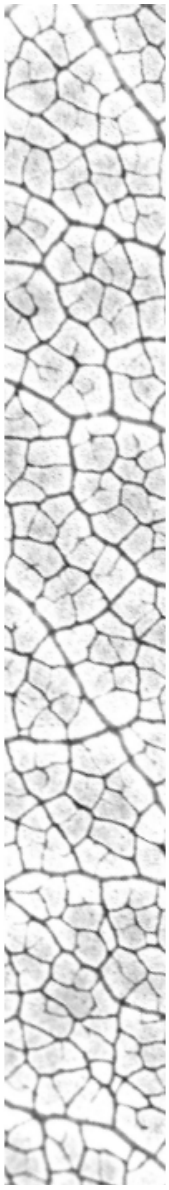

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Effect of *Spirulina platensis* extract on Vascular Endothelial Growth Factor (VEGF) expression in corneal inflammation in rat (*Rattus novergicus*) strain wistar

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Abstract

Background: Spirulina is a microalga known as has anti-inflammatory, anti-angiogenic, antioxidant, antibacterial and radioprotective properties. The aim of this study was to elucidate the differences in VEGF expression in response to various dosages of Spirulina platensis (SP) topical treatment in normal and corneal inflammation in the rat.

Material and Methods: This was an experimental study. SP powdered extract was obtained by maceration method using water as a solvent. Effect on VEGF expression was analyzed after SP aqueous extract topical treatment in corneal inflammation rat model for four times a day, over seven days. Corneal inflammation was induced by basic chemical trauma using 1N NaOH. VEGF expression was analyzed by histopathology: cornea samples were made into microscope slides and stained with immunofluorescent stain. Quantification was aided with confocal laser microscopy.

Results: Significant differences of VEGF expression were observed after topical treatment with 50 µg/mL, 100 µg/mL, and 200 µg/mL dosages. A significant correlation was also observed between SP treatment dosages and VEGF expression.

Conclusion: Topical SP treatments at 50 µg/mL, 100 µg/mL, and 200 µg/mL concentrations have anti-angiogenic effects by reducing VEGF expression in the cornea inflammation model in rats.

Keywords: cornea neovascularization, *Spirulina platensis* extract, Vascular Endothelial Growth Factor (VEGF)

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BACKGROUND

The cornea is a transparent tissue and does not have any blood vessels. It is very vulnerable to many kinds of trauma, such as physical, environmental stress or infection. This leads to inflammation rather easily and, eventually, can damage and/or cause death to corneal cells. Corneal inflammation is the most common eye disease in humans and animals. One of the complications from this condition is neovascularization that leads to corneal clouding and decreases eyesight or even causes blindness (Azar 2006, Eraslan and Toker 2009, Gagen 2011, Rolfsen et al. 2013).

Almost 4% of the US population suffer corneal vascularization. Every year, there are 1.4 million patients with this disease risk, and 12% of those have significant decline in eyesight (Rolfsen et al. 2013, Shakiba et al. 2009).

Corneal neovascularization is the formation of new blood vessels from the limbus to the center of the cornea. This process can occur if there is an imbalance between angiogenic factors with their counterparts, anti-angiogenic factors. Angiogenesis-related to corneal inflammation is a very complex process involving proliferation and migration of vascular endothelial cells, extracellular matrix remodeling, and tubular structure formation. Some growth factors and proteinases are involved in corneal neovascularization. Vascular endothelial growth factor (VEGF) is one of the key angiogenic factors in this process. Significantly higher VEGF concentrations were observed in corneas with neovascularization than normal ones (Qazi et al. 2009, Shakiba et al. 2009).

Spirulina is a microalga in the Oscillatiriaceae family. It is spiral in form and contains a high concentration of phycocyanin, which makes it green-blue in color. Spirulina platensis (SP) is used in many countries as a food or beverage supplement in humans and animals. Spirulina has various nutritional components, such as proteins, essential amino acids, vitamins, minerals, phycocyanins, and polysaccharides. Many studies

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showed that Spirulina has anti-inflammatory, anti-angiogenic, antioxidant, antibacterial and radioprotective properties (Capelli and Cysewsky 2010, Christwardana and Hadiyanto 2012, Saranraj and Sivasakthi 2014).

In this study, several dosages of topical SP aqueous extract were administered to a rat model of corneal inflammation in order to compare VEGF expression in each dosage, and if it alters expression at all. Corneal inflammation was induced by topical administration of 1N NaOH.

MATERIALS AND METHODS

This experimental study was conducted in the Laboratory of Pharmacology and Laboratory of Pathology, Medicine Faculty, Brawijaya University, Malang. VEGF expression assay was conducted in the Central Laboratory of Life Sciences, Brawijaya University. This study was conducted from July 2015–October 2015. Rats (*Rattus norvegicus*) were used as the samples for this study. As many as five rats per group were used and one rat was added to anticipate complications.

SP Extract Production

Powdered SP was used to make the topical administration. Twenty grams of SP powder was soaked in 1L of ultra-pure water and shaken for 24 hours at room temperature. The solution was centrifuged at 5000 rpm for 10 mins at 4°C and then filtered with Whatman no. 1 filter to remove debris. The extract was then freeze-dried until becoming powder and stored at 4°C. The powdered extract was made into solutions of 50 µg/mL, 100 µg/mL or 200 µg/mL and placed in sterile bottles. The solution was used as eye-drops with a volume of 20 drops/mL (Chu et al. 2010).

Sample Treatments

Thirty Wistar rats were divided into five groups of six rats. Group I, or negative control group, did not get any chemical trauma and got distilled water eye drops four times per day for seven days. Groups II–V were treatment groups, which got basic chemical trauma by sticking filter paper, previously soaked in 1N NaOH, on the right eye cornea for 30 seconds and then rinsing with 20 mL of saline solution. Group II, or positive control, got chemical trauma treatment and then the same distilled water eye drop treatment as the negative control. Groups III–V got SP extract topical treatment four times per day of 50, 100 and 200 µg/mL concentrations, respectively. Every rat had feed and drink every day until enucleation on the eight days.

Corneal VEGF Expression Calculation

Cornea samples were obtained randomly from the central defect area of the cornea. VEGF expression was examined for the thickness of the cornea in a 1 mm wide observation area. After blocking in paraffin and deparaffinization, slides were stained with immunofluorescent anti-VEGF-FITC *rabbit polyclonal antibody*. The calculation was conducted by one observer only. Every slide was observed and its VEGF expression calculated in two observation fields at 400x magnification. VEGF expression was quantified based on color density using the Olympus Fluoview FV1000 software (1.7.a version) that was integrated into the confocal laser microscope. Final calculation was based on the mean of two observation fields for every sample (Kumar and Rudbeck 2009).

Table 1. Mean observed expression of cornea VEGF (in intensity/micrometer^2)

	Group I K (-)	Group II K (+)	Group III 50 µg/mL	Group IV 100 µg/mL	GroupV 200 µg/mL
1	59.091	208.444	113.785	82.415	93.360
2	45.852	181.234	112.602	99.203	74.159
3	43.624	194.324	133.511	84.244	72.121
4	53.321	220.495	106.526	87.114	72.503
5	57.633	189.439	102.635	74.136	77.360
6	61.381	215.030	107.892	74.244	72.940
Mean	**52.983**	**201.494**	**112.825**	**83.559**	**77.074**

Table 2. One-way ANOVA analysis of each treatment with VEGF expression

Note	One-way ANOVA significance
VEGF expression	0.000

Data Analysis

Data normality distribution was tested with Kolmogorov-Smirnov test. Homogeneity of the data was tested with Levene's statistic. After the data was confirmed to be normally distributed and its variance equal, One Way ANOVA test was conducted to determine the VEGF expression differences in treatment groups, with α = 5% and multiple comparisons Tukey was used as a post-h°C test. Correlation-regression test was used to infer the relationship between different dosages of topical SP treatment to VEGF expression. Data analysis was conducted in SPSS for Windows 17.0.

RESULTS

Elevated VEGF expression was observed in the positive control group. If the result of positive control compared to treatment groups, it produced a declining trend of the mean 3 treatment groups' VEGF expression (**Table 1**).

Normality test showed a p-value of 0.158 ($p > 0.05$), so data were normally distributed. Levene's test showed a p-value of 0.152 ($p > 0.05$), suggesting variant homogeneity of VEGF expression data. Both tests suggested that the data can be analyzed with ANOVA (**Table 2**).

ANOVA test produced a p-value of 0.000 ($p < 0.05$), indicating a significant difference in VEGF expression in the cornea after treated with SP extract (**Table 2**). Tukey's test was used post h°C as well as multiple comparison tests, which are shown in **Table 3**.

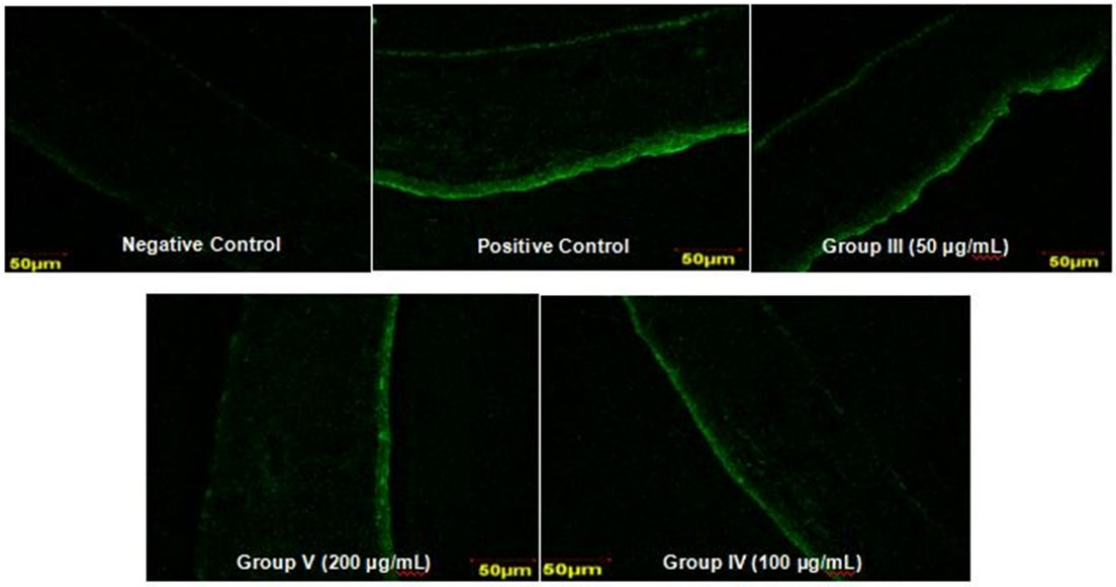

Fig. 1. VEGF-FITC Staining. Green fluorescence is VEGF expressions, pictures were taken by confocal microscopy at 400x magnification

Table 3. Tukey post h˚C test

Group comparison		VEGF expression difference	Sig.	Conclusion
K (+)	K(-)	148.510	0.000	Significantly different
	D1	88.669	0.000	Significantly different
	D2	117.935	0.000	Significantly different
	D3	124.420	0.000	Significantly different
D1	K(-)	59.841	0.000	Significantly different
	K(+)	-88.669	0.000	Significantly different
	D2	29.265	0.001	Significantly different
	D3	35.751	0.000	Significantly different
D2	K(-)	30.575	0.000	Significantly different
	K(+)	-117.935	0.000	Significantly different
	D1	-29.265	0.001	Significantly different
	D3	6.485	0.825	Not significantly different
D3	K(-)	24.090	0.005	Significantly different
	K(+)	-124.420	0.000	Significantly different
	D1	-35.751	0.000	Significantly different
	D2	-6.485	0.825	Not significantly different

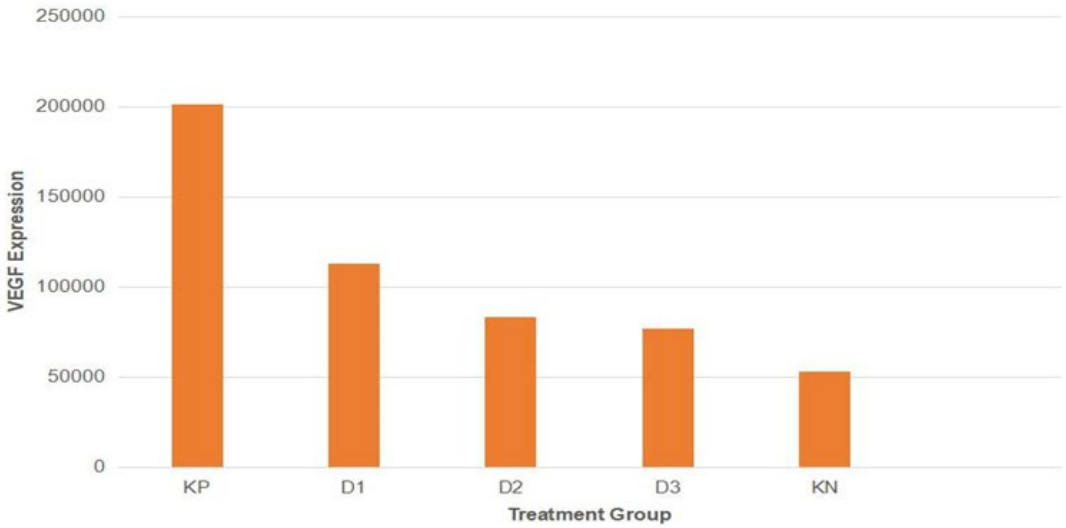

Fig. 2. VEGF-FITC Staining. Green fluorescence is VEGF expressions, pictures were taken by confocal microscopy at 400x magnification

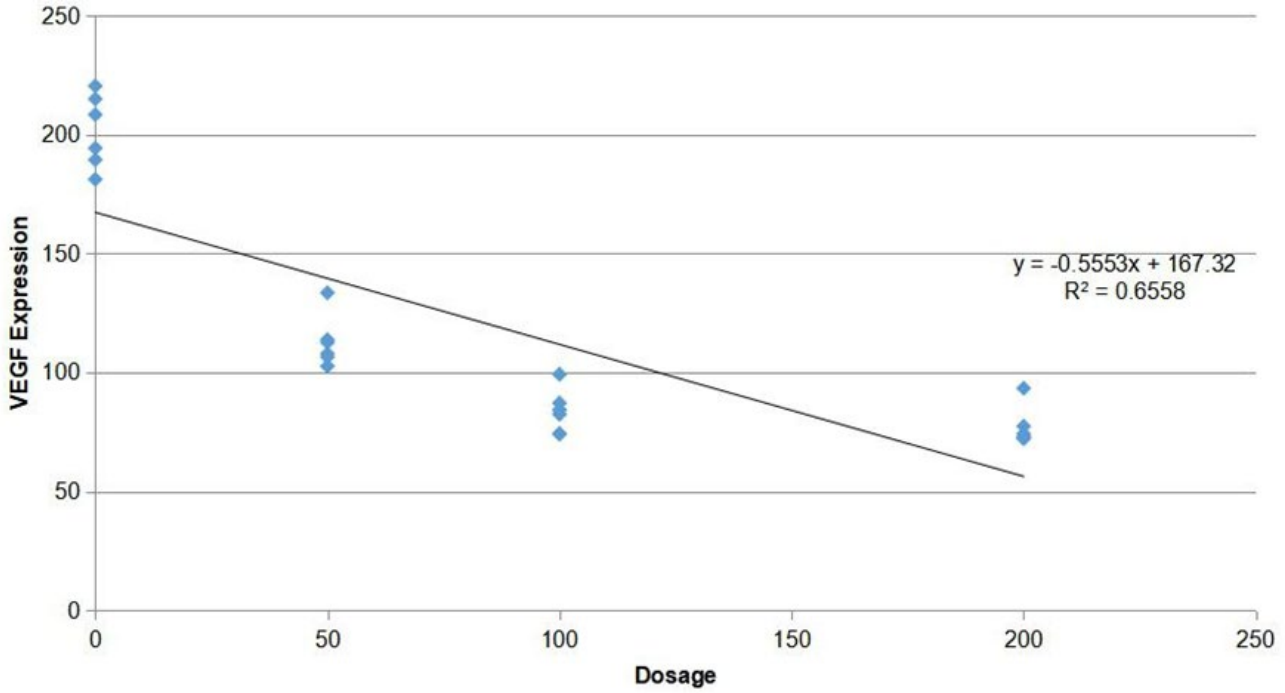

Fig. 3. Regression graph of SP extract topical treatment with corneal VEGF expression

Response plot (main effect) showed the magnitude of the effect of each SP treatment on VEGF expression in an inflammatory rat model. SP extract topical treatment at 200 µg/mL was able to decrease VEGF expression the greatest of all treatments. Mean VEGF expression was not significantly different with 50 and 100 µg/mL SP.

Pearson correlation test produced a p-value of 0.000 ($p < 0.05$), which means there was a significant relationship between SP extract topical treatment and VEGF expression. The correlation was negative, so the relationship was inversely proportional. Regression analysis also had a p-value of 0.00 ($p < 0.05$), which indicated significance for the effect of SP topical treatment to decrease VEGF expression (**Fig. 3**).

The regression graph showed a declining regression line, which means that increasing SP extract concentration will decrease VEGF expression. The decreasing effect was 65.6%, which means the declining of VEGF expression is 65.6% affected by the SP topical treatment dosage and 34.4% is by other factors.

DISCUSSION

Animal Model of Corneal Inflammation

Corneal neovascularization is closely related to the inflammation process, so the inflammation pathway was used to make the animal model. This study used Wistar rats as an animal model and basic chemical trauma as the inducer of neovascularization. Basic chemical trauma is commonly used to induce inflammation as its process is closely related to inflammation in humans and usually causes corneal neovascularization. Previous studies reported that this trauma caused almost 100% corneal neovascularization. Moreover, this kind of trauma usually happens on a daily basis. Up to 5–22% of eye traumas in Australia are caused by chemical trauma, and 30–80% of them are basic chemicals. The usual inducer of this trauma is 1N NaOH, which is easy to procure and has a simple methodology. The result of this trauma can be observed by a whitish, cloudy cornea accompanied with hyperemia and blepharospasm (Ashby et al. 2014, Ortiz et al. 2014, Shi et al. 2011, Yang et al. 2009).

Aqueous Extract of *Spirulina platensis*

Herbal-based medicine, as angiogenesis inhibitors, can be used as therapeutic agents for corneal neovascularization. This study used SP extract to elucidate its effect on the said disease by targeting the VEGF expression pathway. SP was chosen because of its beneficial effect on wound healing and angiogenesis, along with other added benefits. According to Yang et al. (2009 and 2011), SP polysaccharide extract has high potential to inhibit corneal neovascularization. Another study by Saini et al. (2014) observed effectiveness of C-phycocyanin from *Spirulina platensis* in inhibiting colon cancer angiogenesis by suppressing the VEGFR1 pathway (Saini and Sanyal 2014, Yang et al. 2009).

Freshwater SP was used in this study because they grow better in a freshwater environment with less natrium and mineral so the smell is less fishy. Aqueous extract, extraction method by macerating in water solvent, was used because it is a simple method, economically efficient and there is no prior publication about using SP aqueous extract in treating corneal

inflammation in an animal model. Herroro et al (2004) compared SP extraction methods: with hexane, petroleum ether, ethanol and water as a solvent, and discovered that water extraction yielded a better result than others. Water-extracted Spirulina has better antioxidant effects than hexane and petroleum ether extraction. Another study by Singh et al. (2014) also stated that water-extracted SP has better antimicrobial properties than ethanol, methanol and acetone extraction. Syarina et al. (2014) observed better wound healing properties in fibroblast culture with aqueous extract of SP than methanol and ethanol extracts (Herrero et al. 2004, Singh et al. 2014, Syarina et al. 2015, Yan et al. 2007).

SP aqueous extract in this study contains all the substance in SP. The SP extraction method was based on Chu et al (2010). That study stated all the content of the SP extract, and phycocyanin is the most abundant substance. The extract has antioxidant activity 20 times more effective than vitamin C as it has other components than phycocyanin. Those components cooperate to produce better antioxidant effects than one kind of antioxidant (Chu et al. 2010).

Topical treatment was used as it is not invasive and it is predicted that it will not have any systemic effect. Extract was administered for seven days based on the study of Ortiz's et al. (2014), who stated that corneal neovascularization happened between 2 to 12 days after basic chemical trauma, with the highest growth between two to eight days after trauma.

SP Extract Effect on Corneal VEGF Expression

The results of this study suggest that *Spirulina platensis* extract has anti-angiogenic effects by inhibiting VEGF expression. VEGF is one of the main factors which contribute to neovascularization of cornea. Declining VEGF expression in cornea will inhibit the corneal neovascularization process.

VEGF expression calculation showed that positive control had 3.8 times more VEGF expression than the negative control. This is in correlation with Yan et al (2007) study, which observed a significant increase of VEGF expression six hours after the chemical trauma that reached its peak after 12 hours, and then increase again after 96 hours until 192 hours or eight days after trauma. According to Wei et al (2009), VEGF expression in rabbit cornea increased three days after NaOH induction and stayed elevated until 21 days after trauma (Yan et al. 2007, Wei and Zhi 2009).

One-way ANOVA test found a significant difference between treatment groups. Tukey test showed that VEGF expression decreased in treatment groups of 50 µg/mL, 100 µg/mL and 200 µg/mL dosages significantly compared with the positive control, even though the 100 µg/mL and 200 µg/mL groups did not differ significantly. The highest dose of SP was significantly different from negative control.

The candidate of the anti-angiogenic agent, SP extract, was applied by topical administration to corneal inflammation and decreased VEGF expression in the cornea. Yang et al. (2009) also produced the same result with 100 µg/mL dosage, and the extract also suppressed MMP2 and MMP9 expression alongside VEGF while stimulated PEDF expression. This implies the ability of SP extract to suppress angiogenic factors and also stimulate anti-angiogenic factors. This anti-angiogenic effect also affects proliferation, migration and tube formation of *in vitro* vascular endothelial cells. Another study by Ali et al (2015) observed SP extract has anti-inflammatory, antioxidant and anti-angiogenic properties by inhibiting COX-2, TNF-α, IL-6 and VEGF in arthritic rats (Ali et al. 2015, Yang et al. 2009).

The insignificant difference between 100 µg/mL and 200 µg/mL doses need to be considered for the next study as the initial evidence that increasing SP extract dosage will not yield better effects on VEGF inhibition. The other possibility is it may have toxic effects on the cornea. A significant difference of every treatment dosage and negative control indicates that all of the treatment dosages did not decrease VEGF expression until it near normal levels. One probability is the topical treatment time was insufficient. The previous study by Wei et al. (2009) observed that VEGF expression was elevated in rabbit cornea three days after NaOH induction and stayed until 21 days after induction (Wei and Zhi 2009).

SP Extract Dosage Effect on Corneal VEGF Expression

Linear regression test showed that topical SP extract treatment had a significant effect on decreasing corneal VEGF expression. Increasing SP extract is predicted to decrease corneal VEGF expression. The effect will be 65.6%.

The significant effect of topical SP extract treatment to decrease VEGF expression is caused by not only the anti-angiogenic effect but also its anti-inflammatory effect. It is well established that inhibition of inflammation factors will downregulate angiogenic factors. Few studies observed the potency of SP extract as an anti-inflammatory agent, one of them was Yang et al. (2012). They observed that SP polysaccharide extract inhibited inflammation and corneal neovascularization more effectively than amniotic membrane in the chemical traumatized cornea. Another study by El-Shazly et al. (2015) showed that SP extract effectively inhibited corneal neovascularization, decreased fibroblast activation and inhibited active mononuclear cell infiltration compared to topical Bevacizumab in a corneal inflammation model (El-Shazly et al. 2015, Yang et al. 2009, 2012).

Other factors that affect 34.4% of the decrease in VEGF may be in the form of natural anti-angiogenic factors, such as thrombospondin, PEDF, and endostatin. Those factors will be expressed during inflammatory conditions and inhibit VEGF expression. According to Lawler (2012), thrombospondin (Tsp1 and 2) directly inhibits angiogenesis by antagonizing VEGF activity. Wang et al. (2007) reported that VEGF expression is decreased in choroid neovascularization rat model after inter-abdominal injection of endostatin.

However, this study had limitations. The active compound inside the SP extract that contributes to VEGF inhibition was not identified. This study also did not elucidate the effective timing of topical SP extract treatment on corneal inflammation in order to get the optimum effect in decreasing VEGF expression.

CONCLUSION

A significant decrease in VEGF expression was observed after topical SP extract treatment of 50 µg/mL, 100 µg/mL and 200 µg/mL concentrations on rat cornea inflammation model, indicating the significant anti-angiogenic effect of SP extract on VEGF expression.

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