Evaluation of the protective effects of tocotrienol-rich fraction from palm oil on the dentate gyrus following chronic restraint stress in rats

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Exposure to chronic restraint stress has been shown to cause a number of morphological changes in the hippocampal formation of rats. Tocotrienol, an isoform of vitamin E, exhibits numerous health benefits, different from those of tocopherol. Recent studies have demonstrated that tocotrienol prevents stress-induced changes in the gastric mucosa, thus indicating that it may also protect other organs such as the brain from the damaging effects of stress. Therefore, the aim of the present study was to investigate the protective effect of tocotrienol-rich fraction (TRF) extracted from palm oil on the dentate gyrus of rats following exposure to chronic restraint stress. Thirty-six male Sprague Dawley rats were divided into four groups: control, stress, tocotrienol and combination of stress and tocotrienol. Animals were stressed by restraining them for 5 hours every day for 21 consecutive days. TRF was administered via oral gavage at a dose of 200 mg/kg body weight. Our results showed that the plasma corticosterone level was significantly increased in response to stress, compared to the control. The results confirmed previous findings that chronic restraint stress suppresses cellular proliferation and reduces granule cell number in the dentate gyrus. However, TRF supplementation failed to prevent or minimize these stress-induced changes. Therefore, we conclude that TRF at the current dosage is not effective in preventing the morphological changes in the dentate gyrus induced by chronic restraint stress.

Uniterms: Tocotrienol. Tocotrienol rich fraction. Restraint stress. Dentate gyrus. Hippocampus.

A exposição crônica ao estresse por restrição causa série de alterações morfológicas na formação do hipocampo de ratos. Tocotrienol, uma isoforma da vitamina E, apresenta inúmeros benefícios para a saúde, diferente do tocoferol. Estudos recentes demonstraram que o tocotrienol impediu alterações induzidas por estresse na mucosa gástrica, indicando, assim, a possibilidade de que ele pode, também, proteger outros órgãos, como o cérebro, dos efeitos nocivos do estresse. Dessa forma, o objetivo do presente estudo foi investigar o efeito protetor da fração rica em tocotrienol (TRF), extraída do óleo de palma, no giro denteado após exposição crônica ao estresse por restrição. Trinta e seis ratos machos Sprague Dawley foram divididos em quatro grupos: controle, estresse, tocotrienol e combinação de estresse e tocotrienol. Os animais foram estressados por restrição, 5 horas por dia, durante 21 dias consecutivos. TRF foi administrado por gavagem oral na dose de 200 mg/kg de peso corporal. Nossos resultados mostraram que o nível de corticosterona plasmática foi significativamente aumentado em resposta ao estresse em comparação ao controle. Os resultados confirmam os achados anteriores de que o estresse por restrição suprime a proliferação celular e reduz o número de células granulares do giro denteado. No entanto, a suplementação de TRF foi ineficaz para evitar ou minimizar as alterações induzidas por estresse. Assim, concluímos que TRF na dose corrente não é efetiva para prevenir as alterações morfológicas no giro denteado induzida por estresse crônico por restrição.

Unitermos: Tocotrienol. Fração rica em tocotrienol.. Estresse por restrição. Giro denteado. Hipocampo.

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INTRODUCTION

Stress can have negative effects on health and plays an important role in the predisposition to many physical illnesses (Sapolsky, 1996; Wilder, 1995). Exposure to chronic stress has been shown to increase susceptibility to infection, raise the risk of ischemic heart disease, promote tumour growth, exacerbate autoimmune diseases and suppress many aspects of the immune system (Cohen *et al.*, 2007). In addition, chronic stress affects some areas of the brain, particularly the hippocampus. Animal experimental investigations have revealed that exposure to chronic restraint stress results in atrophy of the apical dendrites in the CA3 region of the hippocampus (Magarinos, McEwen, 1995; Magarinos *et al.*, 1997). Furthermore, exposure to such stress suppresses neurogenesis in the dentate gyrus and influences synaptic plasticity in the hippocampus (Pham *et al.*, 2003; Kim, Diamond, 2002). However, these stress-induced changes are difficult or impossible to investigate in humans; hence, the use of animal models is unavoidable and an important element of such investigations (Stefanski, 2001). Of the various experimental models of stress, restraint stress is the most widely used as it is easy to administer, reliable and induces both physical and psychological stress. A number of studies have shown that the effects of stress on the brain, particularly on the hippocampus, can be prevented or at least minimized by certain chemical substances and drugs (Watanabe *et al.*, 1992; Wood *et al.*, 2004).

Tocotrienol is an isoform of vitamin E and tocopherol. Apart from displaying greater antioxidant properties, tocotrienol possesses numerous other functions that are not shared with tocopherol (Sen *et al.*, 2006). More importantly, tocotrienol, particularly alpha-tocotrienol, has strong neuroprotective properties, independent of their antioxidant activity. *In vivo* oral supplementation of tocotrienol has been demonstrated to protect rats against stroke-induced injury (Khanna *et al.*, 2005). Furthermore, tocotrienol exerts neuroprotective activity against various chemical insults, such as glutamate, homocysteic acid and linoleic acid (Sen *et al.*, 2000; Khanna *et al.*, 2006; Sen *et al.*, 2007).

The role of tocotrienol as an anti-stress substance preventing or at least minimizing the effect of stress on the organ system has been promising. Nur Azlina and Nafeeza (2008) demonstrated that tocotrienol-rich fraction (TRF) prevented stress-induced elevation of corticosterone and noradrenalin, the two major hormones released after stressful events. An earlier investigation by the same authors revealed that TRF protected the gastric mucosa from developing stress ulcers and blocked stress-induced changes in gastric acidity and gastrin level (Nur Azlina *et al.*, 2005). However, whether TRF supplementation also has a protective role on the hippocampus is not yet known. Therefore, the present study aimed to evaluate the protective effect of TRF derived from palm oil on the dentate gyrus in rats following chronic restraint stress exposure *in vivo*. The dentate gyrus, an important part of hippocampal formation, was selected as it is often affected by exposure to chronic stress (Pham *et al.*, 2003; Magarinos, McEwen, 1995; Magarinos *et al.*, 1997).

MATERIAL AND METHODS

Thirty-six male Sprague Dawley rats aged 5 weeks were purchased from the Laboratory Animal Research Unit, Universiti Sains Malaysia. They were acclimatised to the researcher and the new environment one week prior to the start of the experiments. Food and water were given *ad libitum* throughout the experimental period, except during experimental sessions. The experimental protocols used in the present study were approved by the Animal Ethics Unit of the Universiti Sains Malaysia (approval number 2010/59/222).

Experimental protocols

Animals were randomly divided into four groups: TRF-treated (TF), TRF-treated plus stress (TF+RS), stress control (RS) and normal control (NC). Treated groups (TF and TF+RS) received TRF at a dose of 200 mg/kg body weight via oral gavage, while the other two groups (RS and NC) received equivalent volume of normal saline via the same route, for a period of 5 weeks. The TF+RS and RS groups were subjected to 21 days of restraint stress, 5 hours daily, beginning in week 3 of the experiment.

Restraint stress was induced by wrapping individual rats with a soft and flexible plastic mesh secured at the head and tail ends with metal clips as described by McLaughlin *et al.* (2007). This prevented free mobilisation of the rats, but without imposing unnecessary pain. All rats were refrained from food and water during the experimental session (9 am to 2 pm). TRF used in this study (Tocomin® Suprabio™ 20%) was derived from palm oil, which consists mainly of tocotrienol and a smaller proportion of tocopherol. It was procured from Carotech Ltd, Malaysia, as a gift.

Body weights (in gram) were measured at the beginning, once a week and at the end of the experiment. In the present study, body weight change was used as a dependent variable and calculated by subtracting the initial body weight from the final one.

Tissue harvesting and processing

At the end of the experimental period, the animals were anaesthetized by intramuscular injection of ketamine (1.0 mL/kg body weight). Blood was collected via cardiac puncture and placed inside glass tubes. After collecting blood, the animals were then infused with 40 ml of normal saline, followed by 40 ml of 15% formalin. The adrenal gland, thymus and the brain were removed, weighed (in milligrams) and preserved in the same fixative. The effects of stress on the adrenal gland and thymus were measured using the relative weight, which was the ratio of the organ weight in milligrams to the body weight in grams.

Following overnight fixation, the brain was divided into two halves by a sagittal cut through the corpus callosum. The cerebral hemispheres were then processed and embedded in paraffin wax, and then serially sectioned using a sliding microtome at 3- to 4-um thicknesses. These sections were stained with cresyl fast violet. For immunohistochemistry, sections were incubated with Ki-67 and GAP-43 primary antibodies.

Measurement of plasma corticosterone level

The collected blood was centrifuged at 4000 rpm to separate the plasma from the blood cells. The plasma obtained was stored at -20 °C until hormonal analysis. The plasma corticosterone level was measured using the rodent corticosterone ELISA kit (Endocrinetech, United States). The absorbance was measured spectrophotometrically at 450 nm with a microtiter plate reader (Varioskan Flash Thermo Scientific). The minimal detectable concentration of corticosterone in this assay was approximately 0.1 ng/mL.

Ki-67 and GAP-43 immunohistochemistry

Selected paraffin sections were first dewaxed in xylene and immersed in water. Following heat-mediated antigen retrieval in Tris/EDTA buffer (pH 9), the sections were incubated with 0.3% hydrogen peroxide in methanol for 20 minutes to block endogenous peroxidase activity. The sections were then incubated with primary antibodies, either with rabbit Ki-67 IgG (Abcam, UK; dilution 1:50) or mouse GAP-43 IgG (Santa Cruz, USA; dilution 1:50) for 30 minutes at room temperature. The sections were then incubated with biotinylated secondary antibodies (anti-rabbit and anti-mouse) for 30 minutes at room temperature (Ultravision One Detection: HRP polymer kit, Thermo Scientific, USA). The reaction was visualised with 3,3-diaminobenzidine (DAB) and then counterstained with Harris haematoxylin. The sections were then dehydrated, sealed with cytoseal XYL (Thermo Scientific, USA) and mounted on coverslips. Negative controls in the experiment consisted of omitting the primary antibody.

Quantification of cellular proliferation

Cellular proliferation in the dentate gyrus was assessed using the Ki-67 antibody. Ki-67 is a nuclear protein present solely in dividing cells and is therefore used as a marker for cellular proliferation (Scholzen, Gerdes, 2000). Quantification of Ki-67 immunoreactive cells was performed using a computer equipped with the Image-Pro® Plus software (Media Cybernetics), which received direct input from a light microscope (Nikon Eclipse E600) via a digital video camera (Sony, Japan). These connections allowed the digitized image of the section to be viewed on a high-resolution screen monitor. The sections were examined under a 40X objective and the positive cells were counted using the manual tagging tool provided in the software. The cells were counted within the subgranular zone of the dentate gyrus or within one cell width from its edge, and expressed as the number of cells per section. Cell counting was performed by the same individual, who was blinded to the treatment group.

Quantification of granule cell number

The number of granule cells of the dentate gyrus was determined using the Image-Pro® Plus software (Media Cybernetics) and a similar setting to that described above. Systematic random sampling procedures were employed to choose areas to be examined. To standardize measurements, only neurons exhibiting well-stained nucleoli were counted. Counting was performed under a high-power 40X objective, using a counting frame measuring 19345 μm^2. The mean numbers of granule cells were averaged and expressed as the number of cells per mm^2.

GAP-43 optical density

Growth associated protein of 43 kDa (GAP-43) is an intracellular protein that plays a role in guiding axonal outgrowth during embryonic development. It is often used as a marker for synaptic plasticity in adults (Hrdina *et al.,* 1998). The measurement of GAP-43 optical density was made using the Image-Pro® Plus software (Media Cybernetics) and the same equipment settings as described above. To ensure reliable optical density measurements, the system was first calibrated for optical density as described by Masliah *et al.* (1990). GAP-43 optical density measurement was performed on the outer molecular layer

TABLE I - Body weight change and relative weight of the adrenal and thymus glands

Group	Body weight change (g)	Relative adrenal weight (mg/g)	Relative thymus weight (mg/g)
Control	114.1 ± 4.9	0.098 ± 0.005	3.23 ± 0.28
Stress	66.9 ± 4.6**	0.143 ± 0.005**	2.23 ± 0.13*
TRF	106.4 ± 4.9	0.087 ± 0.005	3.08 ± 0.12
Stress + TRF	69.8 ± 5.0**	0.130 ± 0.006*	2.23 ± 0.06*

All measurements are expressed as mean ± SEM; ** Significantly less than control at $p < 0.001$; * Significantly less than control at $p < 0.01$; Relative weight = weight of the organ (mg) / body weight (g); TRF- tocotrienol-rich fraction

of the dentate gyrus, with the investigator being blind to the treatment group. The optical density for each section was summed and the average value for each animal was then calculated.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science (SPSS, v12) program. The data are presented as mean ± standard error of the mean (SEM). The differences between the groups were analysed by one-way ANOVA. When appropriate, the main effects of treatment were further assessed using Tukey's *post hoc* tests. A p value of less than 0.05 was considered statistically significant.

RESULTS

### Effects on body weight, adrenal gland and the thymus

To confirm the physiological efficacy of the stress procedure, we measured body weight and the weight of the adrenal and thymus glands. Exposure to restraint stress 5 hours daily for 21 consecutive days resulted in a significant reduction in body weight ($p < 0.001$) when compared to the non-stressed animals. As shown in Table I, concurrent TRF supplementation failed to change this.

As shown in Table I, stress exposure significantly increased the relative weight of the adrenal gland (adrenal hypertrophy) and at the same time, caused a significant decline in the relative thymus weight (thymic involution). As with body weight, concurrent TRF supplementation did not significantly change the relative weight of both the adrenal and thymus glands (Table I).

Effect on plasma corticosterone level

Figure 1 shows the effect of stress, TRF supplementation and their combination on the plasma corticosterone level. Plasma corticosterone amounts in rats exposed to restraint stress and in stressed rats supplemented with TRF were much higher than those in the normal control and rats supplemented with TRF alone. These results indicated that there was a significant increase in the plasma corticosterone level in rats exposed to stress compared to those that were not. However, there was no significant difference in the corticosterone level between the stressed group and the stressed group supplemented with TRF. The finding suggests that TRF supplementation is not effective in mitigating the stress–induced elevation of the plasma corticosterone level.
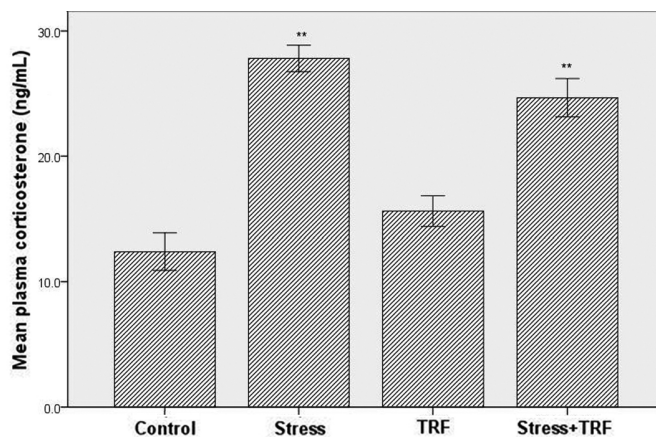
FIGURE 1 – Effects of strees, tocotrienol rich fraction (TRF) supplementation and combination of stress and TRF on the plasma corticosterol level. Value represents mean ± SEM. **indicates significantly different from the control at $p < 0.001$.

Effect on cellular proliferation in the dentate gyrus

The number of actively dividing cells in the subgranular zone of the dentate gyrus was found to be significantly decreased in rats exposed to stress compared to non-stressed rats (Figure 2). To be more specific, there was a marked reduction in the number of Ki-67-positive cells in rats exposed to stress (13 Ki-67-positive cells/section) and in stressed rats supplemented with TRF (17/section), compared to the normal control

(29/section) (Figure 3). Moreover, no significant difference was noted between the stressed group and the stressed group supplemented with TRF (Figure 2), indicating that TRF supplementation failed to alter the rate of cellular proliferation in the dentate.

Effect on granule cell number

The number of granule cells (per mm^2) in the dentate gyrus was significantly reduced in stressed rats relative to non-stressed animals ($p < 0.01$). The number of granule cells in rats exposed to stress (33.78 ± 1.94) and in rats that received both stress and TRF (35.77 ± 2.60) were 31% and 26%, respectively, less than that of the control (48.90 ± 3.29). On the other hand, there was no significant difference in the number of granule cells between animals exposed to stress alone and stressed animals supplemented with TRF (42.56 ± 2.11). This finding suggests that TRF supplementation had no influence on the survival rate of the granule cells in the dentate gyrus.

GAP-43 optical density

Measurement of GAP-43 optical density showed no significant difference between control (OD 0.424 ± 0.014) and stressed (OD 0.437 ± 0.010) rats. Similarly, there were no significant differences between rats treated with TRF alone (OD 0.423 ± 0.011) and those that received both TRF and stress (OD 0.442 ± 0.013). In other words, exposure to chronic stress either alone or in combination with TRF failed to alter the expression of GAP-43 in the outer molecular layer of the dentate gyrus (Figure 4).
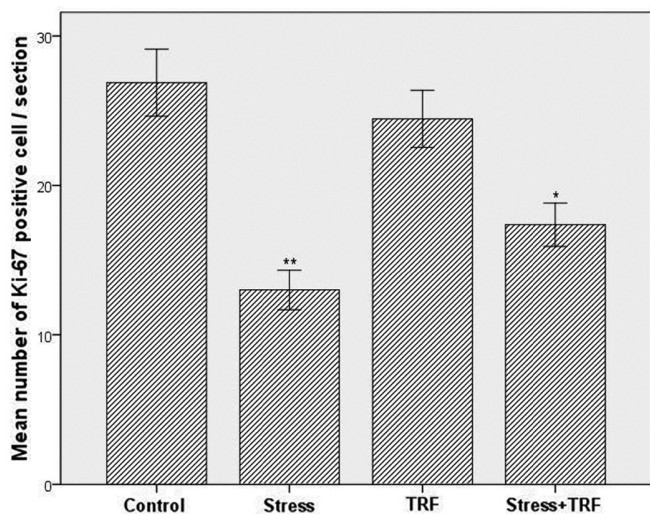
FIGURE 2 – Effects of stress, tocotrienol rich fraction (TRF) supplementation and combination of stress and TRF on the number of Ki-67 immunopositive cells. Value represents mean $\pm$ SEM. * & ** indicate significantly different from the control at $p < 0.01$ and $p < 0.001$ respectively.
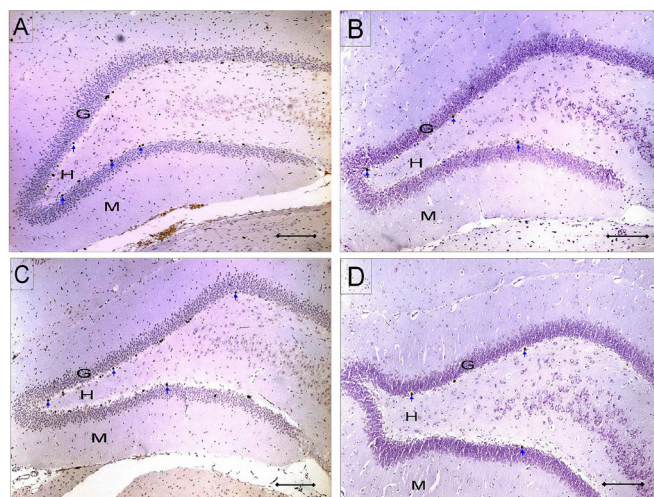
FIGURE 3 – The number of actively dividing cells in the subgranular layer of the dentate gyrus, labelled by the Ki-67 antibody (blue arrows) in the control group (A), stressed group (B), tocotrienol-treated group (C) and the stressed group treated with tocotrienol (D). G – granular cell layer; H – hilus of the dentate gyrus; M – molecular layer. Scale bar represents 100 μm.
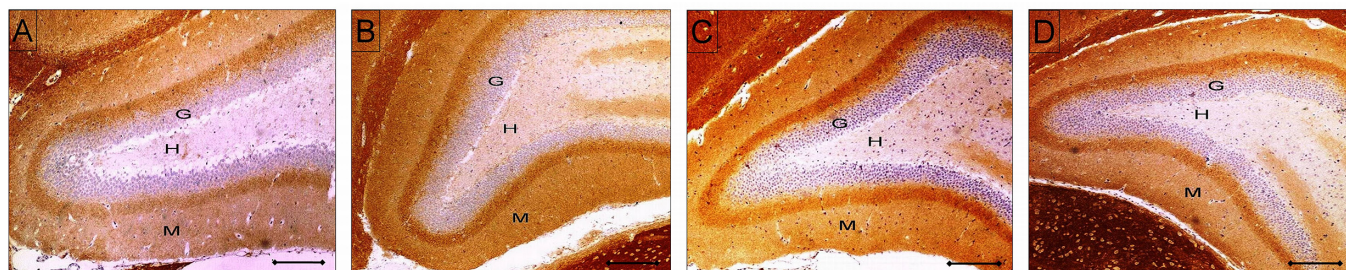
FIGURE 4 –GAP-43 immunoreactivity in the various layers of the dentate gyrus under low magnification in the control group (A), stressed group (B), tocotrienol-treated group (C) and the stressed group treated with tocotrienol (D). Note that GAP-43 immunoreactivity was the strongest in the inner molecular layer and the weakest in the granular cell layer. Measurement of GAP-43 optical density was performed on the outer molecular layer of the dentate gyrus. G – granular cell layer; H – hilus of the dentate gyrus; M – molecular layer. Scale bar represents 150 μm.

DISCUSSION

In the present study, daily 5-hour exposure to restraint stress for 21 consecutive days resulted in a significant elevation in the plasma corticosterone level. The stressed rats also displayed a significantly reduced body weight gain, and experienced adrenal hypertrophy and thymic involution. These results indicated the physiological efficacy of the restraint stress employed in the present study, which is in accordance with previous findings (Bauer *et al*, 2001; Watanabe *et al*., 1992).

Another important effect of chronic restraint stress on rats is its suppressing influence on neurogenesis in the hippocampus, often measured using two mitotic biomarkers, 5-bromo-2-deoxyuridine (BrdU) and Ki-67 (Kee *et al.*, 2002; Rosenbrocke *et al.*, 2005). Although we cannot draw definitive conclusions on the effects of stress on neurogenesis in this study as Ki-67 was not co-localize with the markers of mature neurons (for example NeuN), our results did conclusively show that exposure to chronic restraint stress suppressed the number of proliferating cells in the subgranular layer of the dentate gyrus. Similarly, exposure to chronic stress elicits a significant reduction in the number of granule cells, which is in accordance with previous studies (Jayatissa *et al.*, 2008). However, the present study failed to detect significant changes in GAP-43 optical density in the dentate gyrus after restraint stress exposure. This is in line with the findings of Rosenbrocke *et al.* (2005), who found no significant changes in both synaptophysin and GAP-43 after chronic restraint stress exposure.

We demonstrated that TRF supplementation failed to prevent stress-induced reduction of body weight gain and changes in the adrenal gland and thymus weights. In addition, TRF was not effective in preventing stress-induced elevations in plasma corticosterone levels. Together, these results suggest that TRF supplementation does not directly antagonise the actions of corticosterone. Moreover, TRF supplementation is not effective in preventing the inhibitory effect of chronic stress on cell proliferation in the dentate gyrus. Likewise, TRF failed to influence granular cell numbers in the dentate gyrus after stress exposure. Overall, these data show that TRF supplementation does not provide significant protection against the effects of chronic restraint stress on the dentate gyrus.

The result of the present study is somewhat contrary to the finding of Nur Azlina *et al.* (2005). In their study, TRF supplementation was found to effectively block stress-induced changes in gastric acidity and gastrin levels, thus maintaining gastric mucosa integrity in rats exposed to 4 days of restraint stress. Similar results were reported by Ibrahim *et al.* (2008) using acute water immersion restraint stress. Moreover, Nur Azlina and Nafeeza (2008) showed that TRF could suppress stress-induced increases in plasma corticosterone amounts. Even more surprisingly, TRF was able to prevent the rise in plasma noradrenalin following acute stress exposure. However, the discrepancy between the present and earlier studies could be related to the differences in the duration of stress exposure, although the dose of TRF we used here was 3.3 times higher (200 mg/kg) than that (60 mg/kg) used in the previous studies. The TRF dose of 200 mg/kg used in the present study was carefully selected as numerous prior studies have shown it to be effective. For instance, Tiwari *et al.* (2009) found that a TRF dose as low as 50 mg/kg protected the hippocampus from the damaging effects of chronic ethanol consumption, although the higher dose of 200 mg/kg provided the most protection. Sandra *et al.* (2008), using a slightly higher dose, revealed that 100 mg/kg of TRF was an effective antioxidant in the bone. Similarly, Budin *et al.* (2009) reported that 200 mg/kg of TRF improved dyslipidemia, reduced oxidative-stress marker levels and prevented the progression of vascular wall changes in diabetes mellitus.

In summary, the present study confirmed that exposure to restraint stress for 21 consecutive days induced significant morphological changes in the dentate gyrus of rats. Although TRF has been shown to have numerous beneficial effects on human health (Sen *et al*., 2007), it was not effective in preventing or minimising stress-induced changes in the dentate gyrus of rats in this study.

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