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Infectious Diseases Research 2022;3(2):12. https://doi.org/10.53388/IDR20220525012

Malondialdehyde and superoxide dismutase levels of dogs with complex dermatitis after trigona honey therapy

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Competing interests
The authors declare no conflicts of interest.

Abbreviations
ROS, reactive oxygen compounds; MDA, malondialdehyde; SOD, superoxide dismutase; PUFA, Polyunsaturated fatty acid; ROS-RNS; reactive oxygen and nitrogen species.

Citation
Purwanti NLL, Suartha IN, Suarsana IN, Jayanti PD. Malondialdehyde and superoxide dismutase levels of dogs with complex dermatitis after trigona honey therapy. *Infect Dis Res*. 2022;3(2):12. doi: 10.53388/IDR20220525012.

Executive editor: Na Liu.
Received: 24 March 2022; **Accepted:** 23 May 2022; **Available online:** 25 May 2022.

Abstract
Dermatitis is a combination of various skin disease agents such as bacteria, fungi, ectoparasites, viruses and metabolic diseases with various clinical symptoms or signs. Dermatitis can cause an imbalance between ROS and antioxidants. Exogenous antioxidants are needed to help balance antioxidant and ROS levels in the body by providing trigona honey. This study aims to determine the MDA and SOD levels of dogs with complex dermatitis after administration of trigona honey. This study used 12 dogs with complex dermatitis, with a moderate severity score. Dogs 4-6 months old. Samples were divided into control group (P_0) without honey, fresh honey group (P_1) 5 mL/head/day and capsule honey group (P_2) 0.1 mg/head/day. Honey was given for 4 weeks in the treatment group. Examination of MDA and SOD levels was carried out using the ELISA kit. The results showed that the Trigona honey treatment had a significant effect ($P < 0.05$) in reducing MDA levels and increasing SOD levels in dogs with complex dermatitis. Duncan's test results showed that SOD levels increased significantly at week 4, while week 2 and week 0 did not increase significantly. Trigona honey has anti-inflammatory, antibacterial, antiaging, antiulcer effects, helps in wound healing dermatitis and is an antioxidant. So that trigona honey can reduce plasma MDA levels and increase plasma SOD levels in dogs with complex dermatitis.

Keywords: MDA levels, SOD levels, complex dermatitis, trigona honey

Introduction

Complex dermatitis is a combination of various skin disease agents such as bacteria, fungi, ectoparasites, viruses and metabolic diseases with various clinical symptoms or signs that appear [1]. The inflammatory process in dermatitis is a change in NADPH to NADP with a NADPH oxidase catalyst. In this process O_2 leakage occurs which then turns into superoxide radicals ($^{\bullet}O_2$) which can stimulate the formation of proinflammatory cytokines such as IL-6, granulocyte-colony stimulating factor (G-CSF), IL-1, IL-13, IL-17, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) which can produce excess reactive oxygen and nitrogen species (ROS-RNS) in the body, so that the balance between oxidants and antioxidants is disturbed [2]. The state of imbalance between ROS and antioxidants is known as oxidative stress [3].

Naturally the body has the ability to counteract free radicals by forming endogenous antioxidants produced by the body whose levels can be measured through superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase [4]. One indicator for determining oxidative stress is the level of malondialdehyde (MDA) [5]. Malondialdehyde is the final product oxidation of unsaturated fatty acids by free radicals and the result of cell metabolites components produced by free radicals [6].

Trigona honey is produced by bees of the *Trigona sp.* species and is used as a natural medicine [7]. The active ingredients in honey are alkaloids, flavonoids, phenolics, steroids and terpenoids. Previous studies on in vitro wound healing using honey showed good performance results, especially multifloral honey, compared to controls without honey. In honey, pinocembrin was identified which functions as an antimicrobial and the main mechanism involved in wound healing [8]. Suartha et al. reported that dogs with dermatitis given trigona honey orally were able to repair skin damage in dermatitis lesions. Seen from the decrease in symptoms of erythema and early hair growth at the site of the alopecia lesion. Histologically there was a decrease in the number of inflammatory cells [9]. Febriyanti et al. reported that honey had great effectiveness in accelerating the healing of burns and cuts when compared to the non-treated group with honey. Honey stimulates the immune system by stimulating B lymphocytes and T lymphocytes and activating neutrophils, supplying glucose for respiration and macrophage production [10].

There have been no reports of MDA and SOD levels in dogs with complex dermatitis after administration of trigona honey. Therefore, further research was conducted to determine the levels of antioxidant biomarkers in dogs with complex dermatitis.

Materials and Methods

Ethical approval

The research was accepted in the Animal Ethics Committee of the Faculty of Veterinary Medicine, Udayana University with certificate number B/81/UN14.2.9/PT.01.04/2021.

Sample Preparation

The study used 12 mongorel dogs [11], with complex dermatitis aged 4-6 months in Denpasar-Bali with moderate severity scores.

The sample size was calculated using the Festing formula [11];

E = Total number of samples – Total number of groups

E = (n × t) – t

E = (5 × 3) – 3

E = 12

Note: the range value (E) to be achieved is between 10-20, the number of samples for each group (n), the total number of treatment groups (t).

Microscopic examination of dermatitis was carried out using the deep skin scraping method, culture with Saburent Dextrose Agar (SDA) for fungal examination and bacterial culture examination with Blood Agar (BA). The results of deep skin scraping were positive for the parasitic mite *Sarcoptes scabei* sp in two dogs. Twelve dogs had complex dermatitis due to the fungus *Candida* sp. and *Staphylococcus* sp. after the lactophenol blue staining method. So that it is said to be a complex dermatitis from a combination of skin disease causes. The severity of the score using the scoring procedure Cahyaniarta, et al. [12] Observations of lesion changes were carried out starting from the head, body and tail. Then given a score of light (1.0; pink lesion color), moderate (2.0; pale red lesion color) and severe (3.0; red lesion color). Observation of lesion severity (wound depth, skin color, scab peeling and wound surface size), type of lesion (primary or secondary), spread of lesion.

Then the sample was divided into two dogs without honey treatment (P_0). Five dogs were given fresh trigona honey (P_1) 5 mL/dog/day and the other five were given honey capsules (P_2) 0.1 mg/dog/day. Honey is given orally for 4 weeks. Prior to blood sampling, all dogs were acclimatized for a week. The first blood sample before administration of trigona honey (week 0) was taken as much as 1.5 mL through the cephalic vein, then after giving trigona honey blood collection was carried out in the 2nd and 4th week. Blood samples were stored in EDTA tubes and centrifuged at 3,000 rpm for 15 minutes to separate blood plasma.

Procedure of Malondialdehyde and Superoxide Dismutase levels

Procedure of MDA and SOD levels using the Bioassay Technology Laboratory ELISA KIT. The procedure for checking MDA and SOD levels using the BT Laboratory is almost the same, only the difference is in anti-MDA and anti-SOD antibodies. The test procedure is to first prepare all standard solution reagents and samples and prepare them at room temperature. Second, 50 μL of standard solution (S5-S1) was added to each well. Next, 50 μL of standard diluents (as a blank) was added to the well. Add 40 μL of sample solution into each well. A total of 10 μL of anti-MDA or anti-SOD antibody was then added to each sample well, and 50 μL of Streptavidin-HRP was added to each standard well and sample (not control blank), homogenized by tapping by hand. The microplate was then covered with a sealer and incubated at 37°C for 60 minutes. Third, remove the sealer and the liquid in the well is removed by turning the well over in the sink. The wells were dried by means of the wells facing downwards and tapping on a tissue towel, then 350 μL of wash buffer was added to each well and allowed to stand for 30 seconds. This procedure was repeated 4 times (5 washes in total). 50 μL of substrate A was pipetted into all wells and then 50 μL of substrate B was added. This mixture was then homogenized and incubated for 10 minutes at 37°C in a dark room. Fourth, 50 μL of stop solution was added to all wells. Finally, the optical density reading at a wavelength of 450 nm on an ELISA reader was then carried out to determine the MDA and SOD levels.

Data Analysis

The data were analyzed using the SPSS 25.0 for windows program. Statistical test using ANOVA. If the treatment has a significant effect, then the test is continued with Duncan's test at a level of 5% to determine the effect between treatments.

Result

Malondialdehyde levels

Based on the table below (Table 1), the highest average MDA level was in the control group (P_0) at week 4 of 1.489 ± 0.011 nmol/mL and the average MDA level tended to increase every week. The lowest average MDA level was in the fresh honey group (P_1) 4th week of 0.863 ± 0.234 nmol/mL. The average MDA levels of the fresh honey (P_1) and capsule honey (P_2) groups tended to decrease every week compared to the control (P_0), although in the table it increased at the 2nd week and decreased at the 4th week. This value is still below the average MDA level of the control group (P_0).

The results of statistical analysis of variance showed that the treatment of trigona honey had a significant ($P < 0.05$) effect on reducing MDA (malondialdehyde) levels in dogs with complex dermatitis. Meanwhile, each week of observation was not significantly different ($P > 0.05$) in the decrease in MDA levels in dogs with

complex dermatitis. The Duncan test results on the MDA levels of dogs with dermatitis after administration of trigona honey showed that the control group (P_0) had higher MDA levels than the fresh honey (P_1) and capsule honey (P_2), while the fresh honey (P_1) and capsule honey treatment groups (P_2) the MDA level is lower.

Superoxide dismutase

Based on the table below (Table 2), the highest average SOD level was in the capsule honey treatment group (P_2) at week 4 of 1.611 ± 0.166 ng/mL then followed by fresh honey treatment (P_1) of 1.454 ng/mL. The lowest average SOD level was in the capsule honey group (P_2) the 1st week of 0.328 ± 0.161 ng/mL. The average SOD levels of the fresh honey (P_1) and capsule honey (P_2) groups tended to increase every week compared to the control group (P_0), although the control group increased slightly in the 4th week. But this value is still below the average of the honey treatment group.

The results of statistical analysis of variance showed that the Trigona honey treatment had a significant ($P < 0.05$) effect on increasing SOD levels in dogs with complex dermatitis. Meanwhile, each week of observation had a significant effect ($P < 0.05$). There was a significant interaction between the treatment group and the week of observation on increasing SOD levels in dogs with complex dermatitis. The Duncan test results on the SOD levels of dogs with dermatitis after administration of trigona honey showed that the control group (P_0) had lower SOD levels than the fresh honey (P_1) and capsule honey (P_2).

The picture below is a high-low chart, the upper limit is marked by a red line (0.328-1.611) and the lower limit is marked by a blue line (0-0.328). If the upper limit intersects with the lower limit, the other high-low images show no significant difference ($P > 0.05$), otherwise if they do not intersect, they show a significant difference ($P < 0.05$). Due to the interaction between treatment and week, the Duncan test was continued along with a high-low graph. From the picture above, the 4th week of SOD levels were higher than the 2nd week and 0th week, while the 2nd week to week 0 had lower SOD levels (Figure 1). Figure 2 and Figure 3 are the results of histopathological images after examination of SOD and MDA levels in dermatitis dogs to provide a microscopic picture of the skin before and after giving honey. Then in Figure 4 is a picture of the development before and after giving Trigona honey.

Table 1 The mean of MDA levels in dogs with complex dermatitis

Treatment	The mean MDA levels (nmol/mL) ($\bar{x}$ + SD)		
	Zero Week	2nd Week	4th Week
Control (P_0)	1,123 ± 0,007^{b,12}	1,463 ± 0,012^{b,2}	1,489 ± 0,011^{b,1}
Fresh honey (P_1)	1,135 ± 0,458^{a,12}	1,281 ± 0,144^{a,2}	0,863 ± 0,234^{a,1}
Honey capsules (P_2)	1,055 ± 0,256^{a,12}	1,258 ± 0,228^{a,2}	0,923 ± 0,042^{a,1}

Note: $\bar{x}$ = mean MDA levels; SD = standard deviation, superscripts with different letters in one column showed significant differences, superscripts with different numbers in one row showed significant differences.

Table 2 The mean of SOD levels in dogs with complex dermatitis

Treatment	The Mean SOD levels (ng/mL) ($\bar{x}$ + SD)		
	Zero Week	2nd Week	Zero Week
Control (P_0)	0,481 ± 0,015^{a,1}	0,448 ± 0,009^{a,1}	0,704 ± 0,043^{a,2}
Fresh honey (P_1)	0,417 ± 0,061^{b,1}	0,426 ± 0,071^{b,1}	1,454 ± 0,086^{b,2}
Honey capsules (P_2)	0,328 ± 0,161^{b,1}	0,401 ± 0,083^{b,1}	1,611 ± 0,166^{b,2}

Note: $\bar{x}$ = the mean SOD levels, SD = standard deviation, superscripts with different letters in one column showed significant differences, superscripts with different numbers in one row showed significant differences.

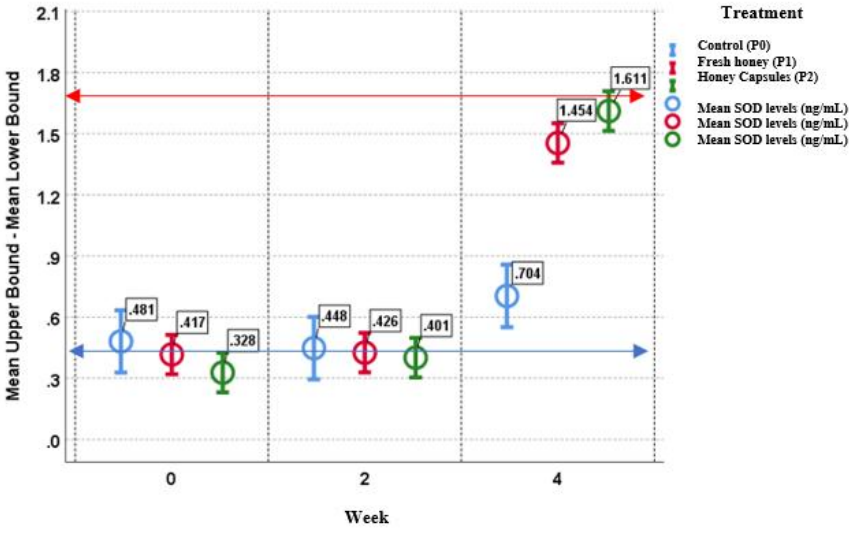

Figure 1 Chart of high-low sod levels in dogs with complex dermatitis for each treatment group

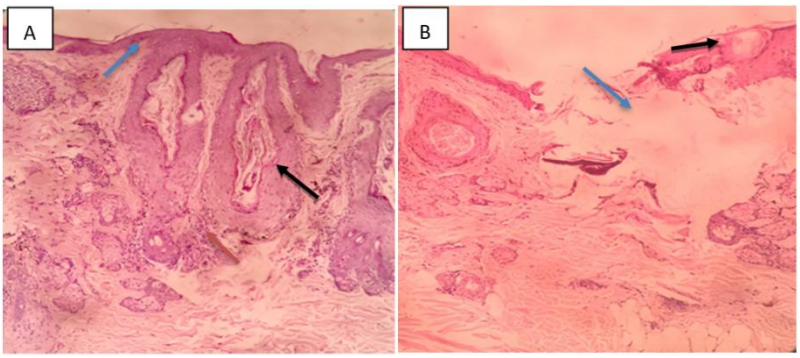

Figure 2 Histopatology result of day 0. (A). There is a Erotion (Blue Arrow) inflammatory cell infiltration (red arrow) and acanthosis (black arrow). (B). The presence of segments of *Sarcoptes scabiei* sp. (black arrow), erosion of the epidermis and dermis (blue arrow)

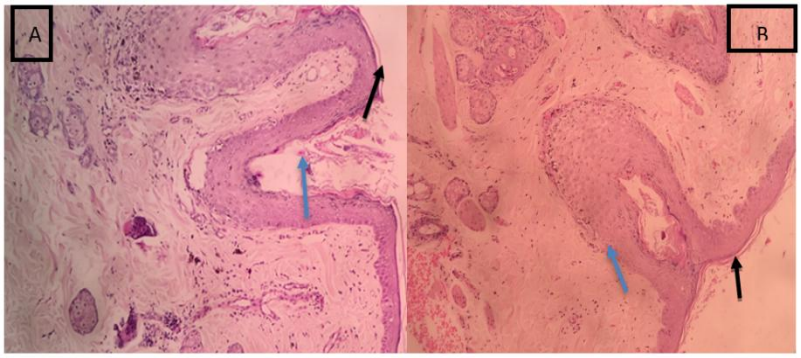

Figure 3 Histopathological results of sample on day 7. (A). presence of debris (blue arrows), the stratum corneum begins to form (black arrows). (B). Histopathological results of sample B1 on the 15th day of the stratum corneum began to close (black arrow), but there was still acanthosis (blue arrow)

Figure 4 Dog with complex dermatitis before being treated with trigona honey (A) and a picture of a dog experiencing dermatitis after being treated with trigona honey (B)

Discussion

Malondialdehyde levels

Malondialdehyde (MDA) is a dialdehyde compound which is the end product of lipid peroxidation in the body, through enzymatic or non-enzymatic processes. MDA is a stable and accurate measurement component of lipid peroxidation, and has helped to explain the role of oxidative stress in several diseases including dermatitis in dogs that play a role in the process of inflammation [13]. Malondialdehyde (MDA) was used to measure the level of oxidative stress in animal cells with dermatitis [14]. Under conditions of oxidative stress, MDA levels will be higher than the normal average [15].

The results showed that the control group in the 4th week had higher MDA levels on average than the fresh honey and capsule honey treatment groups. This indicates that trigona honey can reduce free radicals in the body. MDA levels decreased because vitamin E in honey protects PUFA (Polyunsaturated fatty acid) in the membrane to function optimally because PUFA is a phospholipid and glycolipid content in membranes that are prone to free radicals [16]. Flavonoids act as free radical scavengers because they contain hydroxyl groups [17]. Vitamin C in honey captures $^{*}O_2$, $^{\cdot *}OH$, peroxyl and singlet oxygen radicals so that it can protect membranes and LDL from

peroxidative damage [18].

Trigona honey treatment had a significant effect ($P < 0.05$) on reducing MDA levels in dogs with complex dermatitis, both given fresh honey (P_1) and honey in capsule form (P_2). The treatment of the formula from honey that was formed fresh and after being made into a capsule did not reduce the antioxidant properties of trigona honey. Giving honey to rats with wounds was also able to reduce MDA levels and reduce the wound area of rats with diabetes [19]. Previous studies reported higher MDA levels in dogs with dermatitis compared to a healthy control group without dermatitis [20].

The week of observation had no significant effect ($P > 0.05$) on the decrease in MDA levels in dogs with complex dermatitis. This means that every week the observation of the decrease in MDA levels is not stable from week 0 to week 2 and week 4. This happened because the initial administration of honey was considered a foreign object by the body's immune system so that MDA levels at the beginning of the inflammatory phase were high from week 0. Neutrophils and inflammatory cells when ingesting bacteria undergo reactions in the mitochondria involving NADPH oxidase causing a respiratory burst that produces large amounts of free radical oxygen derivatives such as $^{\cdot}O_2$, H_2O_2, $^{\cdot}OH$. The proliferative phase (from day 4 to day 21) and the remodeling phase (from day 21 to optimal recovery) involve many fibroblast cells undergoing biological oxidation reactions that generate free radicals. Free radicals will initiate lipid peroxidation to produce MDA in wound tissue [21, 22]. According to Yuslianti et al [19] decreased levels of free radicals in wound tissue during the healing process, especially during the remodeling phase. This causes the average MDA level in the level of the control group 2nd week to tend to increase but the value is still below the MDA.

Superoxide dismutase

Superoxide dismutase (SOD) is an enzyme that functions to catalyze the efficient removal of superoxide anions and is an endogenous antioxidant [23]. Naturally, the body has endogenous antioxidants or enzymatic antioxidants to fight free radicals that may not function in the balance of body functions. The enzyme superoxide dismutase (SOD) is the first line of defense against the activation of reactive oxygen compounds (ROS). Superoxide dismutase (SOD) was used to measure the level of oxidative stress in dogs with dermatitis [20]. Under conditions of oxidative stress, the SOD enzymatic system decreased from the normal average [24].

The results showed that the 4th week of capsule honey (P_2) and fresh honey (P_1) treatment groups had a higher average SOD level than the control group. This indicates that trigona honey can increase levels of the antioxidant enzyme SOD. Honey contains flavonoid compounds (pinocembrin, apigenin, hesperitin, chrysin, quercetin, luteolin, myricetin, pinobanksin, galangin, kaempferol), beta carotene, vitamins A, B_1, B_2, B_3, B_5, B_6, C, D, E, K, phenolic acids (chlorogenic, ferulic, caffeic, ellagic, vanillic, benzoic, cinnamic, coumaric acids), uric acid and minerals Fe, S, Mg, P, Cl which can act as antioxidants [25].

The mechanism of honey in increasing SOD levels is the presence of flavonoid compounds that can work as inducers, thereby triggering the expression of antioxidant genes through the activation mechanism of Nrf_2 as the hormone responsible for the synthesis of SOD enzymes [26]. SOD synthesis will increase due to NrF_2 activation along with increasing SOD levels. In other words, NrF_2 activation can help increase the supply of SOD to meet the high demand for SOD due to oxidative stress [27]. The phenol content in honey is very effective as an antidote to peroxyl radicals and has the ability as a reducing agent [28].

The incidence of dermatitis allows a relationship between an imbalance of oxidants or antioxidants and the production of free radicals due to the inflammatory response so that it can cause oxidative stress in animals with complex dermatitis or single dermatitis [20]. Dogs with dermatitis undergo the process of converting NADPH to NADP with the catalyst of NADPH oxidase. In this process, O_2 leakage occurs which then turns into superoxide radicals ($^{\cdot}O_2$) which can stimulate the formation of proinflammatory cytokines that can produce excess reactive oxygen and nitrogen species (ROS-RNS) [2].

Trigona honey treatment had a significant effect ($P < 0.05$) on increasing SOD levels in dogs with complex dermatitis, both given fresh honey (P_1) and honey in capsule form (P_2). Meanwhile, each week of observation was significantly different ($P < 0.05$) and there was a significant interaction between the treatment groups and each week of observation on the increase in SOD levels of dogs with complex dermatitis. The treatment of the formula from honey that was formed fresh and after being made into a capsule did not reduce the antioxidant properties of trigona honey. Giving honey to Pb acetate-induced mice was able to increase SOD levels compared to controls [25]. Research Sharma et al. stated that dogs with complex dermatitis had increased SOD activity compared to healthy controls, whereas in relation to the levels of SOD (SOD level) in the dog's body, the antioxidant status of SOD decreased compared to a group of healthy dogs [20]. This opinion is in accordance with several literature studies which say that higher levels of oxidative stress will increase lipid peroxidation markers presented as malondialdehyde (MDA) and decrease SOD enzyme activity [23, 29, 30].

There was a significant interaction between treatment and weeks, then Duncan's test was continued. If the upper boundary (red line) intersects with the lower boundary (blue line) it is not significantly different ($P > 0.05$), otherwise if it does not intersect it shows a significant difference ($P < 0.05$) (Figure 1). The 4th week was significantly different from the 2nd week and 0th week, while the 2nd week to the 0th week was not significantly different. This is associated with an antioxidant mechanism that scavenges free radicals by donating hydrogen atoms. Antioxidants in honey function as a deterrent but the mechanism is carried out by the SOD enzyme [31]. At week 0 with week 2 there was no significant difference in terms of the inflammatory phase in the body, namely on day 0 then the proliferation phase on days 4 to 21. This phase involves many fibroblast cells that undergo biological oxidation reactions that produce radicals. free [22]. So, SOD levels tend to decrease due to the activity of the superoxide dismutase enzyme to counteract the presence of free radicals in the body [32].

Conclusion

Trigona honey has anti-inflammatory, antibacterial, antiaging, antiulcer effects, helps in wound healing dermatitis and is an antioxidant. So that trigona honey can reduce plasma MDA levels and increase plasma SOD levels in dogs with complex dermatitis.

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