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Research Article

Effectiveness of the Combination of *Spirulina platensis* and *Stichopus variegatus* on Prevention of Caspase-3 Gene Expression of Dementia Rat Models

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ABSTRACT

The death of cells in the brain, especially the hippocampus, manifests in a decrease in memory, language, and behavior. Golden sea cucumber (*Stichopus variegatus*) and blue-green algae (*Spirulina platensis*) are reported to have high antioxidant content which has the potential to prevent cell death in the brain due to oxidative stress thereby preventing memory decline. This study aimed to investigate the effect of the combination of *Stichopus variegatus* and *Spirulina platensis* on preventing *caspase 3* gene expression in pyramidal hippocampal cells in a rat model of trimethlytin-induced dementia (TMT). This study used Sprague Dawley rats, about 2 months old, weighing 180-200 g, divided into 6 treatment groups, with each group consisting of 8 rats. The hippocampus was taken from the right cerebral hemisphere for histological observations of pyramidal *caspase-3* gene expression in the CA1 and CA2-CA3 regions. The combination of *Spirulina platensis* and *Stichopus variegatus* with a dose 200 mg/kg BB can prevent the expression of *caspase-3* in the CA1 and CA2-CA3 areas of the hippocampus. Conclusion: The combination of spirulina and golden sea cucumber extracts has the potential to prevent *caspase 3* gene expression in pyramidal cells of TMT-induced dementia rat models.

INTRODUCTION

The brain is a complex organ that functions as the control center for body systems and cognitive centers. The brain is very vulnerable to oxidative stress. Oxidative stress on the brain will result in decreased brain function, which will cause various kinds of disorders, including dementia (Turana, 2013).

Dementia is a clinical syndrome with a progressive decline in intellectual functioning. Cognitive abilities affected by dementia include changes in personality, emotional regulation, and social behavior. Other changes occur in memory, language, reasoning, decision-making, visuospatial function, attention, and orientation. As a result, these changes can affect daily activities and relationships with those around them. Dementia generally develops in people over 65 years of age, but the disease can also start earlier, as early as 40 or 50 years of age (Diana *et al*., 2016).

Data from the World Health Organization (WHO) and the Alzheimer's Disease International Organization show that the total number of people with dementia worldwide in 2015 reached 47.5 million and amounted to 22 million cases mostly in Asia. In developed countries such as the United States of America, there are currently more than 4 million elderly people with dementia. This number will continue to increase by almost 4 times in 2050 (Koo *et al*., 2019).

The use of dementia models using test animals has been widely conducted. Some of the approaches taken are based on the similarity of the symptoms that occur in test animals to those that occur in humans, including a decrease in memory, changes in markers of oxidative stress

and disturbances in the cholinergic system (Yuliani *et al*., 2021). One of the compounds that can induce dementia in test animals is trimethlytin (TMT) which is derived from alkynes and is a toxic substance found in polyvinyl chloride (PVC) and silicone products such as kitchen utensils, food packaging and fungicides. TMT compounds serve as by-products of plastic stabilizers. TMT toxicity causes damage to the limbic system, cerebral cortex and brainstem and long-term exposure to TMT causes TMT to accumulate in the body (Kim & Cho, 2019). Nerve cells that are sensitive to the toxic effects of TMT are the hippocampus, piriform cortex, entorhinal cortex, amygdala, neocortex and olfactory tubercles (Balaban *et al*., 1988). TMT can increase the formation of reactive oxygen species (ROS) and can increase the formation of hydroxyl radicals, malondialdehyde (MDA) and hippocampal protein carbonyls (Noraberg *et al*., 1998).

Indonesians have a very high diversity of marine life that can be utilized for sustaining health. The search for medicines made from marine biota is necessary because it is associated with the increasing resistance of various diseases to various types of existing drugs. One of the marine biotas that have economic potential is spirulina (*Spirulina platensis*) and golden sea cucumber (*Stichopus variegatus*). *Spirulina platensis* is widely used as a nutritional supplement which is rich in protein and antioxidants. *Spirulina platensis* is a filamentous blue-green alga (cyanobacterium) that occurs naturally in alkaline lakes (Yang *et al*., 2020). Many studies indicate that *Spirulina platensis* and *Stichopus variegatus* exhibit antioxidant, anti-inflammatory, neuroprotective, hepatoprotective, antidiabetic, hypolipidemic, and anti-cancer activities *in vivo* (Grosshagauer *et al*., 2020). Free radicals are caused by oxidative stress and increase the severity of dementia, while the presence of antioxidant activity in *Spirulina platensis* can reduce the high number of free radicals (Cojocaru *et al*., 2013).

Various studies report that compounds with antioxidants are proven to prevent apoptosis (Roni *et al*., 2020). The main mechanism underlying the occurrence of apoptosis is the activity of cysteine aspartic acid protease (caspase). One of the Caspase that plays a role in inducing apoptosis is caspase 3. This caspase can be activated through intrinsic (mitochondrial pathway) or extrinsic (death ligand) mechanisms, with the help of caspase 8 and caspase 9. If caspase 3 is activated then as the executor caspase, it will do its job to apoptotic cells (Moningka, 2019). Previous studies have shown that giving the extract *Spirulina platensis* and *Stichopus variegatus* can prevent the decline in spatial memory of Sprague Dawley rats injected with TMT. Spatial memory decline is related with the number of pyramidal cells (CA1 in CA2-CA3) of the hippocampus (Roni *et al*., 2020).

Alzheimer's disease accounts for more than 50% of all dementia cases worldwide. There is currently no effective treatment available for the disease, so plant-based drugs and dietary interventions have attracted increasing research interest as alternative strategies for the protection and treatment of Alzheimer's disease (Peng *et al*., 2021). Spirulina (*Spirulina platensis*) and golden sea cucumber (*Stichopus variegatus*) have many benefits but few studies have been conducted on the beneficial effects of their combined treatment. This study aimed to examine the effects of the combination of *Spirulina platensis* and *Stichopus variegatus* on dementia, in order to identify their phytochemical potential as an alternative and complementary medicine.

METHODS

Tools

The tools used in this study were a maceration tool, Buchner funnel, rotary evaporator (Heidolph), scales (Ohaus), a set of surgical tools, a set of glassware, a binocular microscope (Olympus), and Optilab (Miconos).

Materials

The materials used were *Spirulina platensis* powder obtained from the Algae Center, Yogyakarta, Indonesia and *Stichopus variegatus* extract obtained from Naturonal Organic Herbal, Yogyakarta, 70% ethanol (Sigma Aldrich, St. Lois, USA). TMT chloride (Sigma Aldrich, St. Louis, USA), 0.9% sodium chloride (NaCl), sodium carboxymethyl cellulose (Na-CMC), and caspase 3 primary antibody (Scytek).

Preparation of Extract Solution

The extract solution was prepared by suspending spirulina powder and golden sea cucumber extract with 1% Na-CMC solution. The volume of the drug solution given to rats weighing 200 grams orally was 2.0 mL.

Treatment of Test Animals

The test animal was a male Sprague Dawley rat, aged 2 months (adult), with 180-200 g body weight (BW). The rats were divided into 6 treatment groups of 8 each, which had received

approval from the Ethics Committee of Ahmad Dahlan University Yogyakarta with Number: 012209148. Before receiving treatment, rats were acclimatized for 7 days to the following conditions: room temperature 24–26°C and humidity 60–65% with a 12-hour dark and light cycle. Mice received food and drink *ad libitum*. The grouping of test animals was as follows:
Normal Control (KN): given a combination of spirulina and golden sea cucumber extracts with Na-CMC solvent orally (p.o).

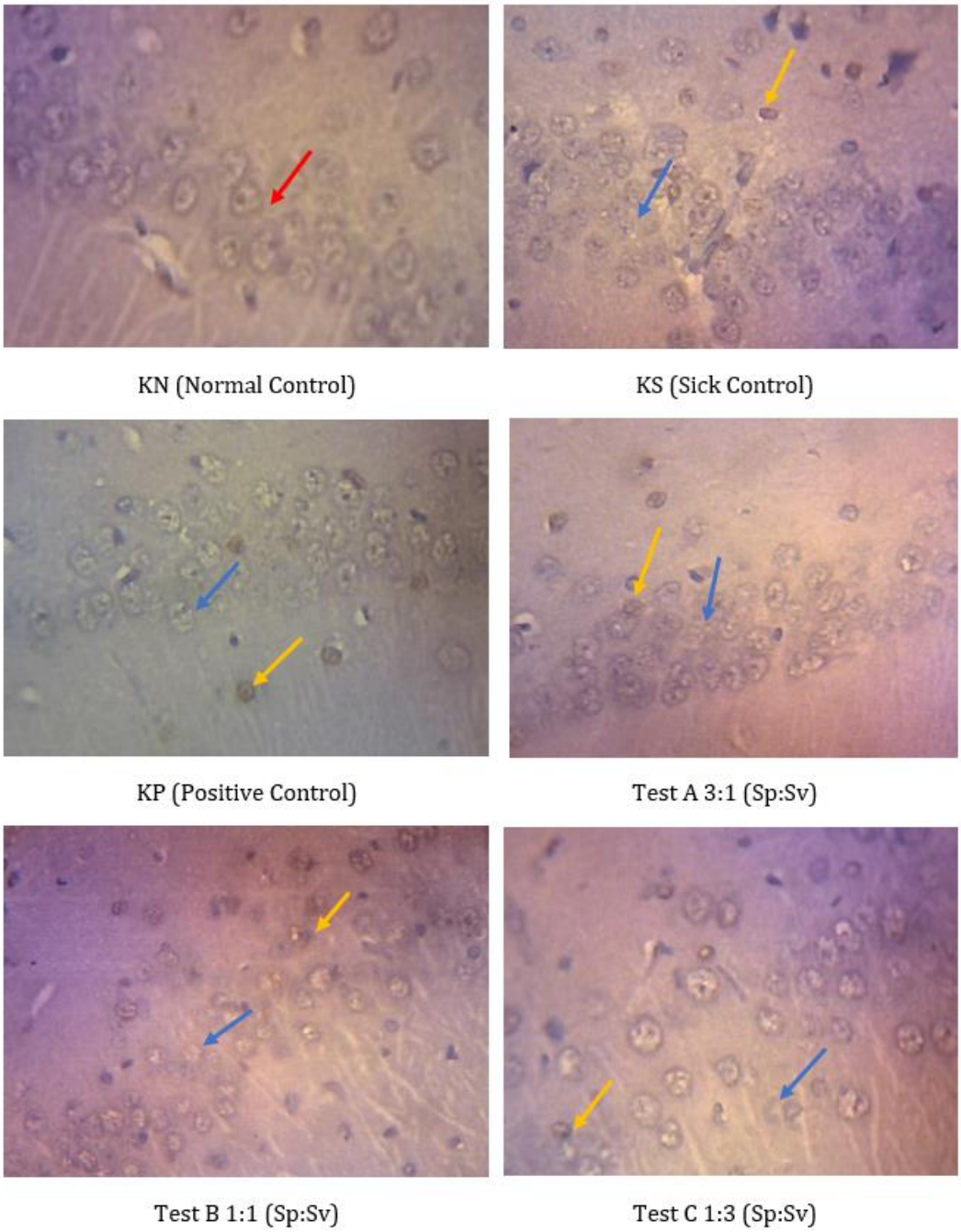

Figure 1. Microscopic appearance of Caspase-3 immunohistochemical staining of pyramidal hippocampus cells in the CA1 area of dementia model rats injected with TMT in all groups. Caspase-3 is not expressed in normal cells (↙). Cells that express the Caspase-3 gene appear brown in the cytoplasm (↙), whereas cells that do not express the Caspase-3 gene are colored blue (↙). 400x magnification.

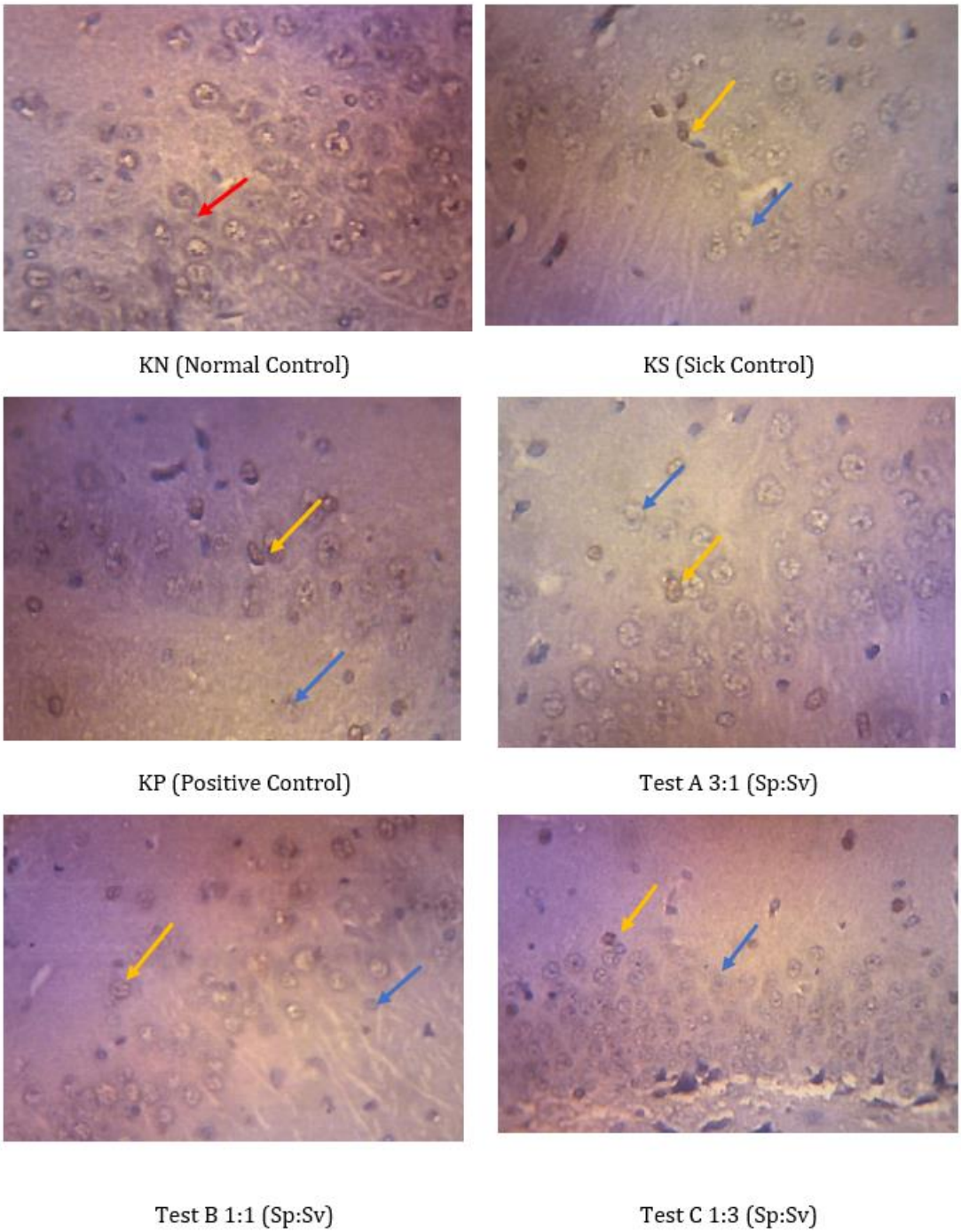

Figure 2. Microscopic appearance of Caspase-3 immunohistochemical staining of hippocampal pyramidal cells in the CA2-CA3 area of dementia model rats injected with TMT in all groups. Caspase-3 is not expressed in normal cells ( ). Cells that express the Caspase-3 gene appear brown in the cytoplasm ( ), whereas cells that do not express the Caspase-3 gene are colored blue ( ). 400x magnification.

Sick Control (KS): given a combination of spirulina and golden sea cucumber extracts by p.o and TMT.
Positive Control (KP): given a combination of spirulina and golden sea cucumber extracts with p.o and citicoline dose of 200 mg/kg BW p.o.
Test A: given a combination of spirulina extract and golden sea cucumber (1:3) at a dose of 200 mg/kg BW p.o.
Test B: given a combination of spirulina extract and golden sea cucumber (1:1) at a dose of 200 mg/kg BW p.o.

Test C: given a combination of spirulina and golden sea cucumber extracts (3:1) at a dose of 200 mg/kg BW p.o.

Administration of treatment extended from day 1 to day 35. Intraperitoneal (i.p) injection of TMT at a dose of 8 mg/kg BW was done in all groups on day 8 of treatment, except the Normal group. On day 36 the rats were euthanized by CO_2 inhalation. The rat brains were removed and put into a formalin buffer for 6 days. The hippocampus was then separated to make paraffin blocks according to the standard method conducted at the Anatomical Pathology Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Caspase 3 Immunohistochemical Staining

Hippocampal paraffin blocks were cut into 3 μm thickness and then placed on poly-L-lysine slides. Furthermore, caspase 3 immunohistochemical staining was conducted using the ULTRATEK HRP Anti-polyvalent (DAB) Staining Complete System (Scytek Laboratories) procedure according to that conducted at the Anatomical Pathology Laboratory of Dr. Sardjito Hospital, Yogyakarta. Observation of caspase 3 expression was done using a binocular microscope connected to Optilab. The number of pyramidal cells expressing caspase 3 protein was calculated based on the total number of 3 microscopic slices of hippocampus tissue from each pyramidal cell in the CA1 area and in the CA2CA3 area.

Data Analysis

Data on total *Caspase 3* gene expression in pyramidal cells in the CA1 and CA2-CA3 regions were statistically analyzed by a one-way ANOVA test followed by LSD post hoc tests with a significance level of $p<0.05$.

RESULTS AND DISCUSSION

In this study the dementia model in rats was performed by TMT injection. TMT is a compound that is neurotoxic. One of the nerve cells that are sensitive to the toxic effects of TMT is the hippocampus. The toxic effect of TMT causes a decrease in the number of pyramidal cells, especially in sensitive areas, namely the CA3 and CA1 regions. TMT can induce an apoptotic cascade (Balaban *et al*., 1988)

A histological test was conducted by observing the expression of the *Caspase-3* gene in the CA1 and CA2-CA3 region of the hippocampus with immunohistochemical staining. The results of immunohistochemical staining are presented in Figures 1 and 2 with 400x magnification scale which includes all groups (Normal, Sick, Positive, Test A, Test B, and Test C). To determine the number of cells expressing the *Caspase-3* gene, a calculation based on 3 microscopic slices of each hippocampus was then performed. Based on the results of immunohistochemical staining showed that the protein pro-apoptotic caspase-3 was expressed in mice injected with TMT and not expressed in the normal group. Specific antibodies forcaspase-3 selectively stained brown in the CA1 and CA2-CA3 regions. Based on the results of immunohistochemical staining showed that the protein pro-apoptotic caspase-3 was expressed in mice injected with TMT and not expressed in the normal group. Specific antibodies for caspase-3 are selectively stained brown in the cytoplasm and cell nucleus in the CA2-CA3 area which is predominantly stained in the cytoplasm only in the CA1 area.

Based on the results of semiquantitative analysis, it showed that mice injected with TMT experienced increased gene expression of *Caspase-3* in pyramidal cells in the CA1 and CA2-CA3 regions. The presence of protein expression of caspase-3 indicates the presence of cells experiencing death through the process of apoptosis (Clerici, 1996). To find out the number of cells that express the gene *Caspase-3* then calculations were done based on 3 microscopic slices of each hippocampus. Based on the results of immunohistochemical staining, it shows that pro-apoptotic protein caspase-3 was expressed in mice injected with TMT and not expressed in the normal group. Specific antibodies for caspase-3 are selectively stained brown in the CA1 and CA2-CA3 regions.

Table 1 shows that there is a significant difference between groups in *Caspase-3* gene expression, $p= 0.000$ ($p<0.05$). The normal control group and test group C (0.007) had a significant difference from the sick control group ($p= 0.001$). Results showed that the combination of extracts *Spirulina platensis* and *Stichopus variegatus* with a dose variation of 1:3 (Sp:Sv) at a dose of 200 mg/kg BW, namely test group C was able to prevent caspase-3 protein expression in the CA1 area.

Table 2 shows that there is a significant difference between groups in *Caspase-3* gene expression, $p= 0.004$ ($p<0.05$). The normal control group and test group C ($p= 0.001$) had a significant difference from the sick control group ($p= 0.005$). Results show that the combination of extracts *Spirulina platensis* and *Stichopus variegatus* with a dose variation of 1:3 (Sp:Sv) at a dose of 200 mg/kg BW, namely test group C was

able to prevent protein expression of *caspase*-3 in the CA2-CA3 area.

Based on the results of statistical analysis, it was shown that mice injected with TMT experienced a significant increase in *Caspase 3* gene expression in the pyramidal cells of the CA1 and CA2CA3 regions. The presence of caspase 3 protein expression indicates the presence of cells that experience death through the process of apoptosis. The mechanism of TMT in causing toxic effects is not fully known with certainty (Kothakota *et al*., 1997). Damage to TMT-sensitive cells is related to oxidative stress, calcium overload and mitochondrial damage. Oxidative damage can be caused by the induction of various radical and non-radical reactive species which include ROS, reactive nitrogen species (RNS) and reactive aldehyde species (Farmer & Mueller, 2013).The most reactive oxygen free radicals in the body are superoxide anion (O_2), hydroxy radicals (OH) and hydrogen peroxide (H_2O_2). TMT increases free radical activity in cells resulting in oxidative stress which is an important factor in neurodegenerative disorders (Liu *et al*., 1999).

Mitochondrial damage may be mediated by the protein stannin (SNN), an 88-amino acid protein encoded by the cDNA of TMT-sensitive cells (Balaban *et al*., 1988) SNNs are likely located on the mitochondrial outer membrane and endoplasmic reticulum membrane. SNN will bind directly with dimethyltin (the result of TMT demethylation), causing release of cytochrome c and activation of various caspase. Calcium overload occurs depending on the extracellular stores present in the mitochondria and endoplasmic reticulum, leading to activation *Calpain* and *Cathepsin D* which triggers apoptosis (Kothakota *et al*., 1997).

Histological observations showed that the expression of protein caspase-3 in the cytoplasm of pyramidal cells in the CA1 area and the cytoplasm and nucleus of pyramidal cells in the hippocampus CA2-CA3 area was more intensive than the normal group indicating that the decrease in the number of these cells was due to cell death through the process of apoptosis. Administration of a combination of *Spirulina platensis* and *Stichopus variegatus* (sea cucumber) with a dose ratio of 1:3 (Sp:Sv) at a dose of 200 mg/kg BW was able to reduce *Caspase-3* gene expression in pyramidal cells of the hippocampus CA2-CA3 area but could not reduce *Caspase-3* gene expression in the CA1 area. The difference in the activity of the combination of *Spirulina platensis* and *Stichopus variegatus* extracts in the CA1 and CA2-CA3 areas may be due to the difference in the effectiveness of the combination of the two extracts for the two areas.

Table 1. Calculation results of the number of cells expressing the Caspase-3 gene in the CA1 region

No	Group	Average±SEM
1	KN (Normal Control)	20.01±2.08*
2	KS (Sick Control)	81.05±1.94
3	KP (Positive Control)	21.57±0.72
4	Test A 3:1 (Sp:Sv)	26.90±0.83
5	Test B 1:1 (Sp:Sv)	31.86±1.12
6	Test C 1:3 (Sp:Sv)	21.86±2.94*

Description: SEM, standard error of measurement; *significantly different from KS ($p<0.05$).

Table 2. Calculation results of the number of cells expressing the Caspase-3 gene in the CA2-CA3 region

No	Group	Average±SEM
1	KN (Normal Control)	17.46±2.84*
2	KS (Sick Control)	74.18±6.84
3	KP (Positive Control)	18.42±0.81
4	Test A 3:1 (Sp:Sv)	25.84±1.31
5	Test B 1:1 (Sp:Sv)	23.09±2.04
6	Test C 1:3 (Sp:Sv)	17.87±1.14*

Description: SEM, standard error of measurement; * significantly different from KS ($p<0.05$).

To determine the number of cells expressing the *Caspase-3* gene, a calculation was performed based on 3 microscopic slices of each hippocampus. Based on the results of immunohistochemical staining, it was shown that pro-apoptotic caspase-3 protein was expressed in mice injected with TMT and not expressed in the normal group. Antibodies specific for caspase-3 are selectively stained brown in the CA1 and CA2-CA3 regions.

Based on the results of immunohistochemical staining, it was shown that pro-apoptotic caspase-3 protein was expressed in mice injected with TMT and not expressed in the normal group. Antibodies specific for caspase-3 are selectively stained brown in the cytoplasm and cell nucleus in the CA2-CA3 region and are dominantly stained in the cytoplasm only in the CA1 region.

Various studies have shown a neuroprotective role for spirulina in nervous system development, aging, and several pathological conditions, including neurological and neurodegenerative diseases. Spirulina's role in the brain highlights how Spirulina exerts beneficial anti-inflammatory and antioxidant effects, acts on glial cell activation, and in the prevention or development of neurodegenerative diseases, particularly Parkinson's disease, Alzheimer's disease, and Multiple Sclerosis. Due to these properties, Spirulina can be considered a potential natural medicine (Trotta *et al*., 2022) The highest amino acid content in golden sea cucumbers is seen in glutamic acid at 6.6049%. Glutamic acid acts as a neurotransmitter controller that affects cognition and is useful in preventing dementia and improving memory (Gianto & Putri, 2017).

Spirulina platensis and *Stichopus variegatus* extracts contain asiaticoside which has high antioxidant activity. Antioxidant compounds have been shown to prevent apoptosis through the activation of intracellular and mitochondrial free radical scavenging by activating antioxidant enzymes such as superoxide dismutase and catalase to prevent cell apoptosis. The process of apoptosis involves caspase 3 which acts as an initiator. Previous studies reported that administration of *Spirulina platensis* and *Stichopus variegatus* could prevent a decrease in the spatial memory of Sprague Dawley rats that were injected with TMT (Roni *et al*., 2020; Trotta *et al*., 2022).

TMT administration can cause hippocampal neuron cell death, which can also be observed in degenerative changes in the brain associated with Alzheimer's disease. Based on research conducted by Yuliani *et al*. (2016), the results indicated that the histopathology of the guinea pig brain in the Alzheimer's model shows degeneration and death of neuron cells and an increase in the activity of glial cells (gliosis) (Yuliani, 2016). In the rodent model, administration of TMT caused loss of neuron cells with reactivation of glial cells in the brain and changes in behavior such as cognitive impairment, hyperactivity, and tonic-clonic seizures (Noraberg *et al*., 1998).

According to Kristianingrum *et al*., (2016), immunohistochemical analysis of rat brain slices induced by TMT at a dose of 8 mg/kg BW intraperitoneally showed brain neuron cell death in rats starting on the 14th day after treatment, especially in the hippocampus and cortex (Kristianingrum *et al*., 2016).

Previous studies reported that citicoline can prevent apoptosis by decreasing caspase-3 expression in the CA2-CA3 region of TMT-induced Sprague Dawley rats (Yuliani, 2016). In this study, citicoline was used because it has a beneficial effect on neurological function in memory (Duarsa, 2016). The mechanism of action of citicoline is to increase the synthesis of phosphatidylcholine and increase the production of acetylcholine (Jasielski *et al*., 2020). Citicoline has three mechanisms of action, namely repairing neuronal membranes through increasing phosphatidylcholine synthesis, then repairing damaged cholinergic neuronal membranes through potentiating acetylcholine production and reducing free fatty acid production at the site of nerve damage (Martí-Carvajal *et al*., 2017) so that it can prevent dementia in test animal models.

CONCLUSIONS

Based on the results it can be concluded that the combination of spirulina and golden sea cucumber extracts has the potential to prevent *Caspase 3* gene expression in pyramidal cells of TMT-induced dementia rat models.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. None of the authors of this paper have any financial or personal relationship with any other person or organization that could

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