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Sea Urchin (*Diadema setosum*) Chocolate Snack on Haemoglobin, Body Weight, and Brain Characteristics in Mice (*Mus musculus*)

A.A. Denaldo[1], A. Syauqy[2] and D. Pringgenies[3*]

[1]*Student Undergraduate, Department of Nutrition Science, Faculty of Medicine, Diponegoro University, Semarang, Indonesia*

[2]*Department of Nutrition Science, Faculty of Medicine, Diponegoro University, Semarang, Indonesia*

[3]*Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang, Indonesia*

This study investigated the effects of *Diadema setosum* gonad-based chocolate products on amino acid content, body weight, haemoglobin levels, neuronal cell count, brain weight, and brain volume in Mus musculus. A particular focus was placed on evaluating neurodevelopmental impacts and nutritional benefits. Amino acid composition of the *D. setosum* gonad product was analysed using high-performance liquid chromatography (HPLC). Male rats were divided into groups receiving 0.5 g, 1.0 g, or 1.5 g/subject/day of gonad chocolate, with or without fish oil, for 70 days. Measurements included body weight, haemoglobin level, brain weight and volume, and neuronal cell count via histological analysis. The gonad product contained high levels of alanine, glutamic acid, and glycine. The highest body weight increase occurred in the negative control group (39.4 g). The highest haemoglobin level was recorded in the P2 group (13.15 g/dL), which received 1.0 g/day of the gonad product. Brain weight peaked in the negative control group (0.485 g), while the highest brain volume was in the P3 group (3.25 mL). The highest neuronal cell count was also observed in P2 (838 cells). ANOVA results showed no significant differences in body weight, haemoglobin, brain weight, or brain volume. However, a substantial increase was found in neuronal cell count ($p < 0.05$). Conclusion: Administration of 1.0 g/day *D. setosum* gonad chocolate significantly increased haemoglobin levels and neuronal cell count in rats, suggesting potential neuroprotective and haematological benefits. Further research is warranted to explore its applicability in human nutrition and cognitive health.

Keywords: amino acid; gonad; HPLC; neuron cell; nutrition

I. INTRODUCTION

Sea urchin is a sea creature whose gonads contain several amino acids such as valine, threonine, glycine, histidine, alanine, glutamic acid, lysine, leucine, I-leucine, methionine, tyrosine, and arginine (Liyana-Pathirana *et al.,* 2002; Camacho *et al.,* 2023). Although there are several types of sea urchin, the content of amino acids in the gonads of the *Diadema setosum* species is known to be the highest compared to other kinds of sea urchin. Amino acids such as Arginine and Histidine are essential amino acids for the growth and development of children, particularly brain development (Wu, 2013; Semba *et al.,* 2016). They are involved in synthesising neurotransmitters for brain and nerve functions. On the other hand, Sea urchin gonads are also rich in polyunsaturated fatty acids (PUFAs) (Kabeya *et al.,* 2017; Wang *et al.,* 2021), reaching up to 40% of the total fatty acid content and long-chain (LC-PUFAs) are vitally important during human brain development. Thus, intake is essential for the development of the brain's nervous system.

*Corresponding author's e-mail: pringgenies@yahoo.com

The administration of *D. setosum* can increase mice's body weight and provide a significant percentage of neurons and haemoglobin (Pringgenies *et al.*, 2016). Thus, the research results are very beneficial for the growth and intelligence of young children. Toddler is a general term for children aged 1-5 years and toddler age is a golden age for parents to stimulate children because a process of brain development is going on related to children's intelligence and creativity (Fox *et al.,* 2011; Haartsen *et al.,* 2016). At this time the brain can develop rapidly both in terms of structural and functional (Gilmore *et al.*, 2018).

The nutrients most influential for toddlers' growth are proteins and fats (Braun *et al.*, 2016; Smith-Brown *et al.*, 2018). Adequate intake of essential amino acids is needed for optimal growth and repair of the toddler's muscle tissue. Amino acids that play a role in this are valine, leucine, and isoleucine. Specifically, the nutrient that supports the growth and development of the brain is omega-3 fatty acids (Dinicolantonio & Keefe, 2020). Brain fatty acids, essential and omega-3 fatty acids, are essential nutrients that must be met. Nutrients play a vital role in the growth and development process of the brain's nerve cells for children's intelligence (Georgieff *et al.,* 2018), and this content is found in the gonads of *D. setosum* (Ning *et al.,* 2022; Zhang *et al.,* 2022). In the Indonesian community, there is a fact that toddlers are fonder of consuming junk food such as candy, fast food, nuggets and chocolate. Commercial chocolate products usually contain low levels of protein and fatty acids, making it easy for children to get full and affecting their appetite. Based on this, it would be interesting to create a nutritious and beloved food product for children with a combination of *D. setosum* gonad chocolate. Therefore, this research aims to investigate the amino acid content of *D. setosum* gonad chocolate products and its influence on the body weight, haemoglobin count, neuron cell number, brain weight and volume of the *Mus musculus* subjects.

II. MATERIALS AND METHOD

A. Materials

The products used in the treatment in this study are gonad sea Urchin chocolate products. The outputs (outcomes) studied are the levels of amino acids in the development, haemoglobin levels, body weight, brain volume and weight, and the number of mouse brain cells, *Mus musculus*. The outcomes studied were amino acid levels during development, haemoglobin levels, body weight, brain volume and weight, and the number of brain cells of *Mus musculus* with ethical clearance letter number: 100/EC-H/KEPK/FK-UNDIP/VIII /2023. The study was divided into several groups; the division of groups included the negative control group (K-), which was only given a standard feed; the positive control group (K+), which was given high-fat meal, fish oil (Maulana *et al.*, 2014). The treatment group (P) sea hedgehog gonad chocolate *D. setosum* involved three treatments, namely 0.5 g, 1 g, and 1.5 g. The experiment was conducted using mice with an average body weight of approximately 20 g. The dosage and treatment (with 5 groups and 3 replications each) referred to the study by Pringgenies (Pringgenies *et al.*, 2016).

B. Production of Sea Urchin Gonad Chocolate

Production of sea urchin gonad chocolate (*D. setosum*) begins with sifting and sieving the dry sea urchin gonad powder using a 100-mesh sieve and then weighing it according to the concentration. Then the chocolate bar is melted by time method. The melted chocolate is mixed with the gonad powder using a mixer until evenly mixed. Add sea urchin gonad extract to the melted chocolate paste with a ratio of 70% chocolate and 30% extract of gonad. The balance between chocolate and gonad is chosen at 70:30, which proved that the dose had the highest protein content. Then the chocolate paste is poured into moulds and stored in the freezer.

C. The Treatment of Samples

Subjects are divided into five treatment groups, each containing three replications. Subjects were assigned to the control group (K-), treatment group I (given 0.5 g of sea urchin chocolate), treatment group II (1 g of sea urchin chocolate), and treatment group III (1.5 g of sea urchin chocolate). Subjects are divided into five treatment groups, each consisting of three replicates. Subjects are placed in the K- (control) treatment group, the I (given 0.5 g of sea urchin chocolate) treatment group, the II (given 1 g of sea urchin chocolate) treatment group, the III (1.5 g of sea urchin chocolate) treatment group.

The sample dosage (*D. setosum* brown gonadal sea urchin) for each treatment is 0.5 g, 1 g, and 1.5 g, and the fish oil dose for the positive control group is 0.2 g, as seen in Figure 2. Pringgenies *et al.* (2016) demonstrated that a diet of 0.2 g of fish oil is the usual dose, so this was administered for the positive control group. Pringgenies *et al.* (2016) The experimental animals were given the central ransom at 0.005 g/subject/day for seven consecutive days, then provided the treatment by their respective groups for 70 days, which is the time when the mice reach adulthood, and the development of their brain tissue is complete.

D. Amino Acid

Analysis of amino acids from the Gonad of Sea urchins uses High-Performance Liquid Chromatography (HPLC). Following that, haemoglobin level analysis, body weight analysis, brain volume and weight analysis and histology analysis were done by referring to Pringgenies *et al.* (2016). Histology reading was done manually by dividing the sample preparation into ten parts. Then the calculation result was summed up to obtain the total number of brain cells obtained from each treatment group.

E. Statistics Analysis

Primary data collection consists of the results of the Hb concentration test, body weight, brain weight and volume, and mouse cell count. Data collection of mouse body weight starts from before treatment until the end of treatment. Meanwhile, data results of the Hb concentration test, brain weight, brain volume, and mouse cell count are conducted after 70 days of treatment. Data were recapitulated using the Microsoft Excel application and submitted to *SPSS software* (*SPSS*, version 25.1). After 70 days of treatment, to determine the significant intervention, all data were tested using *one-way* ANOVA.

III. RESULT AND DISCUSSION

A. Amino Acid Content of Chocolate from Gonad D. setosum

High-performance liquid chromatography (HPLC) analysis revealed that the gonad-based chocolate of *Diadema setosum* contains a rich amino acid profile. The dominant amino acid was alanine (33.15%), followed by glutamic acid (11.36%), glycine (8.47%), and valine (5.68%). Other amino acids identified in smaller amounts include aspartic acid, leucine, serine, isoleucine, tyrosine, phenylalanine, threonine, and arginine (Figure 1).

This composition highlights the gonad's potential as a source of essential and functional amino acids. Alanine is recognised for its role in energy supply and immune system support, while glutamic acid and glycine contribute to neurotransmitter function and brain health (Das *et al.*, 2022).

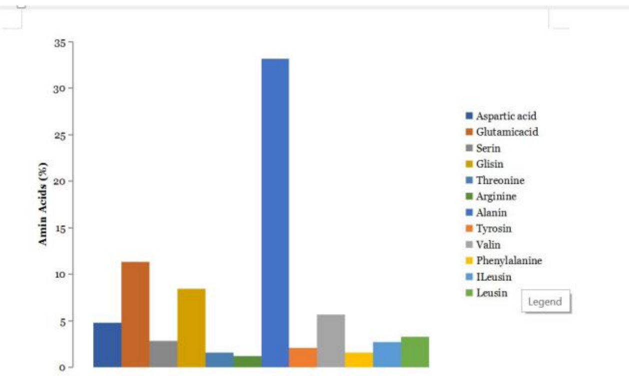

Figure 1. Amino Acid Contents

B. Mice Weight Development

The body weight of *Mus musculus* was measured over a 70-day period across all groups: K– (regular feed), K+ (0.2 g fish oil), and treatments P1 (0.5 g), P2 (1.0 g), and P3 (1.5 g) of gonad *D. setosum* chocolate. The K– group showed the greatest weight gain (39.4 g), followed by P3 (36.6 g), K+ (34.2 g), P2 (33.0 g), and P1 (30.6 g) (Figure 2).

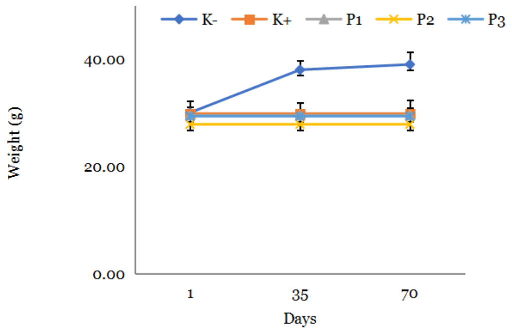

Figure 2. Mice Body Weight Development

One-way ANOVA indicated a statistically significant overall effect of treatment on body weight ($p < 0.05$). However, the Post Hoc Tukey test showed no significant pairwise differences between individual groups ($p > 0.05$). This suggests that while the treatment had a measurable impact, dosage variations did not produce statistically distinct outcomes.

C. Test Haemoglobin Levels

Haemoglobin levels were monitored on days 0, 35, and 70. The P2 group (1.0 g gonad chocolate) showed the highest average haemoglobin level (13.15 g/dL), followed by K– (12.6 g/dL), P3 (12.15 g/dL), P1 (11.75 g/dL), and K+ (10.15 g/dL) (Figure 3).

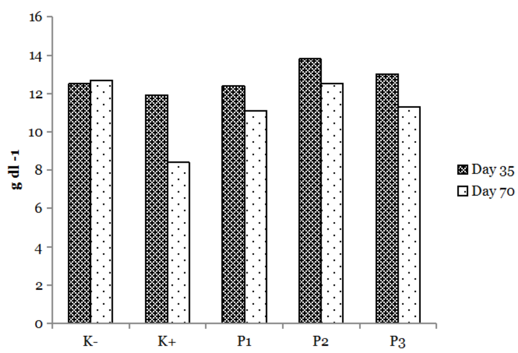

Figure 3. Development of Haemoglobin Levels

The one-way ANOVA revealed a significant overall effect of treatment ($p < 0.05$), although Post Hoc Tukey testing indicated no significant differences between individual groups. These results suggest that the gonad chocolate may support haemoglobin synthesis, likely due to its protein and iron content. In contrast, the low haemoglobin observed in the K+ group could reflect insufficient protein intake, which impairs iron transport and haemoglobin formation (Abbaspour *et al.*, 2014).

D. Brain Weight and Brain Volume Development

The mice's brains were weighed three times on the first day before treatment, day 35 of treatment, and day 70 of treatment. Figure 4 shows that on the first day before treatment (T0), the brain's weight was 0.52 g. On day 35 of treatment, the K- group had the highest weight of 0.47 g, followed by K+ (0.46 g), P3+ (0.46 g), P1 (0.44 g), and P2 (0.44 g). On day 70 of treatment, it was found that the K- group had the highest brain weight of 0.5 g, followed by K+ (0.48 g), P3+ (0.46 g), P2 (0.44 g), and P1 (0.43 g).

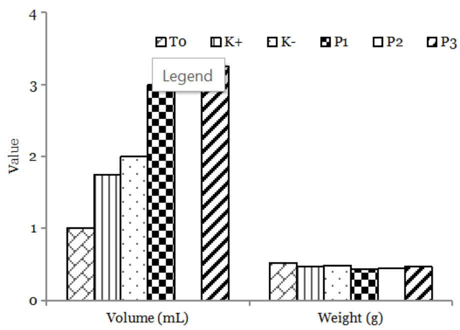

Figure 4. Development of Brain Weight and Volume

Measuring the brain weight of *Mus musculus* subjects from all groups over 70 days under negative control, 0.5 g, 1 g, and 1.5 g of *D. setosum* chocolate treatment and 0.2 g of fish oil treatment in Figure 4 showed that the K- group had the highest average brain weight (0.485 g) compared to P1 (0.435 g), P2 (0.44 g), P3 (0.47 g), and K+ (0.47 g).

Based on the One-Way ANOVA analysis, it showed that there is a significant effect ($P = 0.00 < 0.05$) from the *D. setosum* gonad chocolate treatment on brain weight development. The results from the Post Hoc Tukey test in Figure 4 shows no significant difference between groups with $p > 0.05$ value. The lack of substantial difference indicates that the dosages of gonad chocolate affect brain weight development as it is in average condition.

Brain volume measurements were carried out three times on the first day before treatment, day 35 of treatment, and day 70 of treatment. Figure 4, on the first day before treatment (T0), the brain volume was 1 mL. On day 35 of treatment, it was found that K +, P1, P2, and P3 groups had the same brain volume of 3 mL, followed by the K- group (2 mL). On day 70 of treatment, it was found that the P3 group had the highest brain volume of 3.5 mL, followed by the P1 group (3 mL), P2 group (3 mL), K- group (2.5 mL), and K+ group (2 mL).

Brain volume measurements were carried out three times on the first day before treatment, day 35 of treatment, and day 70 of treatment. Figure 4, on the first day before treatment (T0), the brain volume was 1 mL. On day 35 of treatment, it was found that K +, P1, P2, and P3 groups had the same brain volume of 3 mL, followed by the K- group (2 mL). On day 70 of treatment, it was found that the P3 group had the highest brain volume of 3.5 mL, followed by the P1 group (3 mL), P2 group (3 mL), K- group (2.5 mL), and K+ group (2 mL).

Measurement of the brain volume of mouse *Mus musculus* subjects from all groups for 70 days with control treatment, 0.5, 1, and 1.5 g of gonad *D. setosum* chocolate, and 0.2 g of fish oil shown in Figure 4 revealed that the subject subjected to P3 gonad 1.5 g treatment had the highest brain volume (3.25 mL) when compared with the issues of K+treatment (1.75 mL), K-treatment (2 mL), P1 chocolate gonad 0.5 g treatment (3 mL), and P2 chocolate gonad 1 g treatment (3 mL).

Measurement of mouse brain volume of *Mus Musculus* subjects from all the groups for 70 days with control treatment, 0.5 g, 1 g, and 1.5 g of gonad *D. setosum* chocolate treatment, and 0.2 g fish oil in Figure 4 showed that subjects with P3 gonad 1.5 g treatment had the highest brain volume (3.25 mL) compared to the K+ (1.75 mL), K- (2 mL), P1 chocolate gonad 0.5 g (3 mL), P2 chocolate gonad 1 g (3 mL) treatments.

E. Brain Histology

Observation and measurement of mouse neuronal cells from the control, 0.5 g, 1.0 g, and 1.5 g of gonad *D. setosum* chocolate treatment, and 0.2 g fish oil treatment for 70 days showed that the brain tissue sample from the 1.0 g gonad *D. setosum* (P2) subject had the highest total number of neuronal cells (838) when compared to control- (829), P1 (823), P3 (834), and control+ (828) subjects in Figure 5.

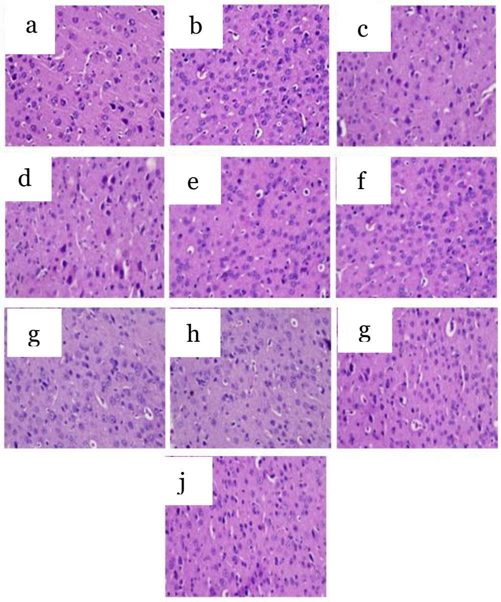

Figure 5. histology results K-.1 (a), K-.2 (b), K+.1 (c), K+.2 (d), P1.1 (e), P1.2 (f), P2.1 (g), P2.2 (h), P3.1 (i), P3.2 (j)

Analysis of the amino acid content of the gonad *D. setosum* chocolate using the HPLC method showed that the highest range is alanine (33.15%) compared to the content of essential amino acids - threonine, valine, I-leucine, and leucine, semi-essential amino acids - histidine, arginine, and non-essential amino acids including aspartic acid, glutamic acid, serine, glycine, and tyrosine.

Amino acids are essential for the functions of the human body, namely for forming the structure of the brain and neurotransmitter substances at the connections of nerve cells. It is known that alanine is a vital source of energy to boost immunity. Furthermore, this essential amino acid is also helpful in supplying power to human muscle, brain, and central nervous system tissues (Das *et al.*, 2022). Glutamate, aspartic, tyrosine, and tryptophan are highly influential in the formation of neurotransmitters, and tyrosine and tryptophan also help regulate and control emotions and intelligence. Tyrosine and tryptophan are the basic building blocks of norepinephrine and serotonin, directly influencing intelligence. Neurotransmitters are chemicals required in the transmission of impulses from one neuron cell to another neuron cell. Other studies have found that amino acids are essential for human development. Amino acids are neurotransmitters that work quickly and accurately and are directed to the brain. Glutamate, aspartate, and glycine are the amino acids that work most effectively as neurotransmitters. Glutamate has been known to play a significant role in maintaining brain development.

The subjects from Group K- in this study were found to have the highest weight gain (39.4 g) compared to issues from the other treatment groups. The administration of *C. setosum* 0.5 g chocolate was found to have the lowest weight gain contribution (30.6 g) compared to doses of 1.0 g (33 g) and 1.5 g (36.6 g) of chocolate, as well as 0.2 g (34.2 g) of fish oil. From the observed weight gain progress, the weights of the P1, P2, and P3 mice groups decreased and increased inconsistently.

Various factors, including nutrition, influence weight gain in an individual. Nutrition is nutrients or nutritional substances found in the feed that enters the individual's body as feed consumption. The weight loss in the chocolate gonad *D. setosum* treatment group was due to sea urchins being rich in healthy but low-calorie nutrition, making sea urchins an appropriate addition to diets to help reduce weight. In addition, chocolate gonads contain omega-3 fatty acids in the sea urchin gonads, which are beneficial in lowering cholesterol levels in the body (Afifudin *et al.*, 2014).

Over the 70-day treatment, haemoglobin levels varied among groups. The P2 group (1.0 g gonad chocolate) exhibited the highest average haemoglobin level (13.15 g/dL), while the K+ group (fish oil) showed the lowest (10.15 g/dL). These results suggest that the protein and iron content of gonad chocolate positively influenced haemoglobin synthesis.

Each feed's content and nutritional value cause differences in haemoglobin levels in each treatment. The high level of Hb in treatment P2 is due to gonad chocolate, which contains a high protein that functions in the transport of iron in the body. The low level of Hb in the K+ group can be caused by inadequate protein intake. Lack of protein intake will cause iron transportation to be inhibited, resulting in iron deficiency. Several iron-binding proteins, including transferrin, lactoferrin, haemoglobin and bacterioferritin, control iron transportation in the digestive system. Iron is a vital part of haemoglobin synthesis (Abbaspour *et al.,* 2014). Therefore, a feed's iron and protein content directly affects the amount of haemoglobin.

Measurement of the brain weight of the subjects from all groups proved that subject K- had the highest brain weight (0.485 g), while the treatment subject P1 (0.5 g of gonad *D. setosum* chocolate) had the lowest brain weight (0.435 g) compared to P2 (0.44 g), P3 (0.47 g), and K+ (0.47 g). In the treatment of varied doses of gonad *D. setosum* chocolate, it was proved that the higher the dosage given, the higher the brain weight of the mice subjects. Group K- had the highest brain weight could be caused by the ratio had sources of energy such as carbohydrates, fats, and proteins as well as other sources of nutrients for synthesising the brain tissue, including synthesis of neuronal cells and oxygen transportation, more significantly than gonad *D. setosum* chocolate and fish oil. In addition to dietary intake, the weight of the mouse brain is also affected by body weight. From the weighing results of body weight, it was found that the K- group had the highest importance, which was 39.4 g. Like the human brain, the mouse brain makes up about 2% of an adult's body weight, receives 20% of the cardiac output, and requires about 20% of the body's oxygen usage (Korobitua *et al.,* 2017).

Measurement of the brain volume of all *Mus musculus* subjects after each treatment for 70 consecutive days showed that the issues from the P3 gonad 1.5-g treatment had the highest brain volume (3.25 mL) compared to the K+ (1.75 mL), K- (2 mL), P1 cocoa gonad 0.5-g treatment (3 mL) and P2 cocoa gonad 1-g treatment (3 mL). Specifically, protein malnutrition can lead to abnormal development with behavioural consequences. The K- group had the lowest brain volume due to inadequate protein intake. Low protein intake reduces brain size, dendritic arborisation, and cell maturation. In addition, low protein intake changes neurotransmitters and brain oxidative status (Chertoff, 2015).

The weight and volume of the brain indicate the number of brain cells. In this case, brain cells include neurons and glial cells (supportive cells). However, it has been explained that glial cells do not directly influence the function of brain intelligence (Verkhratsky *et al.,* 2019). Therefore, the weight and volume of the brain cannot be directly related to the level or quality of intelligence, as there is a possibility that glial cells occupy a higher part of the brain than neurons.

The observations from this study showed that the treatment subject with 1.0 g of gonad *D. Setosum* chocolate (P2) had the highest average number of neurons (838). Therefore, the therapy subject with 1.0 g of Gonad *D. Setosum* chocolate had the highest neuron density compared to all the groups. The one-way ANOVA test for Gonad *D. Setosum* chocolate treatment showed that $P = 0.0 < 0.05$, which means an effect of the treatment on the neurons was found. The results of the study showed that 0.5 g of Gonad chocolate treatment produced the least number of neurons (823) compared to the negative control subject (829), 1.5 g of Gonad chocolate treatment (834), and 0.2 g fish oil (828).

It is believed that sufficient fat and protein intake in the group with 1.0 g of *D. setosum* cocoa treatment contributed to this result. It is known that protein is a source of amino acids, and fat is a source of fatty acids. Proteins and fats are highly valued throughout the growth and development stages of the brain. It is known that the inhibition of amino acid transport points to the fact that the smaller brain has closely coupled cells and glutamate/glutamine, as well as the alanine cycle, playing a significant role in this part of the brain. Glutamine homeostasis in the brain is essential for maintaining excitatory and inhibitory functions.

Glutamine is the primary source of amino acids for synthesising glutamate and GABA. This amino acid acts as a precursor that is neutralised from both neurotransmitters; glutamate released from neurons can be converted to glutamine in glial cells and returned to neurons in this form. Likewise, most of the GABA synthesised in GABAergic neurons comes from astrocytic glutamine. The glutamate/glutamine cycle is not stoichiometric, but this cycle interacts with other pathways, including reactions catalysed by transaminases. The return of ammonia from neurons to astrocytes is achieved through amino acid cycle reactions, such as alanine (Das *et al.,* 2022).

Besides amino acids, the number of brain cells is also affected by fatty acids such as omega-3, PUFA, EPA, and DHA, which can potentially prevent cardiovascular diseases, increase brain capacity and strengthen the body's immune system. It is also known that 60% of the human brain consists of various fats, including unsaturated fatty acids such as omega-3, EPA, DHA, omega-6, AA, and omega-9. Essential fatty acids are also an important dietary intake during brain growth and baby and toddler development (Pringgenies *et al.,* 2020).

IV. CONCLUSION

Amino acid content of *D. setosum* chocolate gonads revealed that the product contains a variety and range of essential amino acids tryptophan (1.60%), valine (5.68%), phenylalanine (1.60%), I-leucine (2.75%), leucine (3.28%); semi-essential amino acids arginine (1.24%), and non-essential amino acids including aspartic acid (4.78%), glutamate acid (11.36%), serine (2.84%), glycine (8.47%), alanine (33.15%), tyrosine (2.10%). The highest weight gain of subjects *Mus musculus* (39.4 g) was obtained from the adverse control treatment, and the lowest (30.6 g) was found in the P1 treatment (0.5 g of gonad chocolate). The highest amount of haemoglobin from all subjects was found in the P2 treatment group (1.0 gonad chocolate g *D. setosum)* (13.15 g/dL), and the lowest was shown by the K+ treatment group (10.15 g/dL). The highest brain weight was found in the K- treatment group (0.485 g), and the lowest brain weight in the P1 treatment group (0.5 g of gonad chocolate *D. setosum*) (0.435 g). The highest volume of brain was found in group P3 (1.5 g of *D. setosum* chocolate) (3.25 ml) and the lowest in the K+ treatment group (1.75 ml). The highest number of subject neuron cells was found in treatment group P2 (1.0 g of D. setosum chocolate) (838) and the lowest in treatment group P1 (823). Treatment with the gonad of *D. setosum* contributes to increased body weight, haemoglobin level, brain weight, brain volume, and several neurons.

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