

Evaluation of eye protection efficacy of sweet potato leaf ethanolic extracts *In Vivo*

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Abstract

Many studies have been verified that human retina contains a large amount of carotenoids such as lutein and zeaxanthin, especially the concentrations of lutein and zeaxanthin in the retina and macula are 1,000 times higher than other tissues in human. Therefore, lutein and zeaxanthin play an important role in the health of human vision. The research and development (R&D) of agricultural functional materials or products for eye protection is urgently needed. In this experiment, except for the normal control group, the other groups were irradiated with light-emitting diode (LED) light at the range of 600-1,000 lux for 12 hours / day for 6 weeks. All BALB/c mice were fed with the normal composition for 6 weeks during the experiment. BALB/c mice in the negative control group and three sweet potato leaf ethanolic extracts (SPLEE) groups were fed SPLEE [0.31, 0.62, and 1.23 mg/kg body weight (BW), respectively] for 6 weeks by gavage during the experiment. During the experiment, the eye status of BALB/c mice in each group were observed every week. At the end of the experiment, the BALB/c mice were sacrificed and their eyes in each group were collected for hematoxylin & eosin (H&E) staining, immunohistochemical (IHC) staining, free radical detection, and the detection of cytokine gene expressions (IL-6; interleukin-6 and TNF- α ; tumor necrosis factor- α) and caspase-3 gene expression. Based on the results of this experiment, no obvious lesions by ophthalmoscopy were observed in the eyes of BALB/c mice in three SPLEE groups. H&E and IHC staining results showed that the consumption of high dose (1.23 mg/kg BW) of SPLEE significantly improved retinal outer nuclear layer (ONL) thickness, which was compared to the negative control group. Other evaluation indicators included retinal out segments-inner segments (OS/IS) thickness, glial fibrillary acidic protein (GFAP) staining, and middle-wavelength opsin (M-opsin) staining, showed no significant difference among three SPLEE groups and the negative control group. The free radical detection results showed that no statistical difference between all groups ($p > 0.05$), however, the trend showed that the free radicals in the high and middle doses (1.23 and 0.62 mg/kg BW) of SPLEE groups were lower than those in the negative control group and the low dose (0.31 mg/kg BW) of SPLEE group. The results of IL-6 and TNF- α gene expression showed that the normal control group and three SPLEE groups were significantly lower than the negative control group ($p < 0.05$), while the gene expression of caspase-3 was not significantly differences between all groups ($p > 0.05$). Taken these results

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together, the high dose SPLEE (1.23 mg/kg BW) has the better potential for improving the retinal ONL thickness and anti-inflammatory effects under LED irradiation.

Keywords: Eye protection efficacy; Functional food; *In vivo* experiment; Sweet potato leaf ethanolic extracts

1. Introduction

Commonly, the eye diseases mainly included cataracts, diabetic retinopathy, dry eye disease, glaucoma, and macular degeneration. At present, the age-related cataracts are a leading cause of vision impairment (the eyes were cloudy) and blindness worldwide [1-5]. In addition, the diabetic retinopathy is a major cause of visual impairment and blindness, the retinopathy develops when the high levels of blood sugar damage the blood vessels in the retina. Dry eye disease is a major cause by insufficient tear fluid to leading discomfort and potential visual problems. Glaucoma is a progressive degeneration disease of the optic nerve to affect the transfer of visual information from eyes to brain. Seriously, this disease can cause poor eyesight or blindness. The macula is the central part of the retina. Age-related macular degeneration is one of the main causes of blindness. Therefore, the visual health is very important for human life. The daily diet may play an important role to prevent the visual injury [1-10].

Currently, the sweet potato has become an important protein source for providing lots of the world's population. The sweet potato also plays a significant role to fight against vitamin A deficiency, is public health significance in developing countries. Additionally, the sweet potato is an excellent source of carotenoid. Therefore, β -carotene is also an important nutrition component in the sweet potato [11]. In this study, we want to understand whether the sweet potato leaf ethanolic extracts (SPLEE) possesses eye protection efficacy *in vivo*.

2. Material and methods

2.1. Chemicals, Reagents and Equipment

Phosphate-buffered saline (PBS; Sigma-Aldrich, Cat. No. P3813), ethanol (Sigma-Aldrich, Cat. No. 493511), saline (Taiwan Biotech Co., LTD, Cat. No. 100-120-1101), Zoletil 50 (Virbac, Carros, France), interleukin-6 (IL-6) primer forward: 5'-GCC AGA GTC CTT CAG AGA GA-3'; IL-6 primer reverse: 5'-TGG TCC TTA GCC ACT CCT TC-3', tumor necrosis factor- α (TNF- α) primer forward: 5'-AGG GGA TTA TGG CTC AGG GT-3'; TNF- α primer reverse: 5'-TGA GTC CTT GAT GGT GGT GC-3'; caspase-3 primer forward: 5'-GAG CTT GGA ACG GTA CGC TA-3'; caspase-3 primer reverse: 5'-GAG TCC ACT GAC TTG CTC CC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer forward: 5'-TGT GAA CGG ATT TGG CCG TA-3'; GAPDH primer reverse: 5'-ACT GTG CCG TTG AAT TTG CC-3', T-PER™ Tissue protein extraction reagent (Cat. No. 78510; Thermo Fisher, Taipei, Taiwan), RNeasy® Plus Mini Kits (Cat. No. 74134; QIAGEN, MD, USA), SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Cat. No. 11736-059; Invitrogen, WA, USA), ROS Detection Cell-Based Assay Kit (DCFDA; Cat. No. 601520, Cayman, MI, USA), GFAP antibody (Cat. No. GTX108711; GeneTex, CA, USA), Opsin 3 antibody (Cat. No. GTX108155; GeneTex, CA, USA), and light-emitting diode (LED) light bulb (600-1,000 lux; Hong Zhao Co., LTD) were applied in this experiment.

2.2. Source of Sweet Potato Leaf Ethanolic Extracts

SPLEE were kindly provided by Assistant Researcher Po-Hsien Lu (Chiayi Agricultural Experiment Station). SPLEE (Tainung No. 57; TN 57) was obtained via 90% ethanolic extraction and its concentration contains 0.163 mg/mL lutein.

2.3. Experimental Animals and Experimental Design

Adult male 30 BALB/c mice [10 weeks old; BW between 25-27 g] with specific pathogen-free conditions were used for this study, were purchased from BioLASCO Taiwan Co., Ltd. (Yilan, Taiwan). The environment was maintained room temperature (24-27°C) and 60%-70% humidity with a photoperiod of 12-hr light/12-hr dark cycle. The study will begin after a week acclimation. The Institutional Animal Care and Use Committee (IACUC) of Agricultural Technology Research Institute inspected all animal experiments and this study comply with the guidelines of protocol IACUC-110059 approved by the IACUC ethics committee. The male BALB/c mice were divided respectively into as the normal control group (n = 6), the negative control group (n = 6), the high dose (1.23 mg/kg BW) of sweet potato leaf ethanolic extracts (SPLEE) group (n = 6), the middle dose (0.62 mg/kg BW) of SPLEE group (n = 6), and the low dose (0.31 mg/kg BW) of SPLEE group (n = 6). All BALB/c mice were fed with standard laboratory diet (No. 5053, LabDiet®; PMI Nutrition International, St. Louis, MO, USA). All BALB/c mice were administrated with distilled water ad libitum during the experimental period. The clinical behaviors, the histopathologic and immunohistochemistry (IHC) examination, and the

expression levels of cytokine genes, caspase 3, and reactive oxygen species (ROS) were observed and detected during the experiment.

2.4. The Clinical Behavior and Histopathologic and Immunohistochemistry Examinations

During the experiment, the BALB/c mice' clinical behavior were observed by the research staffers. The eye lesions (retinopathy) of BALB/c mice were observed and recorded with a veterinarian by using an ophthalmoscope (Funduscopy). At the end of the experiment, the BALB/c mice were euthanasia with Zoletil 50. Their eyes were collected and processed for the further studies. The eye tissues were fixed, sectioned, and H&E stained. GFAP antibody (GeneTex) and Opsin 3 antibody (GeneTex) were used for IHC staining. ROS detection cell-based assay kit (Cayman) was applied to detect the expression level of ROS according to the manufacturer' manual. The histopathologic and IHC examination were performed by a senior pathologic veterinarian with a light microscope.

2.5. The Expression Levels of Cytokines, Caspase 3, and ROS

At the end of the experiment, the BALB/c mice were euthanasia with Zoletil 50. Their eyes were collected and processed for the further studies. The eye tissues were homogenized to detect the gene expression levels of cytokines (IL-6 and TNF- α) and caspase 3 by using reverse transcription polymerase chain reaction (RT-PCR). RT-PCR conditions of the sequential process included 95°C (2 minutes), 94°C (45 seconds), 56°C (30 seconds), 72°C (30 seconds and 40 cycles), and 72°C (5 minutes), sequentially. The levels of ROS were detected 2', 7'-dichlorofluorescein (DCF) production at a wavelength of 488 nm according to the manufacturer' manuals.

2.6. Statistical Analysis

The data were expressed as mean $\pm$ SD. All comparisons were made by one-way ANOVA (Analysis of Variance). All significant differences are reported at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

3.1. The Expression of BALB/c Mice' Clinical Behavior in All Groups

SPLEE were orally administrated to BALB/c mice by gavage in the three SPLEE groups. In this study, the clinical behavior observation indexes of BALB/c mice in each group were normal during the experiments. During the experiments, the BALB/c mice in each group had smooth hair, normal hair color, and the normal activity. Moreover, all BALB/c mice were survival until the end of the experiments. The survival percentage of BALB/c mice was 100% (30/30) (data not shown).

3.2. Histopathologic and IHC Examination of BALB/c Mice' Eyes in All Groups

During the experimental period, the ophthalmoscope was used to observe the eye lesions of BALB/c mice irradiated with LED light every week. Data represents there were no obvious lesions in the eyes of BALB/c mice in the middle dose group and the high dose SPLEE group (data not shown). Under H&E staining, the average thickness of the outer nuclear layer (ONL) in the normal control group was $53 \pm 6.2 \mu\text{m}$, and that in the negative control group was $41 \pm 8.2 \mu\text{m}$. The 47 ± 4.0 , 46 ± 6.0 , and $56 \pm 7.9 \mu\text{m}$ thickness of ONL in the low dose, middle dose, and high dose of SPLEE groups, respectively. From the above results of ONL, it was found that the thickness of the retinal ONL layer in the normal control group and the high dose of SPLEE group was significantly thicker than that in the negative control group ($p < 0.05$) (Figure 1 and Table 1). Additionally, the average thickness of inner segments (OS/IS) in the normal control group was $46 \pm 5.3 \mu\text{m}$, in the negative control group was $40 \pm 6.1 \mu\text{m}$, in the low dose of SPLEE was $37 \pm 3.8 \mu\text{m}$, in the middle dose of SPLEE group was $42 \pm 5.5 \mu\text{m}$, and in the high dose of SPLEE group was $43 \pm 4.8 \mu\text{m}$. From the above results of OS/IS thickness, it was found that the OS/IS thickness in the negative control group and the low dose of SPLEE group was thinner than that in the normal control group. There was no significant difference between the high dose of SPLEE group and the normal control group ($p > 0.05$) (Figure 1 and Table 1).

Under the glial fibrillary acidic protein (GFAP) IHC staining, GFAP positive signals can be seen in the nerve fiber layer and ganglion cell layer of BALB/c mice in each group, but the ONL layer is only positive for GFAP in the negative control group (Figure 2 and Table 1). On the other hand, the various level expression of middle-wavelength opsin (M-opsin) can be seen in the ganglion cell layer, the inner nuclear layer, and the photoreceptor layer. There is no significant different in the three layers of all groups (Figure 3 and Table 1).

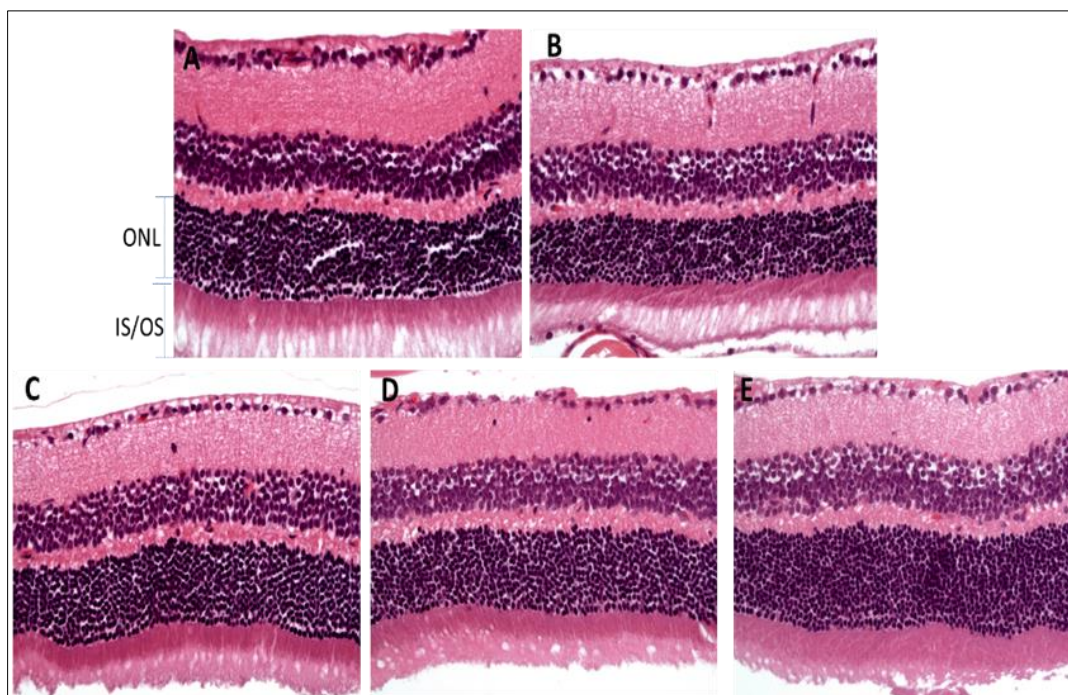


Figure 1 Under the hematoxylin & eosin (H&E) staining, the histopathologic examination of outer nuclear layer (ONL) and out segments-inner segments (OS/IS) of BALB/c mice' retina was performed in each group. (A) the normal control group (B) the negative control group (C) the low dose of SPLEE group (D) the middle dose of SPLEE group (E) the high dose of SPLEE group. 400× magnification

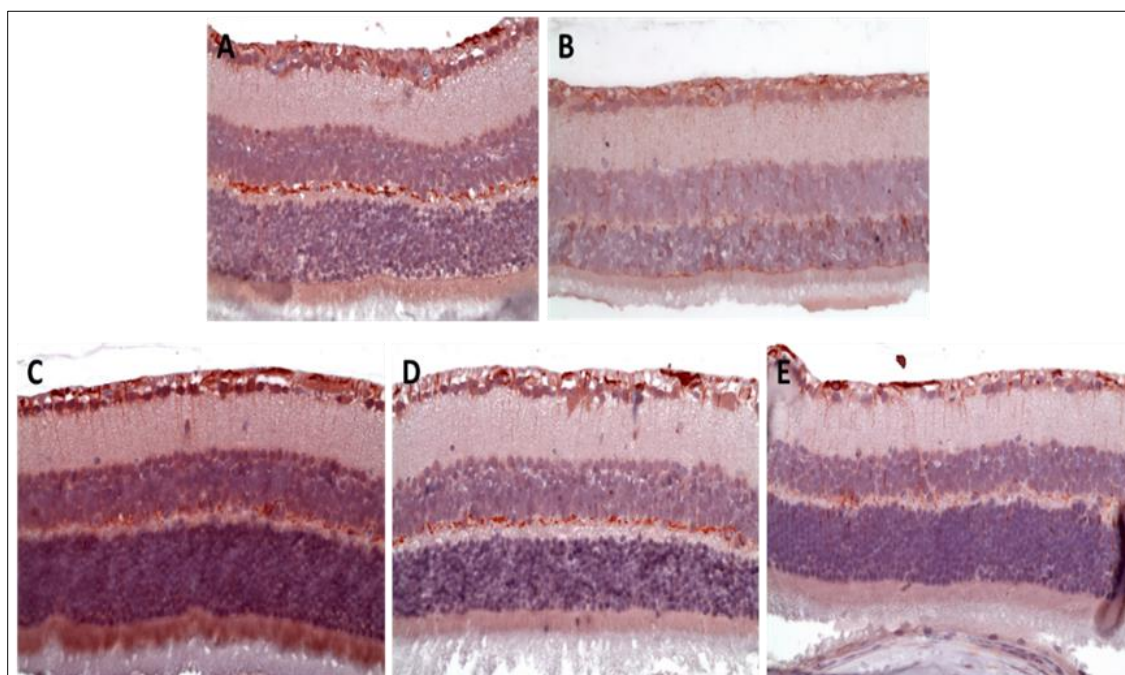


Figure 2 Under the immunohistochemistry staining, the expression of glial fibrillary acidic protein (GFAP) of BALB/c mice' optical microstructure. (A) the normal control group (B) the negative control group (C) the low dose of SPLEE group (D) the middle dose of SPLEE group (E) the high dose of SPLEE group. 400× magnification

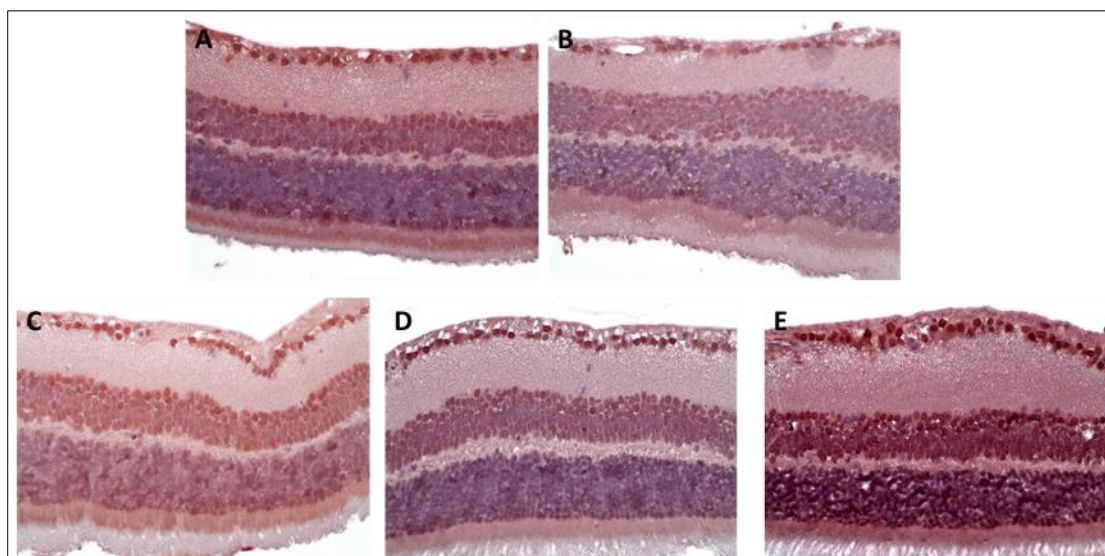


Figure 3 Under the immunohistochemistry staining, the expression of M-opsin of BALB/c mice' optical microstructure. (A) the normal control group (B) the negative control group (C) the low dose of SPLEE group (D) the middle dose of SPLEE group (E) the high dose of SPLEE group. 400× magnification

Table 1 Under H&E staining and IHC staining, the thickness of ONL and OS/IS, and the expression of GFAP and M-opsin of BALB/c mice' eyes were analyzed in each group. * $p < 0.05$ is compared to the negative group

	Normal control group	Negative control group	Low dose	Middle dose	High dose
				SPLEE group	
Thickness of ONL	$53 \pm 6.2^*$	41 ± 8.2	47 ± 4.0	46 ± 6.0	$56 \pm 7.9^*$
Thickness of OS/IS	46 ± 5.3	40 ± 6.1	37 ± 3.8	42 ± 5.5	43 ± 4.8
GFAP	-	+	-	-	-
M-opsin	+	+	+	+	+

The abbreviation: the outer nuclear layer (ONL); the out segments-inner segments (OS/IS), glial fibrillary acidic protein (GFAP); sweet potato leaf ethanolic extracts (SPLEE); '+' is IHC; '-' is IHC; The high dose (1.23 mg/kg BW) of SPLEE group. The middle dose (0.62 mg/kg BW) of SPLEE group. The low dose (0.31 mg/kg BW) of SPLEE group.

3.3. The Gene Expression Levels of Cytokines and Caspase 3 of BALB/c Mice' Eyes in All Groups

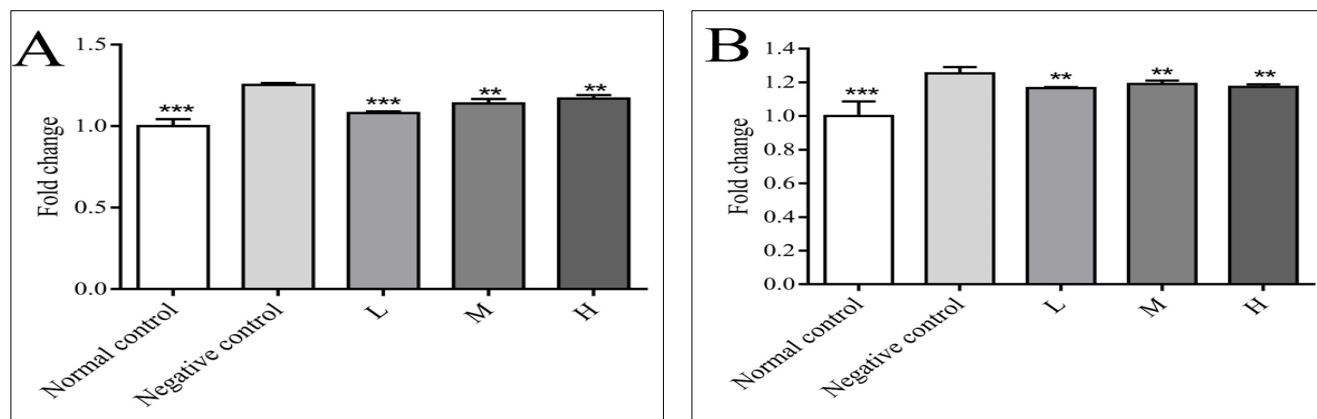


Figure 4 The expression levels of cytokines of BALB/c mice in all groups. (A) IL-6 (B) TNF- α . All data are expressed as mean $\pm$ SD. 'H' is high dose (1.23 mg/kg BW) of SPLEE group. 'M' is middle dose (0.62 mg/kg BW) of SPLEE group. 'L' is low dose (0.31 mg/kg BW) of SPLEE group. ** $p < 0.01$; *** $p < 0.001$

Except for the normal control group, the other groups were irradiated with LED light in the range of 600-1,000 lux for 12 hours every day, and were orally fed with low, middle, and high doses of SPLEE for 6 weeks. At the end of the experiment, all BALB/c mice were sacrificed and their eyes were collected. Based on the IL-6 and TNF- α gene analysis expressions, both IL-6 and TNF- α gene expressions in the normal control group and three SPLEE groups were significantly lower than the negative control group ($p < 0.01$ - $p < 0.001$) (Figure 4), while there was no significant difference in the caspase 3 gene expression between groups ($p > 0.05$) (Figure 5).

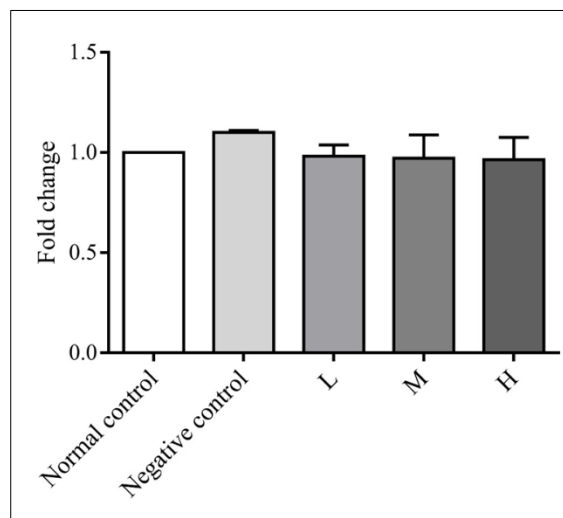


Figure 5 The expression levels of Caspase 3 of BALB/c mice in all groups. All data are expressed as mean $\pm$ SD. 'H' is high dose (1.23 mg/kg BW) of SPLEE group. 'M' is middle dose (0.62 mg/kg BW) of SPLEE group. 'L' is low dose (0.31 mg/kg BW) of SPLEE group.

3.4. The Expression Levels of Reactive Oxygen Species of BALB/c Mice' Eyes in All Groups

Except for the normal control group, the other groups were irradiated with LED light in the range of 600-1,000 lux for 12 hours every day, and were orally fed with low, middle, and high doses of SPLEE for 6 weeks. At the end of the experiment, all BALB/c mice were sacrificed and their eyes were collected. Based on the reactive oxygen species (ROS) analysis expressions, the results showed that ROS in the negative control group were higher than those in the normal control group. Although there was no significant difference in the amount of ROS between SPLEE groups ($p > 0.05$), the trend showed that the levels of ROS in the middle and high dose of SPLEE groups was higher than the negative control group and the low dose of SPLEE group (Figure 6).

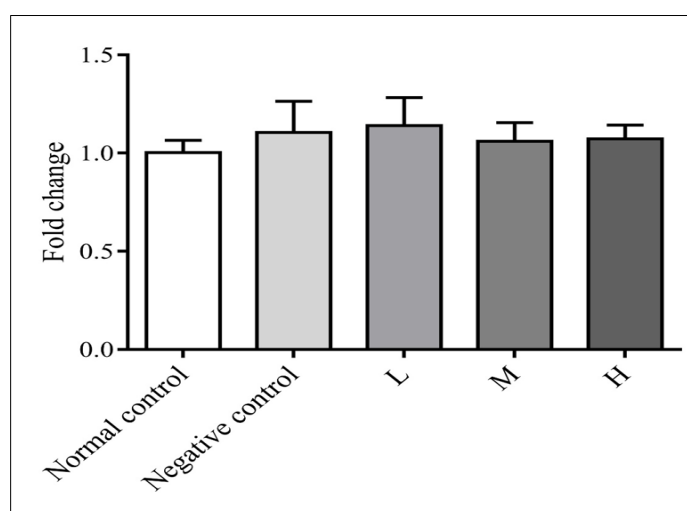


Figure 6 The expression levels of reactive oxygen species of BALB/c mice in all groups. All data are expressed as mean $\pm$ SD. 'H' is high dose (1.23 mg/kg BW) of SPLEE group. 'M' is middle dose (0.62 mg/kg BW) of SPLEE group. 'L' is low dose (0.31 mg/kg BW) of SPLEE group

4. Discussion

The sweet potato possesses nutrients as starch and protein can create new economic and employment activities [11]. They can also increase the nutritional values for the human food systems [11]. Additionally, the sweet potato contains many other nutrients as carbohydrates, minerals, carotenoids, dietary fiber, vitamins, and very little fat. Currently, the sweet potato is a staple food source for many people. Its protein contents were major in the leaves and roots of sweet potato. In addition, the sweet potato has been verified for involved many physiological regulatory functional components as β -carotene and anthocyanins [11].

Globally, vitamin A deficiency (VAD) causes the temporary and permanent eye impairments. Plant foods do not contain vitamin A, however, they contain precursors of vitamin A as β -carotene or carotenoids, which converts to vitamin A via human body. The control of VAD is via improving dietary quality, quantity, and diversification for human consumption. Because the sweet potato is a β -carotene-rich crop, its major carotenoid is all trans- β -carotene, which exhibits highest pro-vitamin A activity. Therefore, sweet potato could be also considered as an excellent novel health care source for people. Based on the sweet potato possesses the high levels of anthocyanin and β -carotene, it is a promising and healthier alternative to synthetic coloring agents in human foods [12-18].

According to the statistical reports in Taiwan, Taiwan people's demand for the eye health care products is increasing. Studies have shown that lutein can remove reactive oxygen species through its antioxidant capacity to protect the macula from blue light damage. Lutein can be regarded as a blue light filter, which can help the macula to reduce the risk of developing cataracts, and improve the condition of glare and the state of the eyes after light stimulation [19-28]. In this study, the trend showed that the free radicals in the high and middle doses (1.23 and 0.62 mg/kg BW) of SPLEE groups were lower than those in the negative control group and the low dose (0.31 mg/kg BW) of SPLEE group. Additionally, the high dose (1.23 mg/kg BW) of SPLEE significantly improved retinal ONL thickness under LED irradiation. Therefore, SPLEE can be like a blue light filter, which can improve the state of the eyes after light irradiation.

In this study, IL-6 and TNF- α gene expression showed that the normal control group and three SPLEE groups were significantly lower than the negative control group. Therefore, SPLEE can be like a suppressor for the blue light-induced damage on BALB/c mice' eyes, which can improve the inflammatory state of the eyes after light irradiation. Taken these results together, the high dose SPLEE (1.23 mg/kg BW) has the better potential for improved retinal ONL thickness and anti-inflammatory effects under LED irradiation.

5. Conclusion

In this experiment, BALB/c mice' eyes were collected for H&E staining, IHC staining, free radical detection, IL-6, TNF- α , and caspase-3 gene expressions. Based on the results of this experiment, no obvious lesions by ophthalmoscopy were observed in the eyes of BALB/c mice in three SPLEE groups. H&E and IHC staining results showed that consumption of high dose of SPLEE significantly improved retinal ONL thickness. The free radical detection results showed that no statistical difference between the groups, however, the trend showed that the free radicals in the high and middle doses of SPLEE groups were lower than those in the negative control group and the low dose of SPLEE group. The results of IL-6 and TNF- α gene expression showed that the normal control group and three SPLEE groups were significantly lower than the negative control group. Taken these results together, the high dose SPLEE has the better potential for improved retinal ONL thickness and anti-inflammatory effects under LED irradiation.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

The Institutional Animal Care and Use Committee (IACUC) of Agricultural Technology Research Institute inspected all animal experiments and this study comply with the guidelines of protocol IACUC-110059 approved by the IACUC ethics committee.

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