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DHA-rich fish oil and Tualang honey reduce chronic stress-induced oxidative damage in the brain of rat model

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ABSTRACT

Background: Exposure to chronic stress induces oxidative damage which alters the dynamic balance between antioxidant and pro-oxidant activities in the brain. Tualang honey (TH) is a Malaysian wild multifloral honey which has been shown to contain high amount antioxidants. DHA-rich fish oil is a form of omega-3 fatty acids found in fish which also possesses some antioxidant activity. This study aimed to evaluate anti-stress activity of DHA-rich fish oil, TH and their combination on several parameters of oxidative stress in chronic stress rat model.

Methods: Fifty male Sprague Dawley rats were divided into (i) control, (ii) stress-exposed, (iii) stress-exposed and treated with TH (1 g/kg body weight twice daily), (iv) stress-exposed and treated with DHA-rich fish oil (450 mg/kg body weight twice daily), and (v) stress-exposed and treated with a combination of TH and DHA-rich fish oil. The chronic stress regimen consisted of a combination of restraint stress and a swim stress test for 28 days.

Results: DHA-rich fish oil and TH significantly ($p < 0.05$) suppressed stress-induced elevation of serum corticosterone and lipid peroxidation, and caused a significant increase in total antioxidant capacity. For glutathione status, only TH significantly reduced stress-induced elevation of oxidised glutathione (GSSG) and normalised GSH/GSSG ratio. **Conclusion:** Both DHA-rich fish oil and TH have protective effects against brain oxidative stress but consuming these substances together does not seem to provide an additional benefit compared to consuming them separately.

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1. Introduction

Psychological stress is a widespread condition experienced by all human beings during all stages of human lifespan. Stress is also a state of threatened homeostasis of the body which results in physiological and biochemical changes that can affect many aspects of human health.¹ Stress is one of the known conditions that stimulate numerous intracellular pathways that ultimately cause the imbalance between oxidants (free radicals) and antioxidants in our body, a condition called oxidative stress.² Oxidative stress is a

harmful process which can damage several cellular structures such as membranes, lipid, proteins and deoxyribonucleic acid (DNA) which if not strictly controlled can lead to induction of several chronic and degenerative diseases as well as fasten the ageing process.³

Fortunately, human body is equipped with a defence mechanism to counterbalance the effect of oxidants which can be divided into enzymatic and nonenzymatic mechanisms. Nonenzymatic antioxidants include low-molecular-weight compounds such glutathione which is one of the major soluble antioxidants found in all cell compartments.^{2,3} Numerous studies have reported the ill effects of stress can be alleviated by exogenous antioxidants acting through various pathways to enhance resistance to stress.^{3,4} Pharmacological interventions using exogenous antioxidants may be a promising stress management strategy for protecting against oxidative stress-induced cell damage.⁴

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List of abbreviation

TH	Tualang honey
DHA	Docosahexaenoic acid
GSH	Reduced glutathione
GSSG	Oxidised glutathione
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substances

Fish oil is one of the most important sources of docosahexaenoic acid (DHA) in our body. DHA is a unique polyunsaturated fatty acid particularly abundant in the brain and is a major constituent of nerve cell membrane phospholipids and therefore is an important component for neuroprotection.⁵ Besides DHA, fish oil also contains another form of omega-3 fatty acids, the eicosapentaenoic acid (EPA). Traditionally fish oil contains higher proportion of EPA compared with DHA in a distinctive ratio of 18:12.⁶ New fish oils containing very high proportion of DHA compared to EPA are increasingly becoming available on the market and studies on this kind of fish oil are still lacking. Moreover, recent evidence showed that DHA and EPA are not equal constituents of omega-3 fatty acids in the fish oil as they have different physiological effects which may elicit different effects of the health.⁷ Numerous studies investigating the effects of omega-3 fatty acid during stress used fish oil that contains a mixture of DHA and EPA at a typical ratio of 12:18. As a result, these studies cannot indicate whether DHA is responsible for the evaluated biological effects, therefore it is important that pure or almost pure DHA is used to discriminate the action of DHA from the EPA.⁷

Several animal studies have demonstrated that omega-3 fatty acids supplementation improved psychological wellbeing as well as memory and learning.^{8,9} In addition, a few studies have indicated that omega-3 fatty acids might be involved in stress responses.^{10,11} For instance, Pérez et al.¹¹ showed that omega-3 fatty acids supplementation prevented the stress-induced elevation of corticosterone and impaired learning. However, whether omega-3 fatty acid or DHA can also alleviate chronic stress-induced oxidative stress is largely unknown as the evidence are conflicting.¹²

Tualang honey is a multifloral jungle honey produced by the Asian rock bees (*Apis dorsata*) which build their hives on Tualang trees (*Koompassia excelsa*) found mainly in tropical rain forests including Malaysia.¹³ Tualang honey has been shown to exhibit numerous medicinal properties, including antimicrobial, anti-inflammatory, antitumour, antioxidant, antidiabetic and neuroprotective properties.^{13–15} Tualang honey has been shown to have more free radical scavenging and antioxidant activity than other local and commercially available honey in Malaysia.¹⁵ Also, Tualang honey can potentially protect against the harmful effects of stress on our body. Studies demonstrated that Tualang honey protected the brain against the stress-induced increased of proinflammatory cytokines¹⁶ and improved pregnancy outcome in rats exposed to chronic stress.¹⁷ Therefore, the present study aimed to investigate the protective effects of these 2 substances, DHA-rich fish oil and Tualang honey on the several brain's oxidative stress markers following exposure to chronic stress using rats as a stress model. This study also assessed if the combination of DHA-rich fish oil and Tualang honey would provide any additive protective effect on the rat brain.

2. Material and methods**2.1. Animals**

Outbred male Sprague Dawley rats aged 5–6 weeks old were obtained from the Animal Research and Service Centre (ARASC) of the Universiti Sains Malaysia, Health Campus. The animals were housed in polypropylene cages in ARASC housing facility and given *ad libitum* access to standard rat chow and water except during the experimental period. This room was on a 12:12 h light-dark cycle and maintained under standard laboratory conditions. The rats were acclimatised to the researchers and environment for one week prior to the start of the experiment. Experimental procedures were performed in strict adherence to the conditions approved by The Universiti Sains Malaysia Animal Ethics Committee (approval number: USM/IACUC/2017/(105) (846).

2.2. Study design

Rats were randomly divided into the five experimental groups with 10 rats per group:

- i) Non-stress control group (C) - rats were not exposed to stress but were briefly handled every day during experimental period
- ii) Stress-exposed group (S) – rats were exposed to stress for consecutive 28 days
- iii) DHA plus stress treated group (DHA) – rats were given DHA-rich fish oil (DHA:450 mg/kg body weight twice daily dose) via oral gavage and exposed to stress for consecutive 28 days
- iv) Tualang honey plus stress treated group (TH) – rats were given Tualang honey (1 g/kg body weight twice daily dose) via oral gavage and exposed to stress for consecutive 28 days
- v) Tualang honey plus DHA plus stress treated group (TH + DHA) - rats received both Tualang honey and DHA-rich fish oil via oral gavage and exposed to stress for consecutive 28 days

In order to minimise possible indirect exposure to stress, rats in the control group were placed in a separate room throughout the experimental period. The doses of DHA and TH used in the present study had been shown to be effective to change brain cytokines under chronic stress condition.¹⁶ Equal amount of normal saline was given to rats in the control and stress groups to ensure that all animals were placed under similar treatment condition during the experiment. Tualang honey (AgroMas) was purchased from Federal Agricultural Marketing Authority (FAMA), Malaysia. The honey was filtered, evaporated to 20 @ (w/v) water content at 40 C and then sterilised by gamma irradiation (25 kGy). DHA-rich fish oil was purchased from General Nutrition Corporation (GNC), Pittsburgh, PA, USA (GNC Triple Strength DHA 1000).

2.3. Stress procedures

All rats were subjected to a combination of restraint stress and swim stress test at a randomly determined order except the control group. Restraint stress was imposed by wrapping the animals with flexible plastic mesh with both ends fastened with metal clips. The plastic mesh was appropriately adjusted to just fit the rats without causing any discomfort. The rats were restrained for 5 h a day for consecutive 28 days. In the swim stress test, rats were individually

placed in plastic containers filled with water (23–25) to a depth of approximately 30 cm for 15 min. During the test, rats were allowed to swim freely but could not touch the bottom of the container either with their feet or tail. At the end of 15 min period, rats were dried with a paper towel and return to their home cages. Swim stress test was employed to minimise the occurrence of habituation associated with restraint stress procedure.¹⁸

2.4. Tissue sample preparation

At the end of the experimental period, rats were sacrificed by decapitation. The trunk blood was collected into a 20 ml tube and allowed to clot at room temperature and then centrifuged at 1000×g for 20 min. The supernatants were aliquoted into 1.5 ml tubes and stored at –80 until analysis. Meanwhile, the brains were removed from the skulls, washed with ice-cold saline and stored at –80 . Cerebral hemispheres were homogenised in sodium phosphate buffer (0.1 M, pH 7.4) using a motor-driven tissue homogeniser fitted with a Teflon pestle (Glas-Col, Terre Haute, Indiana, USA) in ice-chilled glass homogenising vessels at 900 rpm for 90 s, yielding 10% (w/v) homogenates. The homogenates were centrifuged in a centrifuge at 3000×g for 15 min at 4, following which the supernatants were aliquoted into 1.5 ml centrifuge tubes and stored at –80 until used.

2.5. Corticosterone analysis

Serum corticosterone was quantified using a commercial enzyme immunoassay kit supplied by Cayman Chemical (Ann Arbor, Michigan, USA) following the manufacturer's protocol. The resulting concentration of serum corticosterone was expressed as ng/ml using a corticosterone standard curve.

2.6. Total antioxidant capacity

Total antioxidant capacity (TAC) was measured using a commercial kit produced by Cayman Chemical (Ann Arbor, Michigan, USA) following the manufacturer's protocol. The principle is based on the ability of antioxidants in tissue samples to inhibit the oxidation of 2, 2'-azino-di-3-ethylbenzothiazoline sulphonate (ABTS) by metmyoglobin. The capacity of antioxidants in the sample to prevent ABTS oxidation is compared with that of Trolox and the results s quantified as nmol/g wet tissue.

2.7. Lipid peroxidation

The extent of brain lipid peroxidation was determined using a thiobarbituric acid reactive substances (TBARS) assay as described by Chatterjee et al.¹⁹ In this method, the amount of the malondialdehyde (MDA) is used as a biomarker to measure the level of lipid peroxidation. Briefly, 100 µl of 10% brain homogenate or standard was added to the reaction mixture tubes containing 0.2 ml of 8.1% (w/v) sodium dodecyl sulphate, 1.5 ml of 20% (v/v) acetic acid (pH 3.5), 1.5 ml of 0.8% (w/v) thiobarbituric acid and 0.7 ml of distilled water. The mixture tube was then vortexed and kept in water bath at 95 for 60 min. After cooling with an ice-bath for 5 min, the tube was centrifuged at 1000×g for 10 min at room temperature. The supernatant was collected and the absorbance was measured spectrophotometrically at 532 nm. Sample values were quantified from a standard curve using 1, 1, 3, 3-tetraethoxypropane as an external standard. Data were expressed as nmol/g wet tissue.

2.8. Glutathione

Glutathione exists in two forms, one as antioxidant reduced glutathione (GSH) and the other as oxidised form known as glutathione disulfide (GSSG). In the present study, concentration of total glutathione and GSSG were measured separately using commercial kit supplied by Cayman Chemical (Ann Arbor, Michigan, USA) following manufacturer's protocol. This kit utilizes an optimized enzymatic GR recycling method for quantification of glutathione. Each sample was assessed in duplicates and sample concentrations were determined from total glutathione and GSSG standard curves. The amount of reduced glutathione (GSH) was obtained by subtracting GSSG from total glutathione. The levels of GSH and GSSG were expressed as nmol/g wet tissue. The ratio of GSH over GSSG (GSH: GSSG) was calculated and used to indicate redox status.

2.9. Statistical analyses

Statistical analysis was carried out using one way ANOVA. If ANOVA analysis achieved significance, multiple Turkey's post hoc tests were performed. P value of less than 0.05 were considered significant. Data are presented as means ± standard error of the means (SEM).

3. Results

3.1. Serum corticosterone

Analysis of serum corticosterone showed that stress group significantly had higher level of serum corticosterone as compared to the control group ($p < 0.001$) (Fig. 1). This indicated that sufficient amount of stress had been imposed to the animals throughout the study period. Serum corticosterone levels in the DHA group, TH group and combination DHA + TH group were all significantly higher than that of the control ($p < 0.001$), but significantly lower than the stress group ($p < 0.001$). Other comparisons were not significant.

3.2. Total antioxidant capacity

Total antioxidant capacity (TAC) was measured to evaluate

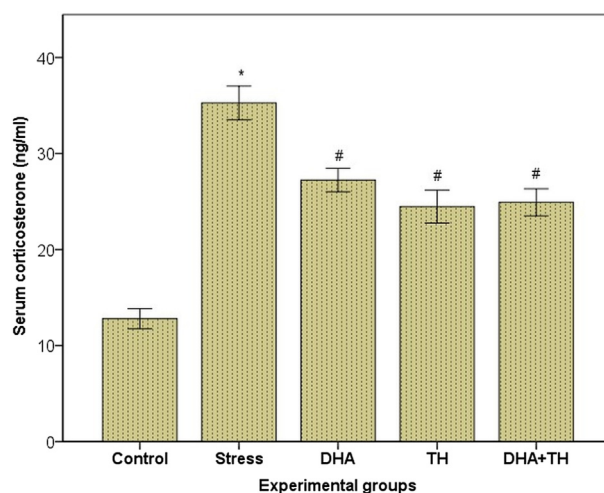


Fig. 1. Serum corticosterone levels (ng/ml) in various experimental groups (n = 10 rats per group). * Significant changes compared to the control group ($p < 0.05$). # Significant changes compared to the stress group ($p < 0.05$).

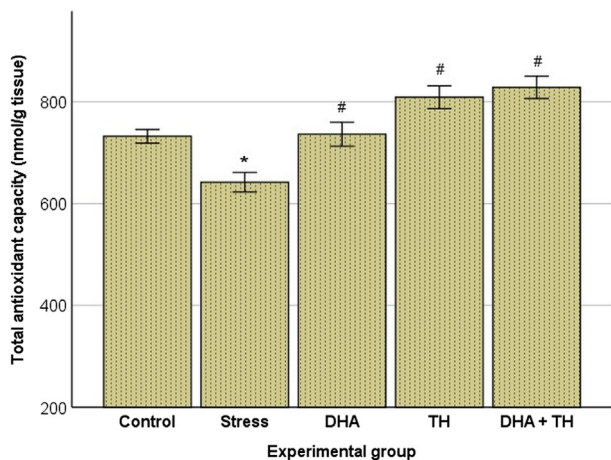


Fig. 2. Total antioxidant capacity in the brain (nmol/g tissue) in various experimental groups (n = 10 rats per group). * Significant changes compared to the control group (p < 0.05). # Significant changes compared to the stress group (p < 0.05).

overall oxidative buffering capacity in the brain. Analysis revealed significant effect of stress on the TAC as revealed by significant reduction in TAC following stress exposure compared to the control (p < 0.05) (Fig. 2). There were also significant effects of DHA-rich fish oil and Tualang honey as indicated by significantly increased in TAC in DHA (p < 0.05) and TH (p < 0.001) groups compared to the stress group. There was no significant change in TAC in combined DHA + TH group compared to the DHA alone and TH alone groups.

3.3. Lipid peroxidation

The amount of lipid peroxidation was measured using TBARS as the marker. Analysis revealed significant effects of stress on the level of TBARS (p < 0.001) (Fig. 3). Stress exposure caused lipid peroxidation as indicated by significant higher TBARS in the stress group compared to the control (p < 0.001). There was significant effect of DHA-rich fish oil and Tualang honey on the level of TBARS as evident by the significant reduction of TBARS in the DHA (p < 0.05) and TH (p < 0.05) groups compared to the stress group. However, no significant change was observed in the combined

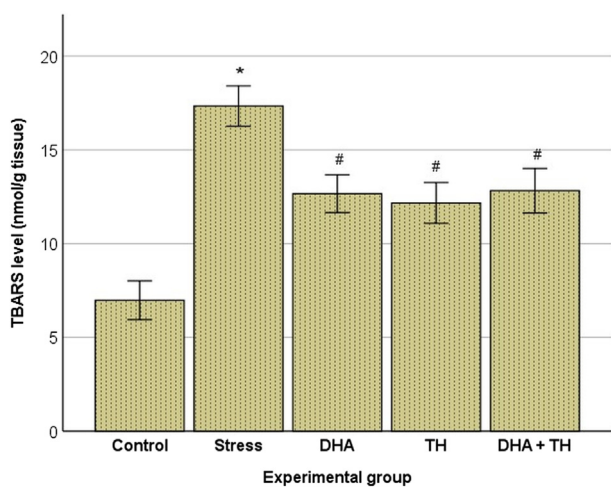


Fig. 3. The amount of thiobarbituric acid reactive substances (TBARS) in brain in various experimental groups (nmol/g tissue) (n = 10 rats per group). * Significant changes compared to the control group (p < 0.05). # Significant changes compared to the stress group (p < 0.05).

DHA + TH group when compared to the DHA alone and TH alone groups.

3.4. Glutathione

In order to gauge the activity of endogenous antioxidant in the brain, reduced glutathione (GSH), oxidised glutathione (GSSG) and the total amount of glutathione (GSH + GSSG) were determined. Analysis of total glutathione did not find significant differences between experimental groups. Analysis of GSH showed a significant reduction of GSH following stress exposure in comparison to the control group (p < 0.05) (Table 1). With regard to GSSG, there was significant effect of stress on the level of GSSG. Stress exposure caused significant increase in GSSG in the brain in all experimental groups when compared to the control (p < 0.05). Analysis also revealed significant effect of Tualang honey on the GSSG level. Tualang honey treatment significantly reduced the GSSG level in the TH groups when compared to the stress group (p < 0.05). However, DHA treatment did not change the GSSG level. Regarding the ratio GSH:GSSG, analysis revealed significant effect of stress on the ratio. Stress exposure significantly reduced the GSH:GSSG ratio in the stress group compared to the control (p < 0.001). There was also significant effect of Tualang honey on the ratio. Tualang honey treatment significantly increased the ratio in the TH group when compared to the stress group (p < 0.01). Treatment with DHA did not influence the GSH:GSSG ratio. These findings suggested that Tualang honey but not DHA seemed to enhance glutathione activities in the brain.

4. Discussion

The present study showed a significant reduction in TAC following exposure to chronic stress which is parallel to the findings of several other researchers.^{20,21} Interestingly, a study has demonstrated that maternal stress exposure during gestation can affect TAC of the mother as well as the born offspring rats.²¹ Reduction in TAC during stress indicates the presence of low antioxidants in scavenging ROS resulting in higher formation of free radicals which in turn can damage the cells. However, TAC only provides limited information about the antioxidant status because the TAC assay does not measure all antioxidant components. For example, the assay does not measure several important antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalases.² Regarding the effect of Tualang honey, the present showed that Tualang honey significantly increased TAC in the brain which is in agreement by studies by Al-Rahbi et al.²² and Azman et al.²³ employing social instability stress and noise stress respectively. Tualang honey used in the present study has been reported to contain a high amount of antioxidants such as phenolic acids and flavonoids which have strong free radical-scavenging activities.¹³ Similarly, the present study showed that DHA-rich fish oil treatment caused an increase in the TAC in the brain which is similar to several previous studies.^{24,25} In addition, the present study indicated that a combination of DHA-rich fish oil and Tualang honey can increase TAC significantly, but the effect was similar to those observed when DHA-rich fish oil or Tualang honey was consumed separately.

Quantification of lipid peroxidation is widely used to indicate oxidative injury in diseases.²⁷ The present study is in parallel with many other studies which shows chronic stress causes lipid peroxidation in the brain.^{3,26} Oxidative degradation of lipid occurs when free radicals attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids resulting in cascades of reactive lipid radicals which ultimately lead to serious damage to the cell membrane.³⁹ Brain membrane lipids are very rich in

Table 1
Concentration of GSH, GSSG (nmol/g wet tissue) and ratio of GSH:GSSG in brain homogenates.

Experimental group	GSH (nmol/g tissue)	GSSG (nmol/g tissue)	GSH:GSSG Ratio
Control	1483 ± 63	115.9 ± 8.1	13.0 ± 0.65
Stress	1301 ± 52 *	185.2 ± 9.2 *	7.1 ± 0.61*
DHA	1384 ± 44	184.4 ± 10.3*	7.6 ± 0.49 *
TH	1475 ± 51	153.7 ± 10.3*#	10.6 ± 0.54 *#
DHA + TH	1466 ± 32	148.8 ± 7.9*#	10.0 ± 0.55 *#

The values are means ± SEM. There were 10 animals in each group. *p < 0.05 compared to the control group. #p < 0.05 compared to the stress group. Analysis were done using AVOVA followed by Tukey post hoc test. GSH, reduced glutathione; GSSG, oxidised glutathione.

polyunsaturated fatty acids, which explained the susceptibility of brain tissue to lipid peroxidation.²⁷ In the present study, Tualang honey treatment seems to protect against stress-induced lipid peroxidation. Our finding supports earlier studies showing chronic noise stress prevented stress-induced elevation of malondialdehyde, a by-product of lipid peroxidation.²³ This effect is believed to be mediated by antioxidant property of Tualang honey which prevented the generation of free radicals and enhances the activities of antioxidant enzymes in the body. The present study also indicated that DHA-rich fish oil can reduce lipid peroxidation and hence can protect the brain against stress-induced oxidative stress. This finding is parallel to other studies investigating the effect of omega-3 fatty acids on the brain²⁸ and also on other organs.²⁹ However, the effect of omega-3 fatty acids on lipid peroxidation is still inconclusive as a number of studies have shown that it can increase lipid peroxidation in many organs^{30,31} while other studies indicated that it does no influence lipid peroxidation at all.³² The extent of lipid peroxidation may be region dependent.³¹ It has been shown the amount of lipid peroxidation decreased in some regions of the brain but remains unchanged in some other regions following omega-3 fatty acids supplementation.³³ It has also been suggested that whether omega-3 fatty acids exhibit antioxidant or pro-oxidant activity in the mammalian brain depends on the experimental protocols and the dosage of omega-3 fatty acids administered.¹² This present study also shows that a combination of DHA-rich fish oil and Tualang honey is not superior to consuming each of these substances separately.

In the present study, chronic stress cause a compromise in antioxidant glutathione defence as indicated by the decrease in GSG level, increase in the GSSG level and reduction in GSH: GSSG ratio which is parallel to the findings of previous studies.^{26,34} GSH plays an important role in antioxidant mechanisms. GSH is the most abundant non-protein thiol that buffers free radical in the brain tissue. GSH protects the cells from oxidative damage by reacting with free radicals to form oxidised glutathione (GSSG). GSH also detoxifies hydrogen peroxide and lipid peroxides via the action of glutathione peroxidase.³⁵ GSSG can be recycled back to GSH by the enzyme glutathione reductase using NADPH as a co-factor which completes the cycle. The ratio of GSH to GSSG often reflects cellular redox balance and is considered as the most sensitive indicator of oxidative stress.²

With regard to Tualang honey, the present study showed that Tualang honey improved glutathione status in the body as indicated by lower level of GSSG and higher value of GSH: GSSG ratio in comparison to the stress only group. This finding is parallel with the work of other researchers.^{22,36} In addition, it has been demonstrated that Tualang honey could influence the activity of several enzymatic glutathione biomarkers such as glutathione peroxidase and glutathione reductase.²² Although not measured in the present study, the activities of these two enzymes are speculated to be affected by Tualang honey resulting in a reduction in GSSG and higher GSH: GSSG ratio. Regarding DHA-rich fish oil, this study failed to show that it can influence the glutathione status in the

brain following exposure to stress. A study in rat model of post-traumatic stress disorder demonstrated omega-3 fatty acid was able to normalise GSSG and GSH: GSSG ratio.³⁷ However, a systematic review on omega-3 fatty acid indicated that while it can significantly affect some oxidative parameters such as total antioxidant capacity and lipid peroxidation, it has no effect of glutathione level in the body.³⁸

Taken together, these set of results indicates that Tualang honey can protect against oxidative stress induced by chronic stress by partly increasing the TAC in scavenging free radicals in the brain. Besides possessing anti-stress activity, Tualang honey has been shown to exert antidepressant-like effect by restoration of hypothalamic-pituitary-adrenal (HPA) axis.³⁶ Therefore, the other possible protective mechanism of Tualang honey during stress condition is by influencing HPA axis, specifically by suppressing the influence of corticosterone and adrenocorticotropic hormone, thus reducing the adverse effect of stress on the body.³⁶ The present study also indicated that DHA-rich fish oil may have important role in preventing oxidative damage following exposure to chronic stress. A previous study showed that rats exposed to chronic stress improves learning, locomotor activity and improved personal behaviour after were given omega-3 fatty acids. On the other hand, rats chronically lacking omega-3 fatty acids exhibited reduced locomotor activity as well as abnormal behavioural responses.^{11,25} Studies in human subjects showed that omega-3 fatty acids supplementation could prevent the occurrence of some psychiatric disorders such as major depression and anxiety disorders possibly through anti-inflammatory and anti-oxidative mechanisms.^{40,41} Although the focus of the present study is DHA, the contribution of the other component of omega-3 fatty acid, namely the EPA should not be underestimated. In fact, a few studies have shown that EPA is far more effective than DHA in preventing some psychiatric disorders such as depression.^{41,42} There is increasing evidence that early intervention with omega -3 fatty acids can minimise unnecessary exposure to conventional medication.⁴²

5. Conclusion

It can be concluded that DHA-rich fish oil and Tualang honey are able to reduce brain oxidative damage following exposure to chronic stress. These effects possibly work by improving antioxidant status in the brain as well as by modulating the HPA axis. However, combined treatment of these two substances is not superior to consuming each of the substance separately. Dietary supplementation with DHA-rich fish oil and Tualang honey can be the potential natural therapy against oxidative stress caused by exposure to chronic stress.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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