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# Comparison of antinociceptive and antioxidative effects of Tualang honey and Vitamin C in a rat model of inflammatory pain

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This study aimed to compare the antinociceptive and antioxidative effects of Tualang honey and Vitamin C in formalin-induced pain in the rat. About 24 Sprague Dawley male rats were randomized into three groups and each group (n=6) received either distilled water (C) or Tualang honey (1.2 g/kg body weight/day) (TH) or Vitamin C (20 mg/kg body weight/day) (VC) for 10 consecutive days. On the tenth day, intraplantar formalin 1% (0.1 mL) was given one hour after the treatment. Rat's pain behaviour was recorded using a digital camera for an hour. The rats were sacrificed two hours post-formalin injection and blood was taken to measure the malondialdehyde (MDA) level, catalase (CAT) and superoxide dismutase (SOD) activities. TH not VC significantly reduced the pain behavior score and improved oxidative stress parameters compared to control. However, MDA level was not significantly different between groups. Interestingly, there was a good inverse correlation between CAT level and mean pain behavior score suggesting the important role of antioxidants in modulating the inflammatory pain responses. In conclusion, TH has better antinociceptive and antioxidative properties compared to VC. The antinociceptive property of TH might be partly contributed by increasing CAT level in this model of inflammatory pain.

**Keywords:** Antioxidant, Inflammatory pain, Oxidative stress, Tualang honey, Vitamin C.

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## Introduction

Tualang honey (TH) is found mostly in the tropical rain forest in the Northern Peninsular Malaysia, Southern Thailand and Borneo. It is produced by Asian rock bees (*Apis Dorsata*). It was shown that TH contains many bioactive compounds which include phenolic acids, flavonoids, vitamins, and enzymes, as well as a small amount of mineral content, particularly copper and iron[1]. TH contains higher level of phenolic acids and flavonoids compared to Manuka honey and other local Malaysian honey[2]. A total of six phenolic acids (gallic, syringic, benzoic, transcinnamic, p-coumaric, and caffeic acids) and five flavonoids (catechin, kaempferol, naringenin, luteolin and apigenin) are found in TH[3].

Several studies have shown that TH has antinociceptive properties. A report has demonstrated that TH administration was associated with increased tail flick latency time when stimulated with noxious heat[4]. Antinociceptive property of TH is not directed towards phasic pain alone but also on tonic pain[5]. Abd Aziz *et al*. (2014) have shown that TH administration led to reduced nociceptive behavior score for both phases in formalin test[5]. Formalin injection in rats induces two phases of pain behavior. Phase 1 behavior is partly due to the peripheral stimulation of nociceptors[6] and formation of prostaglandin in the periphery[7] while phase 2 is contributed by hyperexcitable dorsal horn neurons and ongoing inflammation in the periphery[8].

The antinociceptive effects in formalin test were also seen in the adult offspring when TH was given to the pregnant dams. These effects most probably were mediated by reducing oxidative stress in the central nervous system e.g. spinal cord[9,10]. These findings may indicate that antioxidants are involved in the modulation of inflammatory pain. Reactive oxygen species (ROS) have been reported to be involved in the development and maintenance of central sensitization in chronic pain conditions[11-13]. Hydroxyl radicals, superoxides, peroxynitrites, lipid peroxyl radicals and hydrogen peroxide are examples of

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ROS[14]. Presence of hydrogen peroxide in the periphery is associated with pain or hyperalgesia[15]. In a sciatic nerve ligated model, malondialdehyde (MDA) level was significantly increased in the sciatic nerve compared to control rats[16]. At the central level, hydrogen peroxide was shown to be increased in the lumbar level of spinal cord[17], and trigeminal nucleus[18] following formalin injection at the hind limb and the whisker pad respectively. The hydrogen peroxide is specifically and largely responsible for formalin-induced central sensitization-mediated tonic pain[17].

Vitamin C (VC) is one of the antioxidants present in TH and VC itself has been shown to inhibit nociceptive behavior induced by formalin injection[19]. Whether, the antinociceptive property of TH is contributed by VC alone or due to other components available in TH remained to be investigated. To date, there has been no report that compares the antinociceptive and antioxidative effects of TH and VC in the formalin induced-pain model. In addition, it was not known whether there is any correlation between the oxidative stress parameters and mean pain behavior score. Therefore, the main aim of this study was to compare the effects of TH and VC on mean pain behavior score and oxidative stress parameters in inflammatory pain model. The second aim was to determine whether there was a correlation between the parameters investigated.

## Materials and Methods

### Animals

Twenty-four adult male Sprague-Dawley rats weighing between 220-300 g were supplied by the Animal Research and Service Centre, Universiti Sains Malaysia (USM). All rats were acclimatized for a week and had free access to animal chow and water. They were maintained under standard conditions with 12 hours light/dark cycle and temperature controlled room of 22±1 °C. The experimental procedure was approved by the Animal Ethics Committee, Universiti Sains Malaysia [USM/Animal Ethics Approval/2015/(94)(586)].

The rats were randomized into three groups: control (C), Tualang honey (TH) and vitamin C (VC). Distilled water or TH (1.2 g/kg body weight/day)[4] or VC (20 mg/kg body weight/day)[20] was administered orally by gavage feeding to the rats daily for ten days. TH was supplied by the Federal Agricultural Marketing Authority (FAMA) Malaysia and VC was purchased (HmbG® chemicals, Germany). On the 10$^{th}$ day, the rat's behavior was recorded for one hour using a video camera immediately after injection of 1% formalin (0.1 mL) into the rat's right hind paw. The rats paw diameter was measured using a digital caliper (Duratool, China) before and two hours post formalin injection. The changes in the paw diameter were then recorded. Change in paw diameter was recorded as the paw diameter at 2 hours minus paw diameter before injection.

### Formalin test

The behavioral test was conducted in a testing chamber (26 cm x 20 cm x 20 cm) with a mirror positioned beneath the chamber to allow a good view of the rat's paws[21-23]. The behavior was scored by two observers blinded to the treatment of each rat. The test score was given for every minute and averaged at 5-minute intervals[24]. The test score was based on the behavioral categories described by Dubuisson & Dennis where 0, the injected paw is not favoured (no pain); 1, the injected paw has little or no weight on it with no toe splaying (mild pain); 2, the injected paw is elevated and the heel is not in contact with any surface (moderate pain); and 3, the injected paw is licked, bitten or shaken (severe pain).

### Sacrifice of rats and blood collection

Two hours post formalin injection, the rats were sacrificed following intraperitoneal injection of sodium pentobarbitone (100 mg/kg). Following loss of pinch reflex, the heart was exposed by performing a large incision on the thoracic wall. An 18G branula was inserted into the apex of the left ventricle for blood collection. Samples were processed immediately and centrifuged at 10,000 x g for 2-3 minutes. The serum obtained was kept frozen under -80 °C until further analysis of superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA). The markers were measured using commercially available kits (Northwest Life Science, Vancouver, WA).

### Statistical analysis

The results were analyzed using SPSS version 22 software. Repeated measure ANOVA was used to analyze the behavioral data. Meanwhile, one way ANOVA was used to compare paw oedema and oxidative stress parameters between the groups. Probability values $<0.05$ are considered significant. Correlation analysis was performed to look at the relationship between each oxidative stress parameter and mean pain behavior score. Pearson correlation

coefficient (r) was used to examine correlation between mean pain behavior score and oxidative stress parameters. The strength of the correlation between the two parameters is indicated by the r values in the Table 1. A negative value indicates an inverse correlation.

## Results

### Paw oedema and pain behavior

In this study, changes in the paw diameter were recorded and there was a significantly ($P$ <0.001) smaller change in paw diameter in TH (1.39±0.09 mm) and VC (1.55±0.06 mm) groups compared to control group (2.86±0.17 mm) (Fig. 1). The smaller changes in paw diameter are suggestive of reduced oedema in TH and VC groups. Fig. 2 showed the appearance of the paw diameter after formalin injection.

The pain behavior of the rats was assessed based on the grading mentioned under formalin test section. The rats in control group generally elevated the injected paw, licked or bit the injected paw especially in phase 1 and early part of phase 2. Similar observation was also seen in VC group. For TH group, the biting or licking behavior of the injected paw was rarely seen throughout phase 2 of formalin test. After analyzing the behavioral data following the categories as stated in the formalin test, the three groups demonstrated the typical biphasic pain responses following formalin injection[25]. However, significant differences were seen when compared between the groups.

Generally there was a trend of reduced pain behavior score in TH and VC groups throughout the observation (Fig. 3). The pain behavior score was significantly lower ($P$ <0.05) at minute 5 (phase 1), 20, 45 and 50 minutes (phase 2) post-formalin injection in TH group when compared to control. However, there were no significant differences in the score when compared between VC and control groups or between TH and VC groups.

Table 1 — Correlation coefficient and strength of correlation between two parameters

| Correlation coefficient (r) | Strength of correlation |
|---|---|
| < 0.25 | Poor |
| 0.26 – 0.50 | Fair |
| 0.51 – 0.75 | Good |
| 0.76 – 1.00 | Excellent |

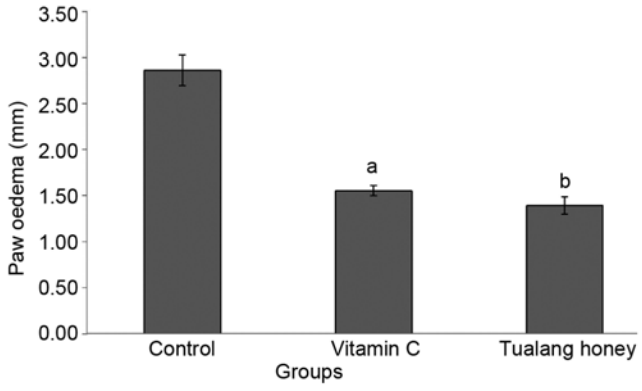


Fig. 1 — The diameter of paw oedema (mm) among control, Vitamin C and Tualang honey groups after formalin injection. Data are presented as mean±SEM (n=6). [a] and [b] indicates $P$ <0.001 when compared to control group.

### Oxidative stress parameters

The present study showed that SOD and CAT levels were significantly higher in the TH group compared to control ($P$ <0.01). SOD level was also significantly higher in VC group ($P$ <0.01) but there was no significant difference in CAT level when compared to control group. However, there was no

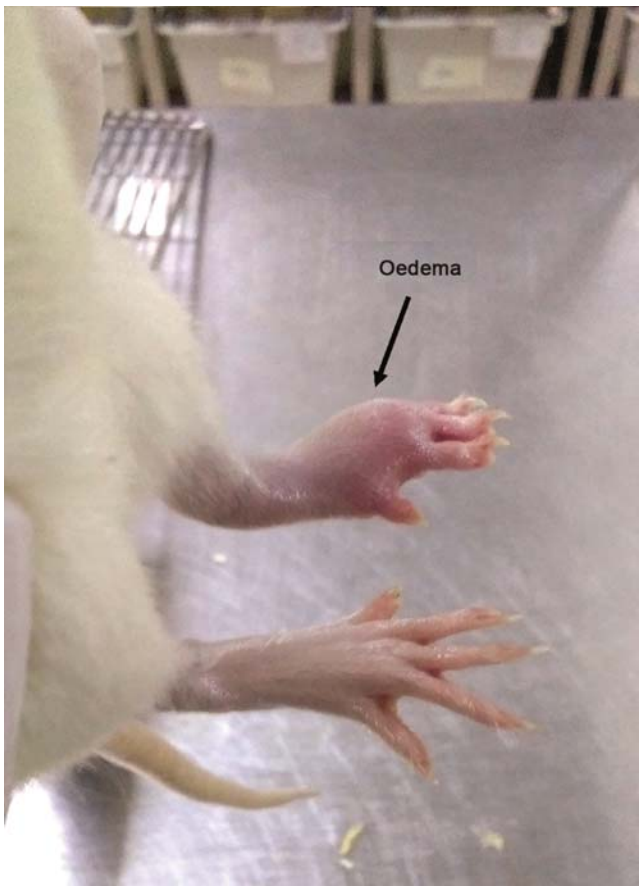


Fig. 2 — Appearance of paw oedema after formalin injection.

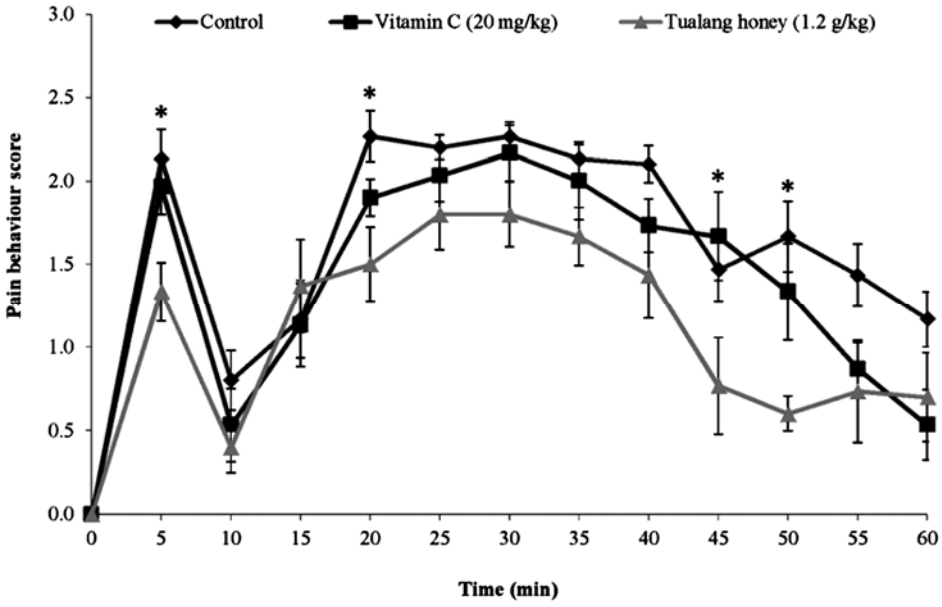


Fig. 3 — The pain behavior score among control, Vitamin C and Tualang honey groups after formalin injection. Data are presented as mean ± SEM (n=6). * indicates $P$ <0.05 when compared between Tualang honey and control groups.

Table 2 — Level of oxidative stress parameters in the groups and its corresponding correlation coefficient with pain behaviour score

| Oxidative stress parameters | Tualang honey (Mean ± SEM) | Vitamin C (Mean ± SEM) | Control (Mean ± SEM) | Correlation coefficient (r) |
|---|---|---|---|---|
| SOD activity (pg/mL) | 94.91 ± 7.29 [a] | 92.12 ± 7.8 [b] | 46.76 ± 9.82 | -0.459* |
| CAT activity (ng/mL) | 116.11 ± 8.57 [a] | 107.07 ± 11.79 | 82.05 ± 6.61 | - 0.736** |
| MDA level (ng/mL) | 2.06 ± 0.22 | 2.15 ± 0.15 | 2.29 ± 0.16 | 0.179 |

Data are represented as mean±SEM for six rats in each group. MDA= malondialdehyde; SOD= superoxide dismutase; CAT= catalase. [a]indicates $P$ <0.05 when compared between Tualang honey and control groups, [b]indicates $P$ <0.05 when compared between Vitamin C and control groups, [*]indicates fair inverse correlation between groups, [**] indicates good inverse correlation between groups.

significant difference in serum MDA when compared between the three groups ($P$ =0.669). All the parameters were not significantly different when compared between VC and TH groups (Table 2). The correlation between MDA level and mean pain behavior score was poor (Fig. 4). There was a fair inverse correlation between SOD levels with the mean pain behavior score (Fig. 5). Interestingly, CAT levels have good and inverse correlation with mean pain behavior score (Fig. 6).

## Discussion

In the present study, the results have shown that there were no significant differences in the parameters investigated when compared between the TH and VC groups. However, when compared to control group, pain behavior score was significantly lower in TH group. In addition, the SOD and CAT levels were significantly higher in the group. The pain behavior score and CAT level were not significantly different in the VC group compared to control. CAT level showed a strong and inverse correlation with the mean pain behavior score suggesting importance of the antioxidant in modulating the pain response in the inflammatory pain model.

Oxidative stress has been shown to contribute to development of pain e.g. back pain in human[26] and neuropathic pain in rats[27]. Formalin injection induces a cascade of cellular events which results in inflammation. Typically, formalin induced inflammation produced two phases of pain behavioral responses contributed by peripheral inflammation (phase 1) and central changes (phase 2)[28]. Inflammatory cells are recruited to the site of inflammation and there is an increase in metabolism with increased usage of oxygen at the inflamed site. The events lead to increase release and accumulation of ROS[29]. The cells also produce soluble mediators e.g. arachidonic acid and chemokines which will enhance the recruitment of inflammatory cells to the inflamed site with production of more ROS.

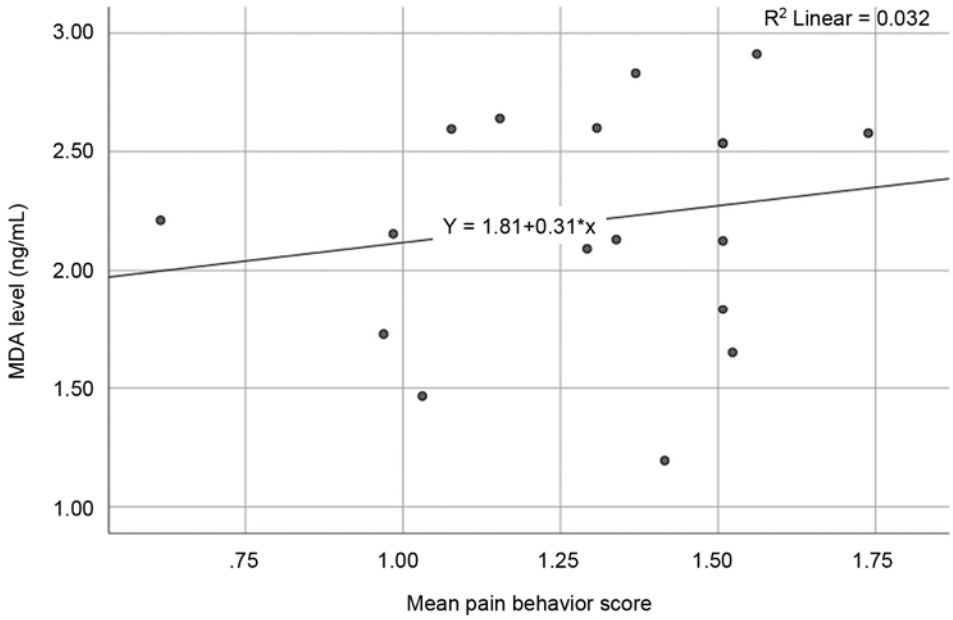


Fig. 4 — The correlation between MDA level and mean pain behavior score for all groups.

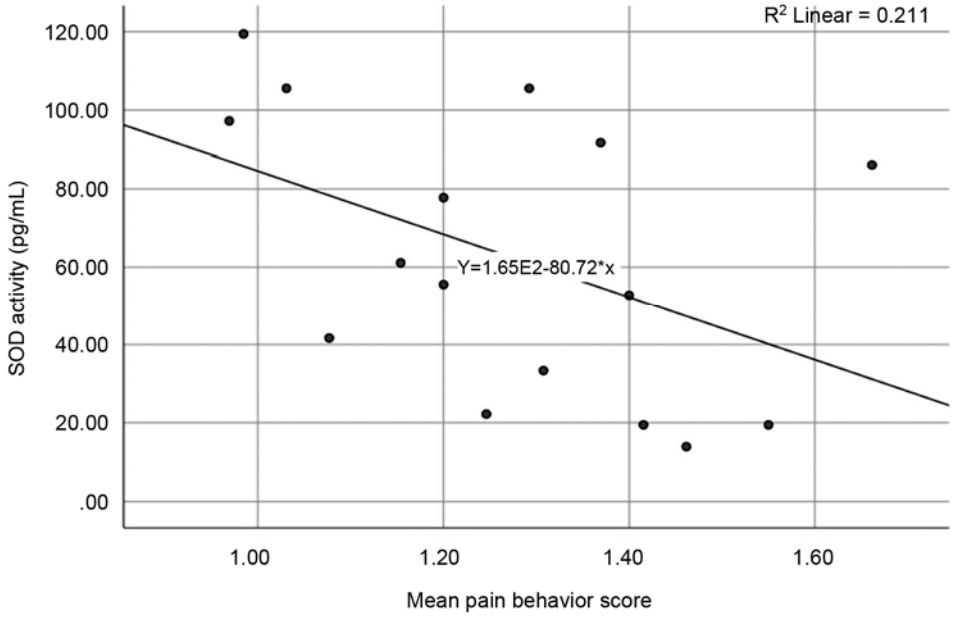


Fig. 5 — The correlation between SOD activity and mean pain behavior score for all groups.

SOD is an antioxidant that removes superoxide radicals by converting it to hydrogen peroxide while CAT converts hydrogen peroxide to water and oxygen. If there is failure of the defensive enzyme systems, the level of free radicals may increases and this may lead to oxidative stress. Lipid peroxidation is one of the events that occurs during oxidative stress and may lead to the formation of MDA. Various studies have used these parameters (SOD, CAT, and MDA) to indicate oxidative stress. Reports have shown that chronic painful conditions in human e.g. rheumatoid arthritis (RA) and osteoarthritis (OA) are linked to oxidative stress[30] and the rat model of RA and OA have shown that exogenous antioxidant administration was associated with either reduction of oxidant or elevation of antioxidants in the blood[31]. Oxidative stress was also shown in the ligated nerve in neuropathic pain model[27] and in the paw tissue of mice injected with formalin.

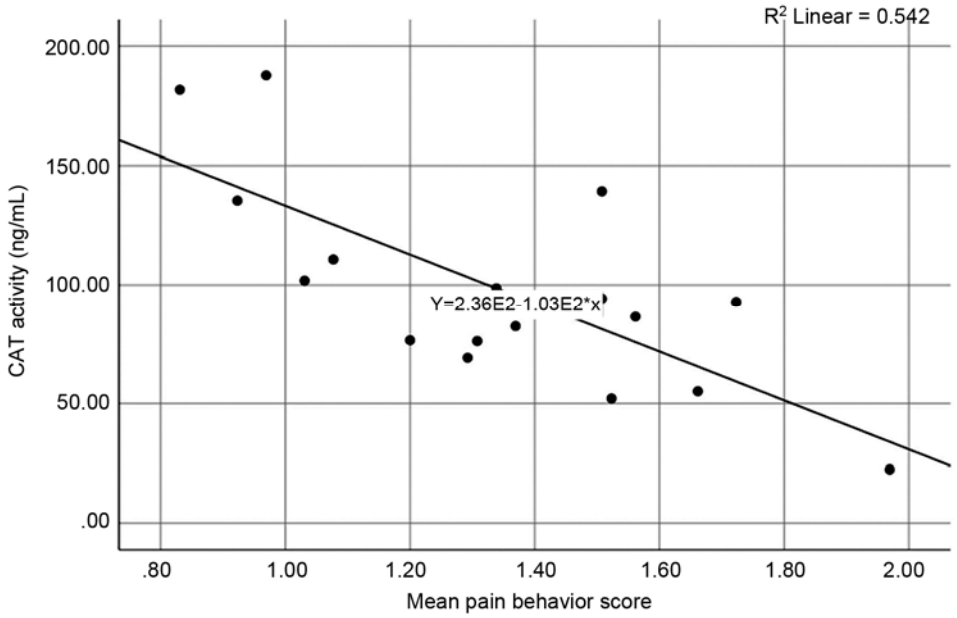


Fig. 6 — The correlation between CAT activity and mean pain behavior score for all groups.

In the present study, MDA levels were not significantly suppressed with the doses of antioxidants used (TH and VC). The results suggest that at the doses used, the antioxidants did not alter the lipid peroxidation in this model of pain. There is also a possibility that the antinociceptive mechanism of TH might not be contributed by suppression of MDA level. The antinociceptive action of TH might be contributed by its high antioxidant content (53.06±0.41 mg of ascorbic acid equivalent per gram of Tualang honey)[2]. However, most of the parameters in VC group were not significantly different when compared to control group. The antinociceptive property of TH might be related to its flavonoid and phenolic contents[2]. In addition, TH has anti-inflammatory property that might contribute to the antinociceptive effects. Studies have shown that the anti-inflammatory effects of honey were associated with decreased polymorph infiltration[32], reduced nitrous oxide and prostaglandin formation[33] and inhibited activation of NF-κB pathway[34]. Activation of NF-κB has an important function in the pathogenesis of inflammation. Moreover, TH has many substances such as flavonoids, phenolic acids, and vitamins including VC which work together to provide a synergistic antioxidant effect[35] compared to VC alone.

Furthermore, the ineffectiveness of VC in reducing pain behavior might be related to the duration of the treatment and dose of VC used in the present study or the differences in quantifying pain behavior compared to previous report[19]. There are variable doses of VC used in the literature. Rosa *et al*. has demonstrated that low dose of VC (3 mg/kg body weight) given via intraperitoneal injection was effective to inhibit formalin induced pain in a group of mice. Another study has reported that intraperitoneal administration of 3 mg/kg of VC for three weeks has increased the pain threshold in neuropathic pain model[36]. Jahan *et al.*, has used a larger dose of VC, 200 mg/kg, which was given orally for thirty days which managed to suppress mercuric chloride-induced oxidative stress in testes of Sprague Dawley rats[37]. Considering the various doses used from previous studies, a few doses of VC can be tested for future studies e.g. 50 mg/kg, 100 mg/kg and 200 mg/kg, to determine the dose which has antinociceptive effects.

To date, few reports have looked at the correlation between antioxidants in the blood e.g. CAT and the pain behavior score[38,39]. The good and inverse correlation between CAT and the mean pain behavior score suggests an important role of CAT in suppressing the pain responses in the rat model of inflammatory pain[39]. This is supported by a study that reported following cholecyctectomy, patients with higher level of plasma catalase required significantly fewer analgesic dose compared to patients with lower catalase level[39]. The present study has shown that TH administration was associated with significant lower pain behavior score together with elevation of CAT

level. There is a possibility that its antinociceptive effects are related to increased CAT level. The study provides a rationale to explore the therapeutic potential of catalase to prevent or reduce inflammatory pain. It will provide a better understanding of the underlying mechanisms which may help to design potential interventions to reduce inflammatory pain.

## Conclusion

In conclusion, at the doses used in the present study, TH has better antinociceptive and antioxidative effects compared to VC. In addition, there was a good and inverse correlation between CAT and mean pain behavior score in the inflammatory model used. This paper suggests that antinociceptive property of TH might be contributed by increased CAT in the inflammatory pain model used in this study.

## Acknowledgement

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## Conflict of interest

The authors declare that there are no conflicts of interest.

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