Original Article

Potential Neuroprotective Effect of Apis dorsata Honey against Morphine Tolerance: An *in vivo* Study

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Abstract

Background: To determine the effects of Apis dorsata honey on the development of morphine tolerance and oxidative stress in rats.

Materials and Methods: A total of 40 male Sprague Dawley rats were injected (subcutaneous) with 10 mg/kg of morphine following oral administration of A. dorsata honey (0.5, 1.5, and 2.5g/kg). On day 15, the rats were euthanized, and the thalamus, spinal cord, and hippocampus were homogenized to assess iNOS and MDA using ELISA kits.

Results: The honey of A. dorsata significantly prevented morphine tolerance to analgesia in the hotplate test on Day 14 ($p<0.05$). The biochemical assessment showed that A. dorsata honey significantly reduced MDA formation in the brain regions compared to the morphine control group at dose 2.5g/kg. Elevation of iNOS caused by chronic morphine intake was reduced in A. dorsata honey co-treatment.

Conclusion: This study suggests the therapeutic role of A. dorsata honey in preventing morphine tolerance via inhibition of oxidative stress.

Keywords: Morphine tolerance, *A. dorsata* honey, Oxidative stress

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Introduction

Morphine is a commonly used opioid in acute and chronic pain management, such as regional anesthesia and joint pain (1). Although morphine possesses a protective effect against clinical injuries (2), prolonged exposure to chronic morphine treatment can lead to tolerance among patients. The tolerance has limited the patient to receive appropriate treatment, as they have to seek medical advice and monitoring for their increasing morphine doses. Thus, it is pivotal to search for a complementary agent to reduce morphine tolerance and to increase the sustainability of the morphine analgesic effect.

The mechanism of morphine tolerance is related to the mu-opioid receptor (MOR) in the brain (3). Studies showed the role of oxidative stress in the development of morphine tolerance, as morphine itself can act as an oxidative stress-causing agent (4). Increased release of the free radical in response to tolerance led to the destruction of cell defense systems, indicating the elevation of oxidative stress (5-7). In addition, neuronal damages were associated with increased tolerance to the drug (8).

Honey is a natural supersaturated solution of

sugar produced by honeybees. They are used traditionally to treat wounds, sore throats, and stomach ulcers (9, 10). Another study also significantly proves that honey acts as an antioxidant and neuroprotective agent (11). Evidence revealed that phenolic contents in honey have an anti-tolerance effect against morphine (12, 13). Malaysian *A. dorsata* honey has higher phenolic content and antioxidant capacity than other honey types such as *gelam*, Indian forest, and pineapple honey (14). Therefore, this study was conducted to evaluate the effect of *Apis dorsata* honey on morphine-induced tolerance using a hotplate test, as well as its antioxidant potential via biochemical analysis.

Methods

Animal: Animals were maintained in Animal Research Laboratory in Faculty of Medicine, Universiti Sultan Zainal Abidin (UniSZA). 40 Sprague Dawley male rats (200-250g each) were kept in a controlled temperature 22±2˚C and 12 hours light/dark cycle with free access to food and water *ad libitum*. The animals were habituated to the testing environment for at least two weeks before the experiment to adapt to the manipulation and minimize nonspecific stress responses. The Animal Experimentation Ethics Committee approved all experiments performed in this study of UniSZA (ID: UAPREC/16/003).

Drugs and honey: Morphine (10mg/ml in 1 ml ampoule) was purchased from Hameln, Germany, and stored in 4˚C. Ketamin, xylazine, and zoletil were prepared into a cocktail based on the ratio described by Cheah et al. (15). *A. dorsata* honey was collected from *A. dorsata* beehives on Tualang tree (*Koompassia excelsa*) in Pasir Akar, Besut, Terengganu, Malaysia by the Department of Agropolis, Faculty of Biosource and Food Industry, UniSZA.

Experimental group: Treatment group was established from the previous study design by Zakaria et al. (16) which consisted of five group (n=8) namely saline, morphine (10mg/kg) + saline, morphine (10mg/kg) + honey 0.5 g/kg, morphine (10mg/kg) + honey 1.5 g/kg and morphine (10mg/kg) + honey 2.5 g/kg. These group are morphine (10mg/kg) + honey 0.5 g/kg; morphine (10mg/kg) + honey 1.5 g/kg and morphine (10mg/kg) + honey 2.5 g/kg. Toxicity test on *A. dorsata* honey was carried out based on Organization for Economic Co-operation and Development (OECD) guideline for testing the chemicals (17).

Induction of morphine tolerance: Morphine tolerance was induced by injection (subcutaneous) of morphine (10mg/kg) twice daily (9 am and 5 pm) for 14 days (18). A preliminary study showed that this dose produces profound analgesia without causing side effects. Meanwhile, in the saline group, rats received 1ml/kg saline via subcutaneous injection. To determine the effect of *A. dorsata* honey on the development of morphine tolerance, *A. dorsata* honey (0.5, 1.0, and 2.5g/kg) was administered using oral gavage 30 minutes before each injection of morphine. Control groups received saline (1ml/kg).

Hotplate test: A hot plate test was carried out 30 minutes after morphine administration. Each rat was placed in a Plexiglas box on a hotplate (Bioseb, France) with a temperature of 55˚C and surface area of 23 x 23cm. Latency time for the rats to lick hind paws or jump (whichever came first) was recorded as post-drug latency (19). The cutoff time was set at the 30s to avoid tissue damage. Before drug administration, the hotplate latency was measured, and the average of the second and the third reading was taken as the baseline latency. Before the experiment, rats were tested for four days to obtain a stable hotplate response, and rats who failed to respond within 30 seconds were excluded from the experiment (20). The result was obtained based on the percentage of the maximum possible effect (%MPE) from the formula below (21):

$$\%MPE = \frac{\text{Post drug latency} - \text{Baseline latency}}{\text{Cutoff time} - \text{Baseline latency}} \times 100$$

A hotplate test was held on days 1 and 14 of the experiment in the morning.

Sample preparation: On day 15, two hours after the last treatment in the morning, the rats were euthanized with ketamine, xylazine, and zoletil (KTX) cocktail and perfused via transcardial perfusion using 1X Phosphate Buffered Saline (PBS) pH 7.4. Thalamus, spinal cord, and hippocampus were dissected and

homogenized in 1X PBS pH 7.4 (1ml PBS/100mg tissue) using motor-driven TissueRuptor (Qiagen). Homogenized samples were centrifuged at 12000 rpm at 4° C using refrigerated micro-centrifuge. The supernatants collected were transferred into micro-centrifuge tubes for immediate use or stored in -80° C for up to 6 months. Malondialdehyde (MDA) and iNOS concentrations in the samples were analyzed using an ELISA kit supplied by Cells Biolabs, Inc. (Catalog number: STA312), Elabscience, Inc. (Catalog number: E-EL-0060), and Cusabio, Inc. (Catalog number: CSB-E08325r).

Data analysis: Statistical analysis was performed using Graph Pad Prism 6. Multiple comparisons in hotplate latency, MDA, and iNOS levels between groups were determined using One Way ANOVA followed by Tukey's post hoc test at 95% confidence intervals.

Results

Effect of *A. dorsata* Honey on the Development of Morphine Tolerance: Hot Plate Test: During the first day of the morphine tolerance development, the saline control group showed the lowest MPE% latency, suggesting no significant analgesic effect. Morphine-induced groups showed a significant increase in analgesic MPE% compared to the saline group ($p<0.05$). The result indicated that the rats experienced analgesic effects induced by morphine. Co-administration of *A. dorsata* honey at three different doses (0.5, 1.5, and 2.5 g/kg) did not inhibit or altered the analgesic latency as all these three treatment groups also showed significant MPE% compared to the saline group (Figure 1). Comparison between the three doses of *A. dorsata* honey with the morphine control group showed no significant differences of MPE% between them. This result suggested no significant effect of *A. dorsata* honey on morphine-induced analgesic effect on the first day of the treatment.

On day 14, saline-treated rats experienced non-significant analgesic effects MPE% compared to day one after 14 days of treatment, indicating no changes in analgesic effect. Meanwhile, the morphine alone group on day 14 demonstrated a significant reduction of MPE% compared to day one treatment, emphasizing the development of the tolerance that was developed following 14 days of morphine administration.

As for *A. dorsata* honey-supplemented morphine groups, all three groups showed significantly higher MPE% of analgesic latency than morphine treatment alone (Figure 1). This result suggested that *A. dorsata* honey co-treatments have prevented the decrease of analgesic effect and the development of morphine tolerance. Among the three doses tested, the intermediate 1.5 g/kg *A. dorsata* honey has the highest prevention on the analgesic tolerance as the treatment gave the highest MPE% value on day 14. However, no significant differences in MPE% between 1.5 g/kg and 2.5 g/kg honey dose.

Effect of *A. dorsata* Honey on MDA Level in Brain Regions of Morphine-tolerant Rats: The result showed that repeated administration of morphine significantly increased the MDA level in the thalamus, spinal cord, and hippocampus compared to the saline group ($p<0.0001$) (Figure 2). Based on ANOVA multiple comparison analysis, co-administration of morphine with *A. dorsata* honey significantly decreased the MDA level in the thalamus and spinal cord at high dose (2.5 g/kg) ($p<0.0001$ respectively). Though not significant in other doses, *A. dorsata* honey reduced the MDA to a lower level than the morphine single group. Meanwhile, all three different doses significantly reduced the MDA formation ($p<0.0001$). Thus, the effect of *A. dorsata* honey in attenuating MDA formation in the brain was significant, particularly in the hippocampus.

Effect of *A. dorsata* Honey on Inducible Nitric Oxide Synthase (iNOS) Production in Morphine-Tolerant Rat Brain Region: Figure 3 shows that morphine injection for 14 days caused an increase of iNOS level in the thalamus, spinal cord, and hippocampus regions. Co-treatment with *A. dorsata* honey significantly reduced the iNOS levels.

Discussion

This current study investigated the effect of *A. dorsata* honey on morphine tolerance. The analgesic MPE%

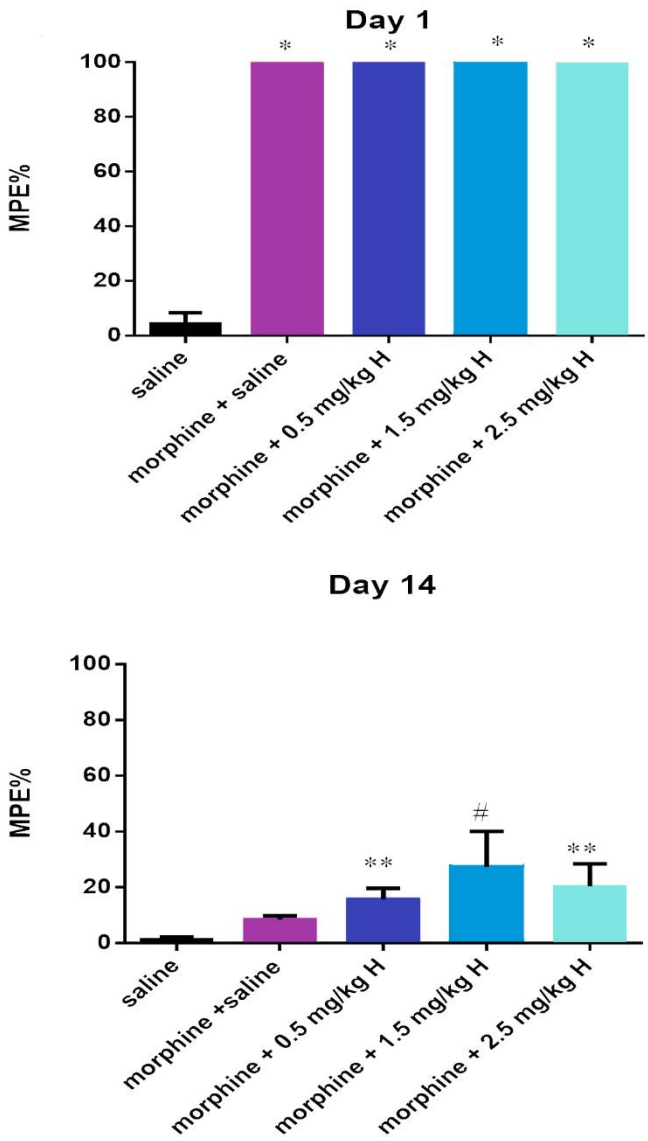

Figure 1. The effect of different doses of *Apis dorsata* honey on the development of morphine tolerance in rats on Day 1 and day 14, respectively. Data represents MPE% mean±S.E.M. (n=8). *p<0.001 compared to the saline group. ** p<0.05 compared to the morphine control group. # p<0.001 compared to the morphine control group. Graphs were obtained using GraphPad Prism 6. The supplement of *A. dorsata* honey prevents the development of tolerance compared to the morphine control group. Dose 1.5g/kg A. dorsata honey shows the highest prevention of tolerance (# p<0.001).

value of morphine alone reduced to the level that has no significant difference compared to the saline group on day 14 of the morphine injection. This outcome showed that the tolerance to the analgesic effect had occurred and was completed on the 14th day of the morphine injection, as suggested by (18).

Recent studies demonstrated the association of oxidative stress in the neurobiology of opioids (22, 23, 4). Prolonged opioid receptor activation can cause excitotoxicity in the synapse (24). This condition is proposed to alter the analgesic cascades and down-regulated the opioid system, causing an irreversible state of tolerance (25, 26).

Based on the result obtained, *A. dorsata* honey possibly prevented the development of morphine tolerance via nourishment of antioxidants to neurons

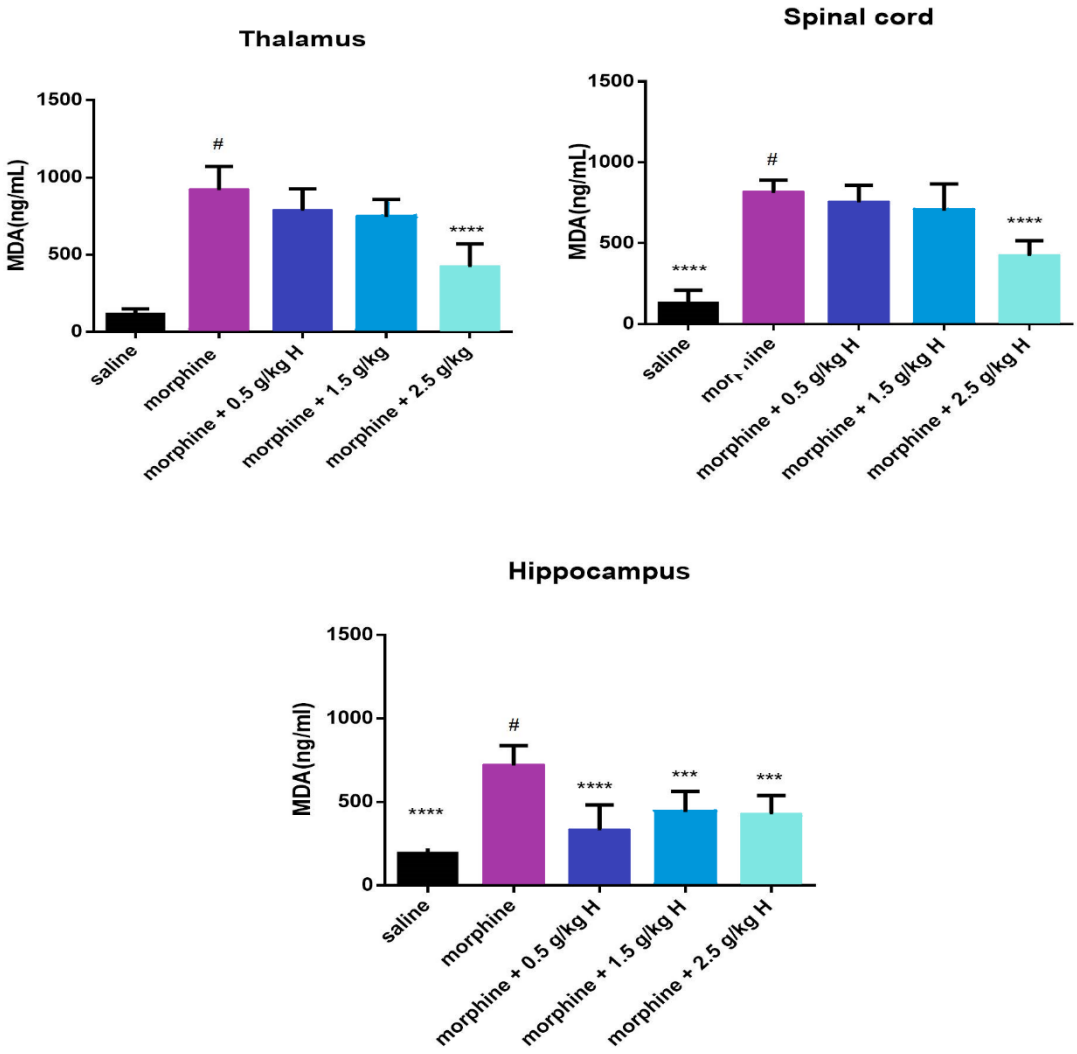

Figure 2. Effect of *A. dorsata* honey supplement on the level of lipid peroxidation marker, MDA in morphine-tolerant rats' thalamus, spinal cord, and hippocampus. One-way ANOVA followed by Tukey's post hoc was used to analyze MDA differences between treatment groups. #p<0.0001 compared to the saline group. Co-administration of *A. dorsata* honey decreased the MDA formation, especially at 2.5 g/kg dose in the spinal cord and hippocampus (****p<0.0001 & ***p<0.001 compared to the morphine control group, respectively).

that decrease oxidative stress. The anti-tolerant effect of *A. dorsata* honey supplements is probably due to honey's antioxidant, analgesic effect, and anti-inflammatory properties (27, 28).

Honey is proven rich in phenolic acids and flavonoids that contributed to antioxidant activity such as gallic acid, benzoic acid, caffeic acid, catechin, luteolin, and apigenin (29). Several honey bioactive compounds were reported to have properties in reducing tolerance, such as ellagic acid and quercetin (12, 13). This finding highlighted the importance of the bioactive compounds on *A. dorsata* honey's anti-tolerant properties. Inhibition of NO radical and RNS, an indicator of oxidative stress formation, can prevent the development of morphine tolerance (4, 30). Co-treatment with honey was demonstrated to reduce

tissue inflammation via inhibition of NO production (31).

defined as the prevention of analgesic reduction after repeated exposure to morphine. Hence, in this study,

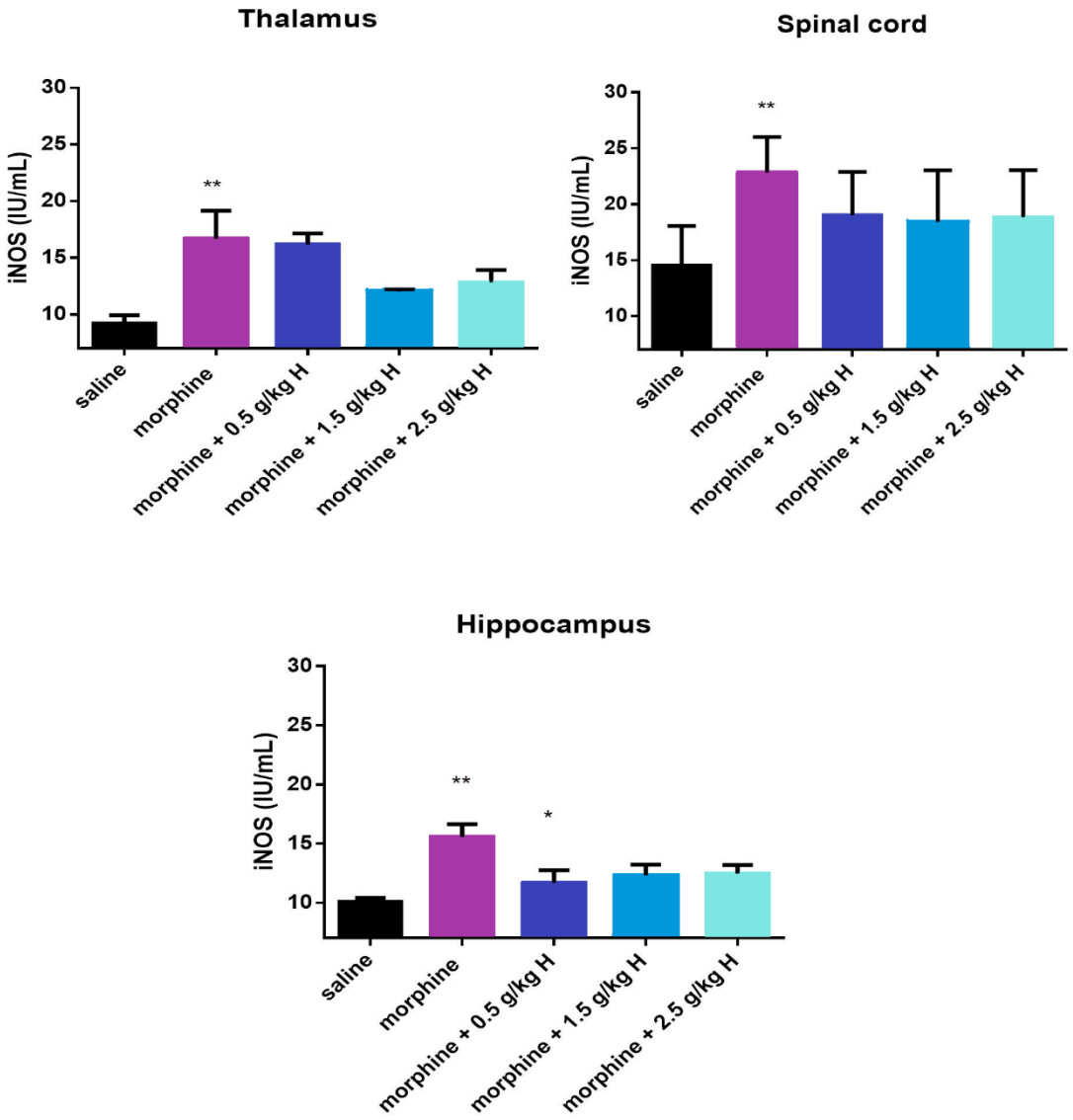

Figure 3. Effect of *A. dorsata* honey on iNOS level in morphine-tolerant rats' thalamus, spinal cord, and hippocampus. One-way ANOVA followed by Tukey's posthoc analysis was used to analyze the differences of iNOS between treatment groups. Data represents iNOS level mean±S.D. (n=8). Morphine control groups in all three regions demonstrated significant-high iNOS levels, **P<0.01 compared to the saline group. The co-administration of *A. dorsata* honey at 0.5 g/kg significantly reduced the iNOS level, *P<0.05 compared to the morphine control group. H= *A. dorsata* honey.

Our result showed that a high dose of *A. dorsata* honey (2.5 g/kg dose) caused a less protective effect on tolerance than intermediate-dose 1.5 g/kg, which gave higher inhibition, though statistically, it was not significant. Protective effect against tolerance is

we hypothesized that the differences between these two ranges of doses probably did not significantly affect tolerance development. Few factors contribute to tolerance development; doses and duration, interaction, metabolism (enzymes), polymorphism

(genetic) (32, 33).

Our current results also indicated that the effect of *A. dorsata* honey on the MPE% was not dose-dependent. One of the explanations for such possibility is the active interaction between compounds in *A. dorsata* honey. Another factor that may be involved is also the interaction of morphine with *A. dorsata* honey phenolic compounds. It is most probably contributed by interactions between more than one phenolic acid and flavonoid of the honey (34). This current result is in line with a previous claim, in which combination with phytochemicals can cause a polyvalent pharmacological action of the drug (35).

In this study, the effect of *A. dorsata* honey gave a preliminary positive finding on its role as an antioxidant in reducing the tolerance of morphine. A possible explanation of this finding is mainly through antioxidant-free radical reaction in glutamate neurotoxicity upon morphine signaling. The result showed that a higher (2.5g/kg) dose gave less prevention of morphine tolerance. In the neuroprotective studies, the significant doses were varied, such as in a study by (36), 1.2 g/kg dose was significant in cerebral neuroprotective. Meanwhile, 0.2g/kg and 1g/kg doses were significant for hippocampal and midbrain neuroprotective effects. As far as this study was conducted, there are not many reports on the optimal dose of *A. dorsata* honey.

The variation of the bioactive contents in different honey contributes to the different rates of biological activities. Nature factors such as climate, floral source, and geographical area of honey sources may vary their chemical and bioactive composition and their biological activity levels (37). Briefly, it is considered challenging to determine the specific flavonoids or phenolic acids in every batch of honey and the efficacy of their antioxidant activities. Thus, it is recommended to analyze bioactive contents in each honey sample to determine active phytochemical content.

Although co-administration of *A. dorsata* honey with morphine significantly prevented analgesic tolerance, the mechanism of *A. dorsata* honey in the morphine tolerance cannot be precisely determined because no *A. dorsata* honey control group was involved, which raises a question of whether the inhibition of tolerance is caused by the additive effect of the honey or the analgesic effect of the honey itself. Hence, the *A. dorsata* honey control treatment group should be beneficial to be included in future investigations.

The outcome suggests that *A. dorsata* honey possibly prevented the increase in ROS/RNS and MDA. Concomitant treatment of honey significantly reduced the MDA formation, especially in the hippocampus, suggesting the protective effect of honey against free radicals. *A. dorsata* honey possibly prevents the development of morphine tolerance via an antioxidant defense mechanism attributed to its bioactive constituents. Previous reports on honey phytochemical contents also showed their protective effects against antioxidant deficits and lipid peroxidation in specific brain regions (38).

At the molecular level, honey phenolic contents possibly participated in the morphine action site. The previous study has compared the activity of antioxidants in morphine action. Langsdorf & Chang (39) proved that antioxidants could reduce ROS and attenuates MOR expression. Based on our result in the hotplate test and MDA level, it can be suggested that the inhibition of the analgesic tolerance is possibly contributed by the *A. dorsata* honey antioxidant mechanism in opioid receptor signaling.

Regarding the doses used, all of the three doses of A. *dorsata* honey significantly reduced MDA levels in the hippocampus of morphine-treated animals. In contrast, the MDA inhibition in the thalamus and spinal cord was only significant at the 2.5g/kg dose of *A. dorsata* honey. The mechanism governing the iNOS release under morphine tolerance can be related to glial activation by morphine. The previous investigation stated that the glial activation in morphine antinociception caused the release of iNOS (40). *A. dorsata* honey significantly reduced the iNOS level at the dose of 0.5 g/kg in the hippocampus (Figure 4). The result suggested that *A. dorsata* honey provides a neuroprotective effect against morphine tolerance to analgesic effect via reducing iNOS generation. One possible mechanism is that honey prevents the increase of NOO^- and $O2_-$ in the glutamatergic pathway of morphine tolerance via antioxidant scavenging activity (41).

The honey antioxidant may simultaneously participate with NO action in reducing opioid receptor

desensitization and eventually suppress morphine tolerance. The previous study has established the significance of antioxidants in attenuating NOS production and morphine tolerance compared to NOS inhibitor single treatment (42).

Furthermore, it can be observed from the overall result that *A. dorsata* honey co-treatment reduced oxidative stress, specifically in the hippocampus, compared to other brain regions. Hippocampus is the site for modulation of behavioral response to nociceptive stimuli and opiate addiction (43). Compared to other regions, morphine treatment caused MOR-induced ROS generation, specifically in the hippocampus, altering the synapse (44).

Taken together, this study proposed the mechanism of *A. dorsata* honey in reducing tolerance to morphine analgesia, possibly via attenuation of morphine-induced oxidative stress in the morphine analgesic pathway. The treatment of *A. dorsata* honey was proven to reduce the formation of iNOS, with the possible reduction of NO radical release in the synaptic cleft. Subsequently, this condition reduces neuronal damage and protects the neuron from the alteration of morphine signaling. *A. dorsata* honey co-treatments potentially prevented the MDA and iNOS increase caused by repeated morphine exposure.

Conclusion

Administration of *A. dorsata* honey reduced the tolerance and oxidative stress biomarkers, demonstrated by the hotplate latency, MDA, and iNOS level. Thus, this study highlighted a new role of *A. dorsata* honey as a potent morphine tolerance-preventing agent other than its well-known antioxidant activity. *Apis dorsata* honey might affect neuronal fate by many signaling pathways that have not been detected yet.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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