % formalin. We believed hydrogen peroxide (H_2O_2) produced from the action of superoxide dismutase (SOD) have been detoxified completely by glutathione peroxidase (GPx) in the liver. Other than CAT, metabolism of H_2O_2 in a body was also taken up by GPx. Thus, in this case, the catalase activity may was not significantly needed. CAT and GPx are an enzyme that causes direct breakdown of hydrogen peroxide produced by SOD to oxygen and water [9]. This indicates that CAT, GPx and SOD working together in the body's defense system against oxidative stress. Banerjee *et al.*, (2001) [4] also concluded that chronic *A. sativum* intake at low dose has the potential to offer direct cytoprotective effects in the event of any oxidant stress but exert significant cellular damage at higher doses. Thus, it is important to recognize the fact that *A. sativum* may not be safe if taken in excess.

Looking at different angle of antioxidant actions, formaldehyde is metabolized by formaldehyde dehydrogenase

(FDH) and this enzyme is dependent on glutathione. Glutathione is a major non enzymatic antioxidant which is found abundant in the body. It is also important for cellular protection by detoxifying free radicals, resulted from FA. This might be also a possible explanation why the level of CAT was not significantly changed.

5.0 Conclusion

Inhalation of 10% formalin for 8 weeks is able to induced oxidative stress in rat liver. Supplementation of 250 mg/kg/day of *A. sativum* for 28 days also lead to further increased the level of malondialdehyde (MDA) in rat liver but has a potential to enhance the endogenous SOD antioxidant status and could be useful in preventing formaldehyde-induced oxidative stress.

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