ejpmr, 2017,4(10), 147-151

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

THE ANTIOXIDANT EFFECT OF RHYNCHOSIA NULUBILIS EXTRACT IN THE XEROPHTHALMIA-INDUCED ANIMAL MODEL

Gil-Hyun Lee[1] and Kyung-YaeHyun[2]*

[1]Depart. of Clinical Laboratory Science, Kyungwoon University, Gumi, Gyungbuk, 730-739, Korea.
[2]Depart. of Clinical Laboratory Science, DongEui University, Pusan, 614-714, Korea.

***Corresponding Author: Kyung-Yae Hyun**

Depart. of Clinical Laboratory Science, Dong-Eui University, Busan, 614-714, Korea.

Article Received on 15/08/2017 **Article Revised on 04/09/2017** **Article Accepted on 25/09/2017**

ABSTRACT

The antioxidant effect of Rhynchosia nulubils extract (RNE) and iNOS protein expression of eyes and lachrymal glands were verified using the xerophthalmia-induced animal model. The animal groups are divided into the normal group (control), xerophthalmia-induced group (vehicle), high-concentration RNE administration group before inducing xerophthalmia that orally administered pre-high dose (250 mg/100g rat weight), low-concentration RNE administration group before inducing xerophthalmia that orally administered pre-low dose (65mg/100g rat weight), high-concentration RNE administration group after inducing xerophthalmia that orally administered post-high dose (250 mg/100g rat weight), low-concentration RNE administration group after inducing xerophthalmia that orally administered post-low dose (65mg/100g rat weight), and positive control group that orally administered omega-3 (85mg/100g rat weight). The nitric oxide antioxidant effect on the cornea specimen showed a statistically significant ($p<0.05$) decrease in all RNE administration groups compared to the vehicle group. For xanthine oxidase inhibitory activity, RNE was concentration dependent and showed superoxide radical scavenging activity, and showed at least a 55% scavenging effect compared to the positive in the 640 μg/mL concentration. For DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, the positive control group showed nearly a 50% antioxidant effect in concentrations of 160 μg/mL or higher compared to ascorbic acid. The iNOS protein expression of eyes decreased statistically ($p<0.05$) in three groups (pre-low, post- high, post- low treat) compared to the vehicle group, and that of lachrymal glands decreased statistically ($p<0.05$) in pre-high and post-high treat group.

KEYWORD: Rhynchosia nulubils extract (RNE), Xerophthalmia, Antioxidant effect, iNOS.

I. INTRODUCTION

Xerophthalmia is a histological change caused by dry ocular surface and is characterized by the pathological change called squamous metaplasia.[1,2] It is reported that at least 70% of people today suffer from xerophthalmia.[3] Such an increase in xerophthalmia among people today is caused by particulate matters from environmental pollution or eye diseases due to inflammation. In particular, the number of smartphone users has exceeded 50 thousand in 2015 since its release in the Korean market.[4] Smartphones provide various application services in addition to voice calls, such as chatting, social networking service (SNS), internet search, games, etc. and are widespread as a commercialized product all over the nation. This led to longer hours of using electronic devices such as smartphones, reducing the number of blinks and causing eye fatigue. In particular, eye fatigue accounted for 31% of physical issues caused by smartphones, which is quite high.[5] Long hours of using smartphones may cause xerophthalmia and deteriorate eyesight.[6] Putting artificial tears or steroids, administering cyclosporine, and wearing contacts or protective glasses for treatment to treat xerophthalmia merely resolve temporary discomfort and reduce the symptoms, but do not bring pathological change.[7] Xerophthalmia is a disease with increased osmol concentration of the tear film, tears that cause instability, and complex causes on the ocular surface, usually accompanied by displeasure, visual disturbance, and inflammation on the ocular surface.[8] Patients suffer from symptoms such as foreign body sensation as if there is sand inside eyes, stiffness, blurry vision as if there is a layer in front of eyes, inflamed eyes for unknown reasons, easily exhausted eyes, burning eyes, and heavy eyelids.[9] Among the visual system symptoms by Visual DisplayTerminal (VDT), those caused by xerophthalmia are generated first[10], with high concerns for eye diseases like damage to epithelialis corneae and keratoconjunctivitis aside from symptoms like fatigue, foreign body sensation, dryness and stinging.[11] Rhynchosia nulubilis is a type of black soybean that is green inside, and is used for edibility in East Asia.[12] Its hulls are black and it is much smaller than regular black soybeans, and its name comes from its

shape that is like a rat's eye. Rhynchosia nulubilis particularly has a great nature of a drug and is widely used for medication in addition to food, and is known to be effective in preventing and treating various adult diseases like diabetes, hypertension, arteriosclerosis, and cardiac disorders.[13] Beans including Rhynchosia nulubilis are known to have the ability to activate and regenerate the antioxidant effect[14], suppress lipid peroxidation and ROS elimination, and suppress DNA oxidative damage.[15] This study will determine the antioxidant effect of Rhynchosia nulubilis and the effect of applying it to the xerophthalmia-induced animal model using Rhynchosia nulubils extract (RNE).

II. RESEARCH METHOD

2.1. Rhynchosia nulubilis extraction

Rhynchosia nulubilis was purchased from Omniherb (South Korea, Daegu), and was washed and pressure-extracted with a handle-type non-pressure extractor (KSNP-BL180-240) and ground with Pin Mil (Pin crasher). The meal was washed twice and dried, and then ground again. 20 times more water was added and heated for 4 hours at 100℃. Then, it was filtered three times with a filter cloth and finally with a filter paper to come up with Rhynchosia nulubilis extract. The filtrate was refrigerated before use in the experiment.

2.2. Animal model

Animals (rats in SD species, 4 weeks old, male purchased from Hyosung Animals) are divided into 8 groups, 8 rats for each group, for the experiment.

The normal group is control,
The xerophthalmia-induced group is vehicle,
The high-concentration RNE administration group before inducing xerophthalmia is pre-high dose (250 mg/100g rat weight),
The low-concentration RNE administration group before inducing xerophthalmia is pre-low dose (65mg/100g rat weight),
The high-concentration RNE administration group after inducing xerophthalmia is post-high dose (250 mg/100g rat weight),
The low-concentration RNE administration group after inducing xerophthalmia is post-low dose (65mg/100g rat weight),
The positive control administered with omega-3 (85mg/100g rat weight).

The pre-groups were orally administered with each concentration of RNE from Day 8 after adapting to the exclusive animal lab for a week, and sacrificed on Day 37. The first xerophthalmia induction in the pre-group was on Day 14 and the second in Day 21. The post-groups were administered with each concentration of RNE along with the first xerophthalmia induction on Day 7 after adaptation and the second xerophthalmia induction on Day 14, and sacrificed on Day 37. Xerophthalmia was induced by injecting 15㎕ of Freund's Adjuvant complete purchased from sigma equally on the first and second induction and for all groups, using an insulin syringe, into the lachrymal glands. RNE was orally administered every day at 10 a.m.

2.3. Measurement of antioxidant effect

2.3.1. Measurement of nitric oxide in the cornea specimen of rats

0.4 mL/g of PRO-PREP(INTRON.) that homogenized the cornea specimens of xerophthalmia-induced animals was mixed and reacted for 30 minutes at 4℃. After that, 100 μl supernatant and Griess reagent [1%(w/v) sulfanilamide, 100μl 0.1%(w/v) naphtylethylenediamine in 2.5%(v/v) phosphoric acid] are mixed and reacted for 10 minutes in 96 well plates, after which the absorbance is measured at 540 nm using ELISA reader. The density curve is obtained by phased dilution of sodium nitrite ($NaNO_2$).

2.3.2. Measurement of xanthine oxidase inhibitory activity

0.2 ml fluid with dissolved 2 mM xanthine was added to 0.1 ml sample solution and 0.6 ml pH 7.5 0.1 M potassium phosphate buffer. Then, 0.1ml 0.2 unit/ml xanthine oxidase was added and the solution was reacted for 5 minutes at 37℃, and 1 ml 1 N HCl was added and the reaction was ended. Then, the absorbance was measured at 292 nm with uric acid generated in the reaction solution. Xanthin oxidase inhibitory activity is represented by the absorbance decrease rate in the sample solution addition group and non-addition group

2.3.3. Measurement of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of Rhynchosia nulubilis

Measurement of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity to measure the antioxidant effect of Rhynchosia nulubilis was done using the property of hydrazyl of DPPH (Diphenylpicryl hydrazyl), where nitrogen is in an unstable state and thus easily accepts hydrogen, thereby reacting to antioxidant matters and accepting hydrogen, losing its orthochromatic property. 1.9 ml 0.2 mM DPPH solution (99.5% ethanol) was added to 0.1 ml (control: 99.5% ethanol) solution made into 7 concentrations (10, 20, 40, 80, 160, 320, 640 μg/mL) by substance of Rhynchosia nulubilis. It was shaken for 10 seconds in Vortex mixer and incubated for 30 minutes at 37℃. Then, absorbance was measured at 517 nm using a spectrophotometer.

The drug for the positive control group was L-ascorbic acid in 6 different concentrations: 10, 20, 40, 80, 160, 320, 640 μg/mL (99.5% ethanol). The antioxidant effect of each sample was represented by the % antioxidant activity (electron donating ability) on DPPH.

2.3.4. Quantitative analysis of protein

The animals were put into inhalation anesthesia with isoflurana and their eyes and lachrymal glands were removed and stored at -70℃. The removed organs were cut fine in cold storage and ground using a homogenizer

after adding Lysis buffer (PRO-PrepTM, protein Extration solution). It was centrifuged for 15 minutes at 13000rpm and the supernatant was obtained to extract protein. Bio-Rad Protein assay Kit (Bio-Rad Hercules, CA, USA) was used to quantify into the same protein amount, and the sample was put in 18.5ul loading and electrophoresis in 10%-12% sodium dodecyl sulfate-polyacrylaminde gel. Through the protein mobilization process in nitrocellulose membrane, iNOS (cell signal, USA) was reacted at 4℃ for 24 hours as the first antibody, and then reacted for 2 hours with the second antibody (Rabbit, cell signal, USA). After that, ECL prime (Amersham Pharmacia Biotech, Buckinghamshire, UK) and molecular Imager Chemi Doc XRS (Bio rad, USA) were used to quantitatively measure the expression. Animal sacrifices were carried out in compliance with the regulation of animal ethics at Dong-Eui University.

2.3.5. Statistical analysis

The experiment results for blood tests were presented in mean ± standard deviation, and were analyzed with ANOVA using SPSS Version 18. The results of the protein quantitative analysis were analyzed using Vision Works Image Software.

III. RESULTS

3.1. Nitric oxide results in the corneas of xerophthalmia-induced animals

Fig. 1 shows the result of measuring the amount of generated NO using Griess reagent. The control group was 2.14±0.20, vehicle 3.92±0.26, pre-low 2.6±0.31, pre-high 2.81±0.12, post-low 2.95±0.41, post-high 3.12±0.25, and positive 2.88±0.33, showing a statistically significant ($p<0.05$) decrease compared to the vehicle group.

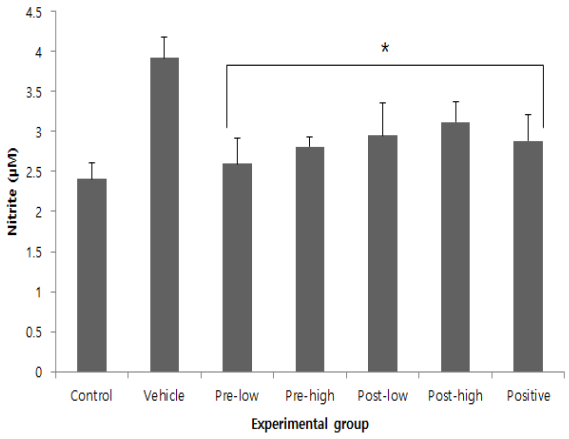

Fig. 1: Measurement of nitric oxide in the cornea of xerophthalmia animals.

3.2. Xanthine oxidase inhibitory activity

Fig. 2 shows the result of xanthine oxidase inhibitory activity. Xanthine oxidase acts as a rate-limiting enzyme in all terminal oxidation of purine and serves as a source of oxidizers like superoxide radical or hydrogenperoxide. Superoxide anion inhibitory effect by the enzymes of xanthine/ xanthine oxidase is shown by the superoxide anion scavenging activity and xanthine oxidase enzyme inhibition and has biological significance by suppressing generation of free radical. The positive control group used BHA (butylated hydroxy anisole) to compare the superoxide radical scavenging effect of 10, 20, 40, 80, 160, 320, 640 μg/mL RNE. As a result, RNE showed a concentration-dependent superoxide radical scavenging activity, and showed at least 55% more scavenging effect than the positive group at the concentration of 640 μg/mL.

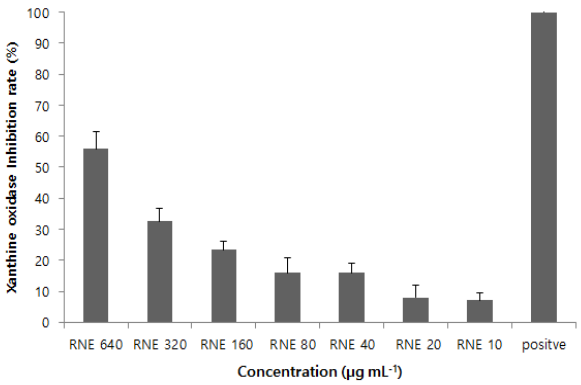

Fig. 2. Xanthine oxidase inhibitory activity of RNE.

3.3. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of RNE

Fig. 3 shows the antioxidant effect of RNE measured in 7 concentrations: 10, 20, 40, 80, 160, 320, 640 μg/mL. The results showed that the positive control group showed nearly 50% antioxidant effect in 160 μg/mL concentration or higher compared to ascorbic acid.

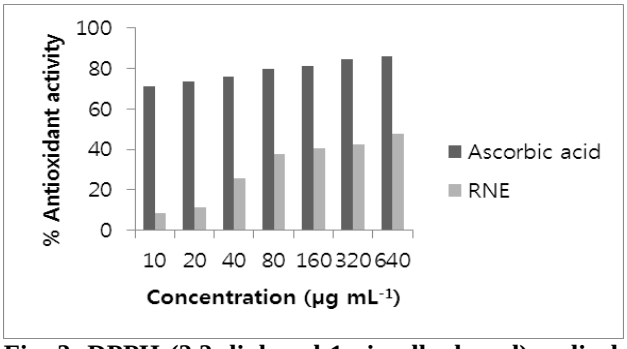

Fig. 3. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of RNE.

3.4. iNOS protein expression in eyes and lachrymal glands

Fig. 4 shows the iNOS moderation changes of RNE in eyes and lachrymal glands. The eyes showed a statistical decrease in expression in three groups (pre-low, post-high, post- low treat) compared to the vehicle group, showing the lowest result in the post-high treat group.

The expression decreased statistically in pre-high, post-high treat group for lachrymal glands.

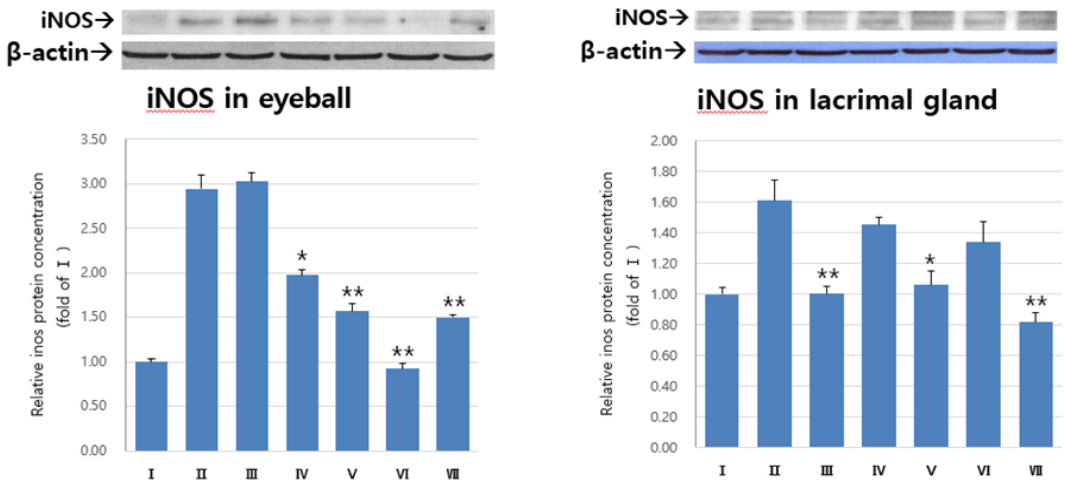

Fig. 4. *;P<0.05, **;P<0.01 compare with group II.

I: control group, II: vehicle, III: pre treatment + high dose RNE(Rhynchosia Nulubilis Extracts), IV: pre treatment low dose RNE, V: post treatment high dose RNE, VI: post treatment low dose RNE VII: omega 3 extract (positive)

IV. CONCLUSION

Use of smartphones or electronic devices that have become a necessity for people today further increases the prevalence of xerophthalmia. Therefore, this study verified the effect of RNE in xerophthalmia-induced rats and determined the antioxidant effect of Rhynchosia nulubilis. Xanthine oxidase inhibitory activity showed a concentration-dependent superoxide radical scavenging activity in RNE, showing at least 55% more scavenging effect than the positive group in 640 μg/mL concentration. This is consistent with the study by Nam et al.[14] For DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, the positive control group also showed nearly 50% antioxidant effect at 160 μg/mL concentration or higher compared to ascorbic acid, which is consistent with the study by Nam et al. The antioxidant effect may have resulted in regenerating and expanding the capillary vessels of eyes.[16,17] Rhynchosia nulubilis was found to have a similar antioxidant effect with that of black soybeans that is already well known.[14]

Nitric oxide synthase (NOS) is an enzyme that synthesizes NO with the existence of NADPH and O_2 that are electron carriers in the body using the amino acid L-arginine as a substrate, classified into nNOS (neuronal type, Type I), iNOS (inducible type, Type II), and eNOS (endothelial type, Type III).[18,19] Unlike nNOS and eNOS expressed as essential components in the body, iNOS is expressed and increased in the state of disease.[18,20] Based on this fact, that in the eyes and lachrymal glands of xerophthalmia-induced animals all decreased statistically compared to the vehicle group, and nitric oxide in corneas also decreased statistically. This study only investigated iNOS protein and thus needs follow-up research for changes in various proteins that increase in pathosis of eye diseases in addition to xerophthalmia. In conclusion, RNE in xerophthalmia-induced rats showed antioxidant effect and a decrease in protein expression that may occur in pathosis. RNE is expected to show effect in reducing xerophthalmia and promote tear generation. It is also effective in eliminating oxides occurred by oxidative stress with its antioxidant activity and may be used as candidate to prevent and treat various eye diseases.

V. REFERENCE

1. Nelson JD, Havener VR, Cameron JD. Cellulose acetate impressions of the ocular surface: dry eye states. Arch Ophthalmol, 1983; 101: 1869-1872.
2. Tseng SCG. Staging of conjunctiva! squamous metaplasia by impression cytology. Ophthalm-ology, 1985; 92: 728-733.
3. Arita R, Morishige N, Sakamoto I, Imai N, Shimada Y, Igaki M, Suzuki A, Itoh K, Tsubota K. Effects of a warm compress containing menthol on the tear film in healthy subjects and dry eye patients. Sci Rep., 2017; 7: 45848.
4. Korea Communication Commission. Wire/wireless telecommunication service subscribers statistics Gwacheon: Korea Communication Commission, 2013.
5. Yun JY, Mun JS, Kim MJ, Kim YJ, Kim HA, Heo BR. Smart phone addiction and health problem in university student. Journal of Korean Association for Crisis and Emergency Management, 2011; 3(2): 94-104.
6. Lee SH. Evaluating the influence of smart phone use on thepsycho-physiological condition Konkuk University, 2011; 41.
7. Safety and Efficacy of Cortisol Phosphate in Hyaluronic Acid Vehicle in the Treatment of Dry Eye in Sjogren Syndrome. Rolando M, Vagge A. J Ocul Pharmacol Ther, 2017; 4.
8. The International Dry Eye Workshop. The definition and classification of dry eye disease: report of the definition and classification subcommittee of the international Dry Eye Workshop. The Ocular Surface, 2007; 5(2): 75-92.

9. Lee BJ, Hong JH, Jung DI, Park MJ. A study on the confidence of dry eye diagnosis methods. Journal of Korean Ophthalmic Optics Society, 2008; 13(1): 15-20.
10. Cho YA, Won JS, An GJ. The effect on the dryness of eye during VDT work. Journal of The Korean Ophthalmological Society, 1996; 37(12): 13-17.
11. Park HJ, Yi K. Relationship between middle school students' computer using time and dry eye. Journal of The Korean Ophthalmological Society, 2002; 43(3): 449-454.
12. Yim J.H., Lee O.H., Choi U.K., Kim Y.C. Antinociceptive and anti-inflammatory effects of ethanolic extracts of Glycine max (L.) Merr and Rhynchosia nulubilis seeds. Int. J. Mol. Sci., 2009; 10(11): 4742-4753 13.
13. Kang, S.A., Jang, K.H., Cho, Y., Hong, K., Suh, J.H., Choue, R. Effects of artificial stomach fluid and digestive enzymes on the aglycone isoflavone contents of soybean and black bean(Rhynchosia Molubilis : Yak-Kong). Korean Nutr. Society, 2003; 36(1): 32-39.
14. Nam, S.H., Kang, M.Y. Screening of antioxidative activity of legume species. J. Korean Soc. Agric. Chem. Biotechnol., 2003; 46(1): 32-38.
15. Toda, T., Sakamoto, A., Takayanagi, T., Yokotsuka, K. Changes in isoflavone compositions of soybean foods during cooking process. Food Sci. Technol. Res. 2000; 6(4): 314-319.
16. Cirino G, Vellecco V, Bucci M. Nitric Oxide-Hydrogen Sulfide: the gasotransmitter paradigm of the vascular system. Br J Pharmacol. Apr 13. 2017.
17. Liu Z, Liu Y, Xu Q, Peng H, Tang Y, Yang T, Yu Z, Cheng G, Zhang G, Shi R. Critical role of vascular peroxidase 1 in regulating endothelial nitric oxide synthase, 12: 226-232.
18. 2017Park HJ, Song M. Leaves of Raphanus sativus L. Shows Anti-Inflammatory Activity in LPS-Stimulated Macrophages via Suppression of COX-2 and iNOS Expression. Prev Nutr Food Sci., 22(1): 50-55.
19. 2017Skov Jensen P, Aalkjaer C, Bek T. Differential effects of nitric oxide and cyclo-oxygenase inhibition on the diameter of porcine retinal vessels with different caliber during hypoxia ex vivo. Exp Eye Res., 2017; S0014-4835(16)30345-1.
20. Zhang HH, Chen JC, Sheibani L, Lechuga TJ, Chen DB. Pregnancy augments VEGF-stimulated in vitro angiogenesis and vasodilator (NO and H2S) production in human uterine artery endothelial cells. J Clin Endocrinol Metab, 2017; 7.