

Combination of *Spirulina platensis* powder and *Stichopus variegatus* powder against Bcl2 expression in the hippocampus of dementia Rats

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ABSTRACT

Spirulina (*Spirulina platensis*) and golden sea cucumber (*Stichopus variegatus*) are known to have antioxidant activity that has the potential to prevent neurodegeneration disease. The aim of this study was to examine the effect of the combination of spirulina and golden sea cucumber on Bcl2 gene expression in pyramidal hippocampus cells of trimethyltin-induced dementia (TMT) rats. The study used *Sprague Dawley* rats which were divided into six groups, namely the normal control group (CMC-Na and NaCl 0.9%), pain control (CMC-Na and TMT), positive control (citicoline dose 200 mg/kg BW and TMT) and and test control injected with TMT and given a combination of spirulina (S) and golden sea cucumber dose (G) with three ratios of SG-3:1, SG-1:1 and SG-1:3 in a single dose of 200 mg/kg BW. Sample and citicoline were given on days 1-28, while TMT injection was given a single dose of 8 mg/kg BW on day 8. On day 36, the rats were sacrificed, brains were removed and the right hemispherium cerebri was fed to 10% formalin in pbs. After 6 days, the hippocampus was separated for immunohistochemical observation. The test result data was statistically analyzed with a *one-way* ANOVA test then followed by post hoc *tukey* to see the differences between groups. Results showed the combination of spirulina and golden sea cucumber can increase the expression of the Bcl2 gene in the hippocampus. The combination of spirulina and golden sea cucumber (SG-1:3) at a dose of 200 mg/kg BW had the ability to increase Bcl2 expression almost the same as citicoline with the number of Bcl2 cell expressions being 27.51 ± 0.70 in the CA1 region and 69.96 ± 1.97 in the CA2-CA3 region. So, it can be concluded that SG-1:3 has the potential to prevent dementia.

Keywords: Bcl2, dementia, *Spirulina platensis*, *Stichopus variegatus*, trimethyltin

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INTRODUCTION

Dementia is a clinical syndrome characterized by progressive cognitive decline that appears as memory loss, communication and language disorders, agnosia, apraxia and impaired executive function (reasoning, judgment and planning) (Duong et al., 2017). Dementia symptoms are caused by damage to a part of the brain called the hippocampus, which has a central role in memory (Lavenex & Lavenex, 2013). Oxidative stress participates in the development of dementia. Oxidative stress is an imbalance between free radicals and antioxidants. The most abundant type of free radical in the body is *Reaction Oxygen Specific* (ROS) (Singh, 2022). Excess ROS can cause apoptosis.

Apoptosis is cell death that occurs regularly to ensure a homeostatic balance between the rate of cell formation and cell death. The process of apoptosis can occur when the amount of antiapoptotic and proapoptotic proteins in the cell is out of balance. Apoptosis is regulated by several genes, including Bcl-2 which plays a role in preventing apoptosis (anti-apoptosis) (Yuliani et al., 2021).

B-Cell lymphoma-2 (Bcl-2) is a protein that provides key functions in cell health through the mechanism of apoptosis (Callens et al., 2021). Bcl-2 can inhibit the release of cytochrome-c from mitochondria and activation of caspase thereby preventing apoptosis. Caspase is a proapoptotic protein that acts as an executor in the apoptotic cascade (Zhao et al., 2014). For this reason, an antioxidant agent is needed to neutralize free radicals that can cause apoptosis.

Spirulina (*Spirulina platensis*) and golden sea cucumber (*Stichopus variegatus*) are two marine resources that have high potential as antioxidants. Research by Jadaun et al. (2018), shows spirulina plays a role in the prevention of increased oxidative stress during apoptosis, increased mitochondrial membrane potential as well as increased Bcl2 expression. Research by Su et al. (2018), showed that sea cucumbers also showed inhibition of caspases-9 and -3 so as to prevent apoptosis.

The development of herbal-herbal combination therapy or known as polyherbal therapy is widely carried out in the treatment of disease. Drug combinations often produce promising effects in the treatment of diseases (Aslam et al., 2016). Research by Safithri et al. (2021), shows methanol extracts of *S. platensis*, golden sea cucumber, and their mixtures have antioxidant activity.

This study aimed to determine the effect of the combination of *Spirulina platensis* and *Stichopus variegatus* on increasing the expression of the Bcl2 gene in pyramidal hippocampus cells of dementia rats.

MATERIALS AND METHOD

Materials

The tools used in the study were oral needles, 1 mL injection syringes, a set of surgical tools and scales (Ohaus), glass tools (*pyrex*), stirring rods, binocular microscopes (Olympus), optilab (Miconos). The main ingredients used in this study were *Spirulina platensis* powder obtained from PT. Algaepark Indonesia Mandiri, Klaten, Jawa Tengah, Indonesia (Batch No. 11723265) and *Stichopus variegatus* powder (hydrolisat) obtained from CV Rigo Alam Sejahtera, Bogor, Indonesia (Batch No. ST012308). Primary antibody (Bcl2) and secondary antibody (*Biotinylated universal*).

Methods

Sample solution preparation

The sample solution was prepared by suspending spirulina powder and golden sea cucumber powder with a 1% CMC-Na solution. Oral administration of the sample at a dose of 200 mg/kg BW. The volume of the drug solution given to rats weighing 200 grams orally was 2.0 mL (Yuliani et al., 2021).

Test animal preparation

The test animals used were *male Sprague Dawley* rats aged about 2 months (150-200 grams). The use of animals in research has been approved for preclinical research by the Research Ethics Committee of Universitas Ahmad Dahlan, Yogyakarta, Indonesia (approval number 012209148). A total of 48 rats were acclimatized for seven days and divided into six groups.

1) Normal control (CMC-Na and NaCl 0.9%)

- 2) Pain control (CMC-Na and TMT 8 mg/kg BW)
- 3) Positive control (citicoline 200 mg/kg BW and TMT 8 mg/kg BW)
- 4) SG-3:1 (Combination of spirulina and golden sea cucumber 3:1 dose 200 mg/kg BW and TMT 8 mg/kg BW)
- 5) SG-1:1 (Combination of spirulina and golden sea cucumber 1:1 dose 200 mg/kg BW and TMT 8 mg/kg BW)
- 6) SG-1:3 (Combination of spirulina and golden sea cucumber 1:3 dose 200 mg/kg BW and TMT 8 mg/kg BW)

CMC-Na, citicoline and sample were administered orally for 28 days, while NaCl and TMT were injected intraperitoneally on day 8.

Hippocampus preparation

Rats are sacrificed by putting them in a container and then flowing with CO₂ gas. The rat was then dissected on its head. After that the brain is taken. Right hemispherium cerebri was introduced in a 10% formalin fixation solution in PBS for 6 days. The hippocampus is then carefully separated from the hemispherium cerebri, then inserted into pots of tissue again (Yuliani et al., 2021).

Paraffin block creation

Paraffin block making is carried out at the Pathology Laboratory, Gadjah Mada University, Yogyakarta. The hippocampus is put in gauze, dehydrated, and immersed in ethanol solutions of 70, 80, 90 and 100%. each for 60 minutes at room temperature. The next process is purification using xylol for 15 minutes. After the *clearing* process, the infiltration process with liquid paraffin is carried out 3 times, each for 60 minutes in an incubator at a temperature of 60°C, then stored at room temperature so that paraffin blocks are formed (Yuliani et al., 2021).

Bcl-2 immunohistochemical procedure

Immunohistochemical staining was carried out at the Pathology Laboratory, Gadjah Mada University, Yogyakarta, using an indirect method. The formed paraffin block is cut horizontally using microtomes with a thickness of 3-4 µm placed on the glass of the *poly-L-lysine* object. Immunohistochemical review uses primary antibodies (Bcl-2) and secondary antibodies (*Biotinylated universal*). The staining results were observed using a binocular microscope connected to a digital camera with a magnification of 400x. Observations were made on hippocampus pyramidal cells in the CA1 and CA2-CA3 areas of 2 tissue slices per hippocampus. In this staining, Bcl2 protein expression is marked with brown color in the cytoplasm and nucleus, while cells that do not express Bcl-2 protein will appear blue in pyramidal hippocampus cells. The Bcl2 cell intensity criterion is based on the average number of cells from 4 CA2-CA3 fields of view (400x magnification) (Yuliani et al., 2021).

Data Analysis

The normality test is performed using the Saphiro-Wilk test and the homogeneity test is performed with the Levene test. Statistical analysis using *one way* ANOVA and followed by Tukey HSD *posthoc* test to see differences between groups.

RESULT AND DISCUSSION

Dementia is a syndrome that can be caused by a number of progressive diseases and affects memory, thinking, behavior and the ability to carry out daily activities (Prince et al., 2014) In this study using trimethyltin (TMT) as a model of dementia in rats. TMT is a toxic organotin compound that selectively induces acute neuron death in the *dentate gyrus* of the hippocampus followed by impaired cognition (Melliou & Chinou, 2014). A collection of evidence suggests that TMT toxicity induces neurodegeneration of the hippocampus and results in cognitive impairment, mental confusion, memory defects, and seizures. Thus, TMT is a useful tool to prevent the most common neurodegenerative disorders such as dementia (Mitrović et al., 2021; Lee et al., 2016; Pompili et al., 2020; Dragić et al., 2021).

Based on the results of immunohistochemical painting, it shows that qualitatively antibodies specific to Bcl2 are selectively painted brown in the cytoplasm and cell nucleus in the CA2-CA3 region and predominantly painted in the cytoplasm only in the CA1 area (Figures 1 and 2). Previous research has said that CA3 and CA2 regions contain relatively higher concentrations of bcl-2 than CA1. Area CA3 holds a strategic position in the hippocampus because it receives sensory information through the main collateral pathway of Schafer (Aboutaleb et al., 2015). In addition, quantitatively (Tables 1 and 2) showed that compared to the normal control group TMT injection can decrease the amount of Bcl-2 gene expression in hippocampus pyramidal cells in the CA1 region and significantly in the CA2-CA3 region. According to Widiyanti et al. (2014), in normal conditions, Bcl2 cells are found in the outer mitochondrial membrane, endoplasmic reticulum and nuclear membrane to determine the response of a cell to the apoptotic stimulus through the intrinsic pathway. In addition, the TMT group rats qualitatively showed less Bcl2 expression compared to the citicoline and extract groups. TMT causes selective neuron death in Cornu Ammonis (CA) pyramidal cells in the hippocampus (Yuliani et al., 2021). So, it can be said that TMT can reduce the amount of expression of the Bcl2 gene in the pyramidal cells of the hippocampus.

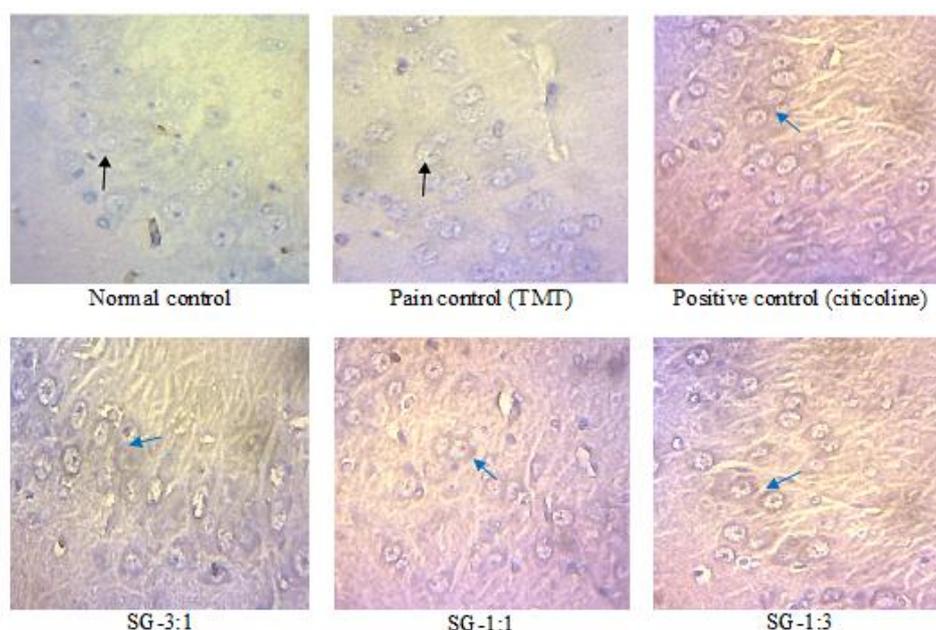


Figure 1. Microscopic image of Bcl2 immunohistochemical painting on hippocampus pyramidal cells in the CA1 region of a mouse model of TMT-induced dementia. Normal cells are expressed in blue (↗), the expression of Bcl2 protein in the cytoplasm is presented in brown (↘). 400x magnification

Table 1. Calculation of the number of cells expressing Bcl2 in the CA1 region

Group	Mean ± SEM
Normal control	12.67 ± 0.85
Pain control (TMT)	10.07 ± 0.47
Positive control (citicoline)	33.32 ± 1.73*
SG-3:1	25.76 ± 1.47**
SG-1:1	26.24 ± 0.58*
SG-1:3	27.51 ± 0.70*

Description: *significantly different from pain control ($p < 0.05$), **significantly different from positive control ($p < 0.05$)

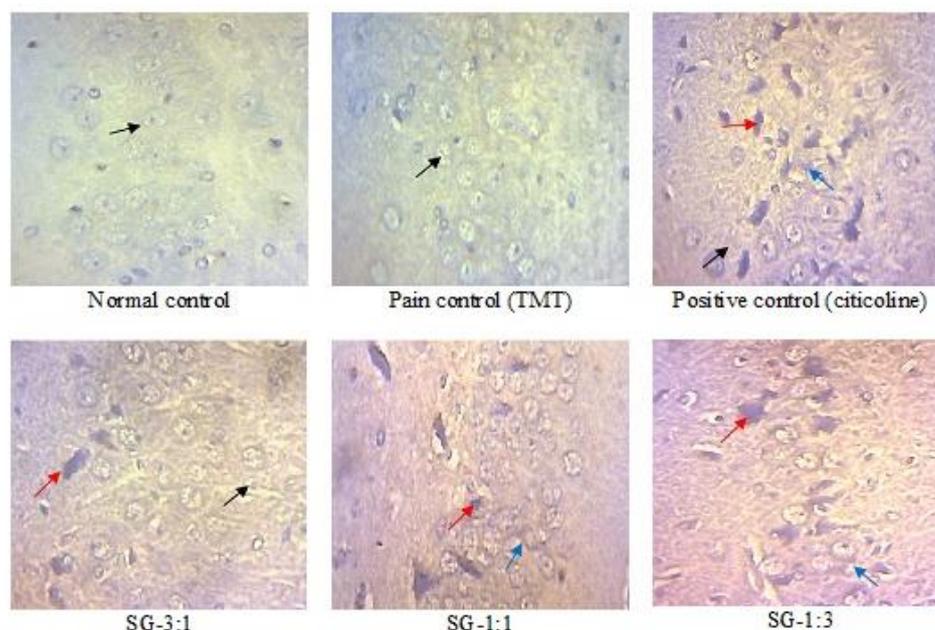


Figure 2. Microscopic image of Bcl2 immunohistochemical painting on hippocampus pyramidal cells in the CA2-CA3 region of a mouse model of TMT-induced dementia. The expression of Bcl2 protein in the cytoplasm (↗) and nucleus (↘) is presented in brown. Normal cells are expressed in blue (↖). 400x magnification

Table 2. Calculation of the number of cells expressing Bcl2 in the CA2-CA3 region

Group	Mean ± SEM
Normal control	23.94 ± 0.33
Pain control (TMT)	13.01 ± 0.53*
Positive control (citicoline)	71.57 ± 1.78 [#]
SG-3:1	57.73 ± 1.44 ^{#†}
SG-1:1	57.89 ± 1.69 ^{#†}
SG-1:3	69.96 ± 1.97 [#]

Description: *significantly different from normal control ($p < 0.05$), [#]significantly different from pain control ($p < 0.05$), [†]significantly different from positive control ($p < 0.05$)

Intraperitoneal TMT injection increased the rate of ROS production in rats in sensitive areas of the hippocampus and increased the rate of ROS-induced oxidative damage, which contributes to activating the apoptosis signaling pathway (Kang et al., 2016). ROS causes mitochondrial dysfunction by inhibiting the synthesis of *adenosine triphosphate* (ATP). The decrease in ATP causes cytochrome-c which interacts with *apoptotic protease-activating factor-1* (Apaf-1) and caspase-9 to form apoptosome. Apoptosome act as activators of caspases 3, 6 and 7 causing apoptosis. Bcl-2 can inhibit the release of cytochrome-c from mitochondria and activation of caspase thereby preventing apoptosis. Caspase 3 is a proapoptotic protein that acts as an executor in the apoptotic cascade (Zhao et al., 2014).

TMT-injected rats differed significantly with citicoline and extract. So, it can be said that the combination of spirulina and golden sea cucumber in this study increased the expression of Bcl2 in pyramidal hippocampus cells. Citicoline is not significantly different from SG-1:3 in both CA1 and CA2-CA3 areas, so it can be said that SG-1:3 has a greater effect in increasing Bcl2 expression than SG-3:1 and SG-1:1. The larger content of golden sea cucumber at SG-1:3 shows a better effect than SG-3:1 and SG-1:1. This may be due to golden sea cucumber samples being used in the form of peptide hydrolysates which can show better antioxidant potential than spirulina powder. In addition, according to research (Windari et al., 2019) golden sea cucumber and the combination of spirulina and golden sea cucumber (81 and 284 mg/kg BW) have antioxidant activity with smaller MDA levels and large SOD and catalase activity compared to spirulina alone. The combination of spirulina and golden

sea cucumber with these three comparisons was chosen because in several previous studies it has been known that both spirulina and golden sea cucumber at doses of 200 mg/kg BW showed good activity in preventing dementia (Ghanbari et al., 2019; Li et al., 2019). In addition, previous studies also showed a combination of spirulina and golden sea cucumber which has antioxidant activity and decreased MDA levels which are important in improving memory function (Windari et al., 2019; Safithri et al., 2021). So that a combination of spirulina and golden sea cucumber was carried out at a dose of 200 mg/kg BW in three concentration ratios. Based on this explanation, it can be said that the combination of spirulina and golden sea cucumber in this study increased Bcl2 expression in pyramidal hippocampus cells.

Spirulina's ability to increase Bcl2 expression according to Jadaun et al. (2018), because spirulina can play a role in the prevention of increased oxidative stress during apoptosis, increased mitochondrial membrane potential as well as increased Bcl2 expression. Several scientific publications have explained its positive effects on various pathologies, one of which is neuroprotective (Trota et al., 2022). On the certificate of spirulina analysis indicates the content of karatenoids and vitamin E (tocopherol) as described by El-Shall et al. (2023), Spirulina has a large amount of natural antioxidants including polyphenols, carotenoids, and phycocyanin. Spirulina also has a wide variety of antioxidants such as superoxide dismutase (SOD), provitamin-A, vitamins C and E (Wang et al., 2013). Carotenoids act as antioxidants to block triggers of apoptosis and ROS-related mitochondrial dysfunction (Park et al., 2020). In addition, it is known that the content of *Phycocyanin* which is a blue-green pigment in *Spirulina platensis* can increase antioxidant enzyme activity and also suppress the expression of caspase-9 and caspase-3 by providing significant protection from mitochondrial membrane permeability and increasing ATP production and restore Bax/Bcl2 balance and also weaken the release of caspase-3 and caspase-9 (Li et al., 2020).

As for golden sea cucumbers, although there is no supporting data related to the analysis of chemical content, but the material used in this study is peptide hydrolysate obtained from golden sea cucumber and in previous studies it has been explained that protein hydrolysate and peptides obtained from sea cucumbers show antioxidant potential. Sea cucumbers contain various bioactive compounds, namely phenolics, polysaccharides, proteins (collagen and peptides), carotenoids, and saponins, which are abundant in these marine invertebrates and exhibit antioxidant activity (Hossain et al., 2022). According to research by Windari et al. (2019) stated that golden sea cucumber and the combination of spirulina and golden sea cucumber (81 and 284 mg/kg BW) have antioxidant activity with smaller MDA levels compared to spirulina. Research Su et al., (2018) states that sea cucumbers can inhibit caspases-9 and -3 so as to prevent apoptosis. Inhibition of caspases occurs due to the presence of Bcl2 as an antiapoptotic. The peptide effect of sea cucumber can improve memory function (Xu et al., 2020).

Citicoline as a comparison that has a value almost equal to SG-1: 3 can induce a significant reduction of cells undergoing apoptosis, citicoline weakens cell death caused by oxidative stress. Previous studies reported that citicoline may prevent apoptosis through decreased caspase 3 expression in CA2-CA3 regions (González-Pacheco et al., 2014). Research Sugianto et al. (2013), states citicoline can reduce inflammation and apoptosis by decreasing the expression of procaspase -1, -2, -3, -6, -8 and caspase-3 and increasing the excretion of Bcl2 hippocampus Nashine & Kenney (2020). The mechanism of citicoline in repairing neuronal membranes is through increasing phosphatidylcholine synthesis, then repairing damaged cholinergic neuronal membranes through potentiation of acetylcholine production and reducing free fatty acid production at the site of nerve damage (Pathan, 2012), so as to prevent dementia in test animal models.

CONCLUSION

The combination of spirulina (*Spirulina platensis*) and golden sea cucumber (*Stichopus variegatus*) can increase the expression of the Bcl2 gene in trimethyltin-induced dementia rats. The combination of spirulina and golden sea cucumber (SG-1:3) dose of 200 mg/kg BW was able to increase hippocampus Bcl2 expression with the number of Bcl2 cell expression almost the same as citicoline in both CA1 and CA2-CA3 regions, so that it has the potential to prevent dementia.

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