

ISSN: 0976-3031

Available Online at <http://www.recentscientific.com>

CODEN: IJRSFP (USA)

International Journal of Recent Scientific Research
Vol. 8, Issue, 5, pp. 17097-17103, May, 2017

International Journal of
Recent Scientific
Research

DOI: 10.24327/IJRSR

Research Article

THE POTENCY OF HIBISCUS SABDARIFFA LINN. ON DECREASED MEMORY FUNCTION RELATED TO THE LEVEL OF BDNF AND CREB IN HIPPOCAMPUS OF OVERTRAINED RATS

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DOI: <http://dx.doi.org/10.24327/ijrsr.2017.0805.0281>

ARTICLE INFO

Article History:

Received 18th February, 2017

Received in revised form 10th

March, 2017

Accepted 06th April, 2017

Published online 28th May, 2017

Key Words:

Overtraining, *H. sabdariffa*, memory, BDNF and CREB

ABSTRACT

Athletes suffering from overtraining syndrome is quite high, and is often related to learning and memory disorders, caused by oxidative stress. This study was conducted to determine the molecular mechanism that bridges the preventive effect of learning and memory disorders by *Hibiscus sabdariffa Linn.* by measuring levels of BDNF and CREB in hippocampal tissue of rats given overtraining exercise. 20 male rats *Rattus norvegicus* (200-250 g) and 8-10 weeks of age were divided into 4 groups randomly, 1) the control group (C), 2) control group with *H. sabdariffa* (C-Hib), 3) group given overtraining aerobic exercise (OT), 4) the group treated as OT group plus the provision of *H. sabdariffa* dose 400 mg/kg/day (OT-Hib). There was a decreased memory function in the OT group based on the travel time and total number of error compared to the C and C-Hib group, although there was no significant difference in the travel time between the OT and the C and C-Hib group ($p > 0.05$). The highest CREB level was found in the OT-Hib group that was significantly different with the OT group ($p < 0.05$). There was a significant decrease of BDNF levels in the OT group and OT-Hib compared to the C-Hib group ($p < 0.05$). In the OT-Hib group, the MDA levels still higher significantly and GPx levels lower significantly compared to C and C-Hib group ($p > 0.05$). In overtraining rats, provision of *H. sabdariffa* can activate CREB but the BDNF still declined and memory function not much improved.

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INTRODUCTION

An athlete will experience a long period of training to deal with a game that aims to improve the physical condition and technical skills in accordance with their sport. However, if the load exceeds a given workout ability then the athlete can experience the overtraining syndrome that is characterized by decreased physical, emotional, and immunological capacity.

The incidence of athletes suffering from overtraining syndrome is quite high. Previous research showed that about 10-20% of elite endurance athletes, 60% of long-distance runners, and 50% of football players suffer from the syndrome overtraining (Kreher *et al.*, 2012 and Morgan *et al.*, 1987). This is why many athletes experience a loss in performance. The decline in performance is often related to learning and memory disorders (Smith., 2000). Learning and memory are required by movement techniques to correct mistakes and develop a strategy in competition. Learning and memory disorders will

cause the athlete to make repeated mistakes so that performance is not optimal. Therefore, prevention efforts should be made so that an athlete is not impaired in learning and memory due to overtraining syndrome. Many theories have been introduced as a cause of overtraining syndrome including oxidative stress (Mc Cord., 2000). This happens because strenuous or long-term exercise increases oxygen consumption and the production of reactive oxygen species (ROS) causing an imbalance between ROS formed and the capacity of the body's antioxidant defense system. Therefore, athletes who perform strenuous exercises require external antioxidants to prevent overtraining. One of the antioxidant efforts is *Hibiscus sabdariffa Linn.* or red tea that contain anthocyanins, beta carotene, ascorbic acid, thiamine, riboflavin, flavonoids, and niacin (Maryani and Kriatiani., 2008). It was shown in previous studies that *H. sabdariffa* 400 mg/kg/day in mice can prevent the syndrome overtraining since plasma MDA decreased and IGFBP3 did not increase, therefore

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IGFBP3 can be used as an indicator of overtraining syndrome (Ilyas et al., 2014). However the mechanism of *H. sabdariffa* in preventing disorders associated with the disturbance of mechanisms or proteins involved in learning and memory is still unknown.

The process of learning and memory associated with synaptic plasticity, especially in the hippocampus. BDNF is a growth factor that plays a role in regulating the plasticity of synapses and inducing long-term potentiation and these two roles shape the long-term memory (Mc Cord., 2000). Our previous study found that overtraining could decrease BDNF levels and impaired memory in rats (Nilasanti et al., 2014). BDNF is regulated by a cellular transcription factor that is cyclic AMP response element binding protein (CREB). CREB interacts with CREB binding protein (CBP) to activate transcription of target genes, i.e. BDNF. CREB plays a role in synaptic plasticity and long-term memory (Mizuno et al., 2002; Brodie., 2004; Rosethorne et al., 2008; Bitner., 2012). This study was conducted to determine the molecular mechanism that bridges the preventive effect of learning and memory disorders by *H. sabdariffa* by measuring levels of BDNF and CREB in hippocampal tissue of rats given overtraining exercise.

MATERIAL AND METHODS

Experimental Animals

20 male rats *Rattus norvegicus* weighing 200-250 g and 8-10 weeks of age were randomly divided into 4 groups: 1) control group (C); 2) control group with *H. sabdariffa* (C-Hib); 3) the group of rats given overtraining aerobic exercise (OT); 4) the group of rats given overtraining aerobic exercise plus *H. sabdariffa* 400 mg/kg/day (OT-Hib). Before and during treatment, the rats were treated according to the ethics guide for the care and use of laboratory animal issued by the National Institute of Health. The rats were kept in cages (6 animals per cage) in accordance with standard treatment such as eating, drinking ad libitum, light-dark cycle of 12h each, room temperature $23\text{C}\pm 1^{\circ}\text{C}$. Rats underwent an acclimatization process for 2 weeks prior to treatment to reduce stress at the time of the study. In particular, the animal obtained the same treatment as reported in our previous study (Ilyas et al., 2017). During this period we also introduced all the experimental animals to the water-E maze test to minimize stress at the time of testing of the capability of memory. The introduction to the water- E maze was done every day for 2 consecutive weeks. We placed the rats on the start point and let them swim to locate the stairs. The rats were allowed to swim for a maximum of 1 minute, 3 times each session. This research passed the Health Research Ethics Committee of the Faculty of Medicine-RSCM no. 403c/UN.2F1/ETIK.IV/2015.

Extract *Hibiscus sabdariffa* Linn.

H.sadariffa extract was obtained from the Research Center of Spices and Medicinal Plants, Agricultural Research and Development Agency, Ministry of Agriculture of Indonesia. The process of making the extract starts by mixing 1 kg of calyx pieces with 5 liters of water for 3 hours. The mixture is allowed to stand for 24 hours at room temperature and is finally filtered through paper filter. The extract of *H sabdariffa* was given at a dose of 400 mg/kg/day to C-Hib and OT-Hib groups

for 11 weeks. Before experiments, the rats were weighed to determine the amount of *H.sabdariffa* to be administered orally once a day using a cannula with a gastric feeding tube.

Overtraining Procedure

Overtraining is aerobic exercise using a treadmill with duration and speed increased gradually over 11 weeks referring to the procedure used by Hohl (Hohl et al., 2009). Before treatment, the rats went through adaptation for one week, i.e., running on the treadmill with the speed and duration increased gradually not exceeding 10m/min and a maximum of 10 min.

Memory Test Procedures

Measurement of memory was performed before and after treatment in each group using the water-E maze procedure modified by Surjono (Surjono., 1997). Water-E maze consists of three E-shaped arms made of glass with a size of 30cm x 60cm x 70cm. There is a ladder placed inside the water-E maze as a motivator to achieve the target. The water-E maze consists of main trench (U) and 3 arm trenches perpendicular to the main trench (Figure 1). The length of the main trench is 125cm, the middle trench is 35cm and the two side trenches are 25cm, each. The width of each trench is 25cm and the height of the maze is 60cm. Water-E maze is filled with water until the rats cannot touch the bottom of the maze. In each memory test, rats performed three repetitions in a row without a break. The test is based on the time reaching the target in the form of steps and the number of mistakes done. The types of mistakes made are divided into backing error = B (flipping the direction of motion, turning toward the correct target and going back to the start), selection error = S (direction of motion opposite to the target but not entering the door barriers), zoning error = Z (direction of motion off the door entry barriers) (Figure 1). Total error is the sum of the three types of errors made (B + S + Z), for each treatment group the average of total errors was calculated and the average time to reach the target. Memory test was performed in all groups of experimental animals. The tests were performed initially, i.e., at the beginning of the study by each of the 4 groups (C, C-Hib, OT, OT-Hib), and then before physical exercise was given to the training groups, every week from the end of week 1 up to the end of week 11 during the study.

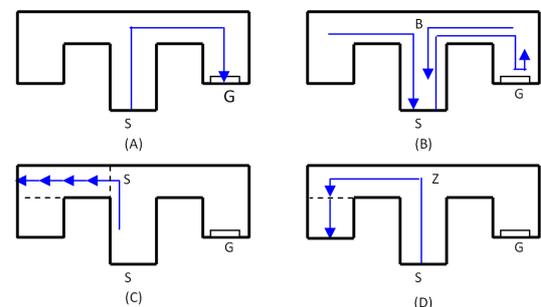


Figure 1 Direction of movement of experimental animals; S = start, G = goal; (A) correct direction of movement; (B) backing error, (C) selection error, (D) zoning error.

After testing the rats decapitated to take brain tissue, followed by isolation procedures and hippocampus homogenizing. The scalp was incised to take intact brain tissue. Brain was cut into three parts. Hippocampus sections were taken from the middle part of the brain. The hippocampus was wrapped in aluminum

foil and stored in a refrigerator at a temperature of -80°C . Making homogenates by adding a solution of 1 ml PBS 0.1M at 100mg hippocampus. The homogenate was centrifugated at a speed of 5000 rpm for 5 minutes to obtain the supernatant.

Measurement of parameters

Malondialdehyde (MDA) and Glutathione peroxidase (GPx) measurements

Using the supernatant of brain tissue MDA levels were measured with Will's method (Will., 1966), and GPx levels were determined by GPx Backpack Kit [RS 505] (Ammerman et al., 1980). The measurements were done in the Department of Biochemistry and Molecular Biology of Medical Faculty, Universitas Indonesia.

BDNF measurement

BDNF protein was measured using ELISA kit (ABNOVA). Samples of homogenates of the hippocampus in standard dilution buffer were incubated on plates at 37°C for 90 minutes. BDNF antibody was added and incubated for 60 minutes followed by washing with 0.01 M TBS. Afterwards, ABC working solution was added, incubated for 30 min and washed 5 times using 0.01 M TBS. Coloring agent TBM was added and incubated in the dark for 30 minutes. TBM reaction was stopped and read at wavelength $\lambda = 450\text{nm}$.

CREB protein measurement

CREB protein was measured using a rat CREB ELISA kit (MYBIO SOURCE/MBS2504589). Samples of homogenates of the hippocampus in standard dilution buffer were incubation on plates at 37°C for 90 minutes. Biotinylated detection ab antibody was added and incubated for 60 minutes, followed by washing 3 times. Afterwards, HRP conjugate was added, incubated for 30 minutes, and washed five times. Substrate reagent was incubated for 15 minutes, then stop solution added and read at wavelength $\lambda = 450\text{nm}$.

Statistic analysis

Data are shown as mean values \pm SD. One way ANOVA analysis was used followed by Post Hoc LSD. Statistical significance was set to $p < 0.05$.

RESULTS

Effect of *H. sabdariffa* on Memory Function

E-Maze test results show an increase in travel time (Figure 2) and in the number of errors (Figure 3) in the OT group vs controls (C) and (C-Hib).

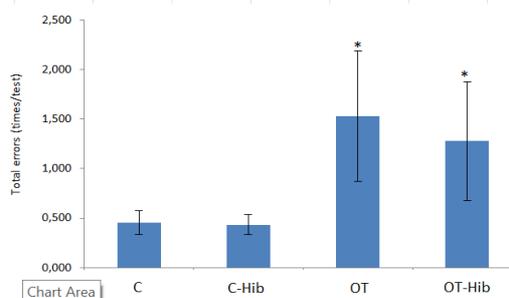


Figure 2 Effect of *H. sabdariffa* on memory function based on travel time in the overtraining model; C = control; C-Hib = control with *H. sabdariffa*; OT = overtraining; OT-Hib = overtraining with *H. sabdariffa*. Each bar shows the average and standard deviation of 5 rats per group.

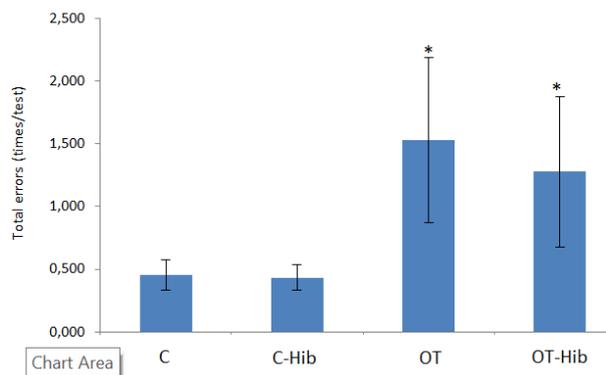


Figure 3 Effect of *H. sabdariffa* on memory function based on the total number of errors in the overtraining model; C = control; C-Hib = control with *H. sabdariffa*; OT = overtraining; OT-Hib = overtraining with *H. sabdariffa*. Each bar shows the average and standard deviation of 5 rats per group; *= significant vs C and C-Hib, $P < 0.05$.

Differences in travel time are statistically not significant between all groups, whereas the increase in the number of errors differs significantly in the OT and OT-Hib groups vs. C and C-Hib ($p < 0.05$). The number of errors decreases in the OT-Hib group vs the OT group but this difference is not significant (Fig. 3). In the overtraining group (OT), there is a significant decline in memory function, which is slightly, but not significantly, ameliorated by *H. sabdariffa* in the OT-Hib group.

Effect of *H. sabdariffa* on BDNF Levels in the Hippocampus

Analysis of BDNF measured by ELISA indicated that there are decreased levels of BDNF in the hippocampus in the groups of overtraining (OT) and overtraining with *H. sabdariffa* (OT-Hib) vs. control with *H. sabdariffa* (C-Hib) ($p < 0.05$). (Figure 4). The slight increase of BDNF in OT-Hib vs OT is statistically not significant.

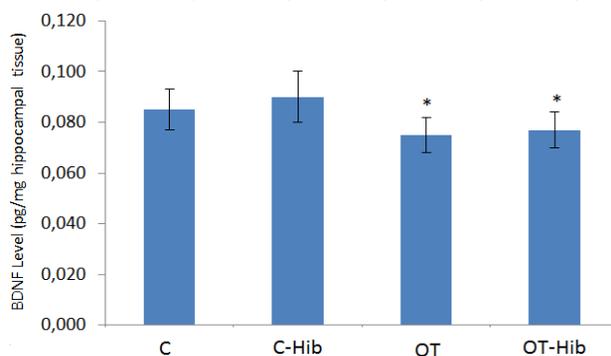


Figure 4 Effect of *H. sabdariffa* on levels of brain derived neurotrophic factor (BDNF); C = control; C-Hib = control *H. sabdariffa*; OT = overtraining; OT-Hib = overtraining with *H. sabdariffa*. Each bar shows the average and standard deviation of 5 rats per group; *= significant vs C-Hib, $P < 0.05$.

Effect of *H. sabdariffa* on CREB Levels in Hippocampus

Based on the results of ELISA measurements, there are significantly decreased levels of CREB in the hippocampus in the group of overtraining (OT) vs control groups (C) and (C-H) (Figure 5). Giving *H. sabdariffa* to rats in overtraining exercise (OT-Hib) increased CREB significantly vs OT ($P < 0.05$) even to higher levels than controls and C-Hib.

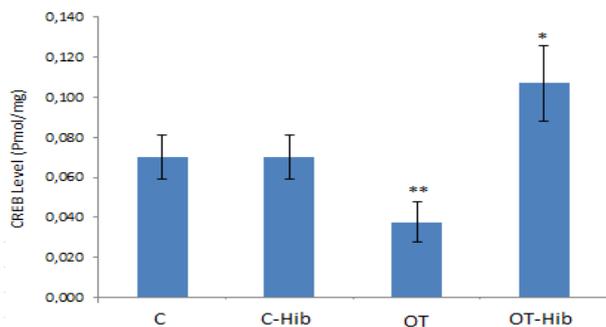


Figure 5 Effect of *H. sabdariffa* on cyclic AMP response element binding protein (CREB) levels; C = control; C-H = control with *H. sabdariffa*; OT = overtraining; OT-Hib = overtraining with *H. sabdariffa*. Each bar shows the average and standard deviation of 5 rats per group; *= significant vs OT, **= significant vs C, C-Hib, OT-Hib (p<0.05).

Effect of *H. sabdariffa* on MDA Levels and GPx Activities in Hippocampus

Table 1 shows that the OT group has the highest levels of MDA (0.21 ± 0.01 nmol/mL) compared with other groups. MDA levels in the OT differ significantly from the C (0.17 ± 0.012 nmol/mL) and C-Hib (0.17 ± 0.026 nmol/mL), with $p < 0.05$. Giving *H. sabdariffa* to the overtraining group (OT-Hib) can prevent increased levels of MDA (0.18 ± 0.03 nmol/mL) which did not differ significantly from groups C and C-Hib.

The OT group (7.5 ± 1.83 U/mg protein) had the lowest levels of GPx in comparison with other groups, differing significantly from group C-Hib (9.93 ± 0.59 U/mg protein), whereas the difference with group C (8.80 ± 1.12 U/mg protein) was not significant. In group OT-Hib the level of GPx (8.69 ± 0.35 U/mg protein) was not significant different from groups C (8.80 ± 1.12 U/mg protein) and C-Hib (9.93 ± 0.59 U/mg protein).

Table 1 The levels of MDA and GPx activities

| Group | MDA levels [nmol/mL] | GPx activities [U/mgprotein] |
|----------------------|----------------------------------|------------------------------|
| C | 0.17 ± 0.012 | 8.80 ± 1.12 |
| C-Hib | 0.17 ± 0.026 | 9.93 ± 0.59 |
| OT | $0.21 \pm 0.01^*$ vs C and C-Hib | $7.5 \pm 1.83^{**}$ vs C-Hib |
| OT-Hib | 0.18 ± 0.03 | 8.69 ± 0.35 |
| Significance, p<0.05 | *significant difference | *significant difference |

Abbreviations: C, control group; C-Hib, control-*H. sabdariffa*; OT, overtraining exercise; OT-Hib, overtraining exercise-*H. sabdariffa*.

DISCUSSION

The aim of this study was firstly to see the effect of overtraining exercise on memory function related to the levels of BDNF and CREB and secondly to see the potency of *H. sabdariffa* on decreased memory function related to the level CREB and BDNF in hippocampus of rats in our overtraining exercise model that caused by oxidative stress.

All experimental animals performed the water-E maze memory test according to Surjono and subsequently, we calculated the average total errors committed and the average of travel time to reach the target (Surjono, 1997). However, to know the mechanism, it is important to know about the interference of

molecular mechanisms involved in learning and memory, determined by the levels of CREB and BDNF.

In the water-E maze memory test presented in Figures 2 and 3 the average travel time and number of errors were highest in the OT group. Our findings are consistent with the symptoms of athletes experiencing memory impairment such as the inability to find a standardized achievement/performance, loss of concentration, repetition of the same mistakes, decreased capacity of differentiation and correcting errors of technique, and changes in the retention learning (Smith, 2000; Fry et al., 1991). The results are also consistent with the findings by other studies (Eich, 2009; Rosa et al., 2007) that the strenuous exercise impairs memory. This is due to impaired LTP process by prolonged overtraining-caused oxidative stress (Wang and Michaelis, 2010).

Administration of *H. sabdariffa* extract to rats in the OT group slightly reduced travel times but this test did not reveal significant differences between all groups. The number of errors was also reduced in the OT-Hib group as compared to OT, although not significant and still significantly higher than controls.

Anyway, our study showed that reduced memory function could be detected in the overtraining exercise group and slightly reversal improvement by *H. sabdariffa* extract in the OT-Hib group. Although *H. sabdariffa* in this study was likely to reduce oxidative damage caused by exercise overtraining, it is still not optimal for preventing interference with the function of memory.

The CREB and BDNF are interacted proteins because they activate reciprocally. The CREB regulates the gene transcription of BDNF genes through the MAPK pathway while BDNF phosphorylates the CREB after activating the BDNF by the Trk pathway. Physical exercises can activate the CREB via MAPK and PI3-K / Akt pathways since they are the major signaling pathways activated by TrkB receptors (Molteni et al., 2002; Shen et al., 2001). However, overtraining exercise can lead to stress and inflammation and subsequently will impact various protein signaling including the CREB (Cunha et al., 2006).²³ Studies by Shen and Aquiar, showed that the CREB levels were lower in high intensity exercise groups compared to controls (Shen et al., 2013; Aquiar et al., 2010).

Oxidative stress caused decreased levels of CREB protein and mRNA in cultured rat hippocampal neurons (Pugazhenthil et al., 2003). This is because the provision of hydrogen peroxide in cultured hippocampal tissue will reduce the binding of CREB in DNA (Zou and Crew, 2006). In this study, we found significantly decreased CREB protein in the group of overtraining (OT) and we assume that it was caused by increased ROS that can penetrate the brain barrier causing oxidative stress in the brain. The mechanism of oxidative stress in the brain is still unknown, but possibly due to increased IL-1 β resulting in damages, atrophy, and dysfunction of nerve cells (Lynch, 2003).

Such damages or atrophy of nerve cells in the hippocampus may lead to a decrease in the amount of protein such as the CREB and the provision of *H. sabdariffa* can counteract this effect in rats with overtraining exercise. In this study, we

observe significantly increased levels of CREB in the OT-Hib group vs. OT. The direct effect of *H. sabdariffa* on the CREB is still not known. Flavonoids and anthocyanins can increase neurogenesis in the hippocampus of rats. One study, found effects of anthocyanins in blueberries on hippocampal CREB phosphorylation and BDNF levels (William *et al.*, 2008). Similarly, other study found an increase in CREB in rats fed flavonoids (Rendeiro *et al.*, 2013). Flavonoids can activate the ERK-CREB-BDNF pathway (Chen and Russo, 2005). In addition to the flavonoid effect, the increased CREB is caused by moderate physical exercises as well. The provision of *H. sabdariffa* is able to eliminate the ROS that is developed by overtraining exercise. The physical exercise stimulates the activation of various signaling pathways such as MAPK and PI3-K / Akt and they activate the CREB (Nibuya *et al.*, 1995). They also showed that physical exercise influences the up-regulation of various signaling molecules such as CaMKII, MAPK I & II that phosphorylate the CREB and the signaling is activated by BDNF-induced TrkB receptors (Nibuya *et al.*, 1995).

Neurotrophin BDNF contributes to synaptic plasticity and long term potentiation to form long term memory. This study found that there are decreased BDNF levels in the overtraining group. Overtraining exercise can lead to oxidative stress-induced damage to proteins and DNA (Marqonis *et al.*, 2007). DNA damage can impair multiple genes encoding a protein defective transcription process, in particular the expression of several proteins in the brain, including the disruption of BDNF (George and Osharechiren, 2009). It was evidenced by that overtraining exercise induces chronic oxidative stress and causes decreased levels of BDNF in the hippocampus (Dong *et al.*, 2013).

As we know, overtraining exercise causes increased levels of MDA, a marker of oxidative damage of cell membranes, indicating exercise-induced ROS, which can trigger oxidative damage in the brain. Our study found increased levels of MDA and decreased activity of GPx in the overtraining group. The oxidative damage due to increased ROS interacts diversely with the endogenous antioxidant enzyme GPx in the brain (Halliwell and Gutteridee., 1998; Marinho *et al.*, 1963; Cohen and Hochstein, 1963; Katayama *et al.*, 1997).

In this study, *H. sabdariffa* reduced oxidative stress in overtraining exercise. Nevertheless, it does not yet seem optimal for preventing interference with the function of memory. In the E-maze test rats with overtraining exercise fed *H. sabdariffa* still had a significantly higher number of errors than controls although the travel time was not significantly different from controls. *H. sabdariffa* may prevent or reduce oxidative damage in rats with overtraining exercise, since in our study, increased MDA levels in the OT group were decreased again to almost normal level of controls. This is due to flavonoids, especially anthocyanins contained in *H. sabdariffa* flower petals (Tsai *et al.*, 2002). The high antioxidant potency is also supported by other studies suggesting that the antioxidant activity of Roselle calyx inhibits lipid peroxidation rate (Thadeus., 2006).

In addition, the provision of *H. sabdariffa* can maintain levels of GPx in overtraining exercise group. This is because the potential of flavonoids that can increase GPx activity through increased gene expression of endogenous antioxidants. Flavonoids activate nuclear factor erythroid 2 relates factor 2

(Nrf2) resulting in an increase in genes involved in the synthesis of endogenous antioxidant enzymes, such as GPx (Amin and Hamza, 2005).

CONCLUSION

In a state of overtraining, provision of *H. sabdariffa* could activate CREB and slightly improve memory function, although the BDNF was still declined.

Acknowledgement

We express our thanks to Prof. Dr. Hans-Joachim Freisleben for his valuable help during preparation of our manuscript. Also to DRPM UI for having funded this study through a grant from Universitas Indonesia 2013 (Hibah Riset Madya UI 2013).

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How to cite this article:

Ermita I.Ibrahim Ilyas *et al.* 2017, The Potency of *Hibiscus Sabdariffa* Linn. On Decreased Memory Function Related To The Level of Bdnf and Creb In Hippocampus of Overtrained Rats. *Int J Recent Sci Res.* 8(5), pp. 17097-17103.

DOI: <http://dx.doi.org/10.24327/ijrsr.2017.0805.0281>
