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Original Article

Anticataract activity of ethanolic extract from *Hippobroma longiflora* (L.) G.Don leaves: *Ex vivo* investigation

[Actividad anticatarata del extracto etanólico de las hojas de *Hippobroma longiflora* (L.) G.Don: Investigación *ex vivo*]

Eva Imelda[1,2,3], Khairan Khairan[4], Rodiah R. Lubis[5], Pati Kemala[1], Utari Zulfiani[6,8], Suci Rahayu[8], Ghazi M. Idroes[7], Sarra M. Adev[9], Nur B. Maulydia[1], Rinaldi Idroes[4,8*]

[1]Graduate School of Mathematics and Applied Sciences, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia.
[2]Department of Ophthalmology, General Hospital Dr. Zainoel Abidin, Banda Aceh 23126, Indonesia.
[3]Department of Ophthalmology, Faculty of Medicine, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia.
[4]Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia.
[5]Department of Ophthalmology, Faculty of Medicine, Universitas Sumatera Utara, Medan 20222, Indonesia.
[6]Department of Chemistry, Faculty of Science and Data Analytics, Institut Teknologi Surabaya, Surabaya 60111, Indonesia.
[7]Department of Occupational Health and Safety, Faculty of Health Sciences, Abulyatama University, Banda Aceh 23372, Indonesia.
[8]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia.
9Medical Programme, Faculty of Medicine Universitas Brawijaya, Malang 65145, Indonesia.
*E-mail: rinaldi.idroes@usk.ac.id

Abstract

Context: In Aceh, *Hippobroma longiflora*, known as kitolod by the locals, is believed to possess medicinal benefits such as anticataract.

Aims: To investigate the anticataract activity of ethanolic extract from *H. longiflora* leaves *ex vivo*.

Methods: The dried fine powder of *H. longiflora* was macerated using ethanol at room temperature, and the concentrated extract was prepared in concentrations ranging from 100 to 300 µg/mL. The cataract model was established by using goat lens induced with H_2O_2 and later exposed to the extract for treatment. Anti-cataract activity was assessed by observing the change in lens opacity. Further, malondialdehyde levels and activities of endogenous antioxidants (superoxide dismutase, glutathione peroxidase, and catalase).

Results: The apparent cataract opacity reduced after the exposure of the extract was observed macroscopically. The cataract eyes experienced a significant increase ($p<0.05$) in malondialdehyde level and a significant reduction ($p<0.05$) in the activities of superoxide dismutase, glutathione peroxidase, and catalase. The pathologic conditions could be reversed by introducing the extract with a concentration as low as 100 µg/mL ($p<0.05$).

Conclusions: The ethanolic extract of *H. longiflora* leaf has a potential anticataract effect by ameliorating oxidative stress-related biocompounds.

Keywords: catalase; cataract; glutathione peroxidase; malondialdehyde; superoxide dismutase.

Resumen

Contexto: En Aceh, se cree que la *Hippobroma longiflora*, conocida como kitolod por los lugareños, posee beneficios medicinales como anticatarata.

Objetivos: Investigar la actividad anticatarata del extracto etanólico de las hojas de *H. longiflora ex vivo*.

Métodos: El polvo fino seco de *H. longiflora* se maceró utilizando etanol a temperatura ambiente, y el extracto concentrado se preparó en concentraciones que oscilaban entre 100 y 300 µg/mL. El modelo de catarata se estableció utilizando cristalino de cabra inducido con H_2O_2, y posteriormente expuesto al extracto para su tratamiento. La actividad anticatarata se evaluó observando el cambio en la opacidad del cristalino. Además, se analizaron el nivel de malondialdehído y las actividades de los antioxidantes endógenos (superóxido dismutasa, glutatión peroxidasa y catalasa).

Resultados: Se observó macroscópicamente la reducción de la opacidad aparente de la catarata tras la exposición del extracto. Los ojos con cataratas experimentaron un aumento significativo ($p<0,05$) del nivel de malondialdehído y una reducción significativa ($p<0,05$) de las actividades de superóxido dismutasa, glutatión peroxidasa y catalasa. Las condiciones patológicas pudieron revertirse introduciendo el extracto con una concentración tan baja como 100 µg/mL ($p<0,05$).

Conclusiones: El extracto etanólico de la hoja de *H. longiflora* tiene un potencial efecto anticatarata al mejorar los biocompuestos relacionados con el estrés oxidativo.

Palabras Clave: catalasa; catarata; glutatión peroxidasa; malondialdehído; superóxido dismutasa.

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AUTHOR INFO
ORCID:
0000-0003-3028-6022 (KK)
0000-0003-1861-0563 (RRL)
0000-0002-3079-6535 (PK)
0000-0001-6217-9067 (GMI)
0000-0003-2264-6358 (RI)

INTRODUCTION

A cataract is a clouding of the eye's lens that can gradually cause visual impairment (Imelda et al., 2022). Cataracts are often associated with age, where, with increasing age, the lens of the eye can turn cloudy due to the process of oxidative stress so that vision becomes blurry (Hossain et al., 2021). Oxidative stress is the main factor in the onset and development of cataracts (Beebe et al., 2010; Berthoud and Beyer, 2009). Free radicals compete with intracellular molecule electrons, leading to lipid peroxidation, protein modification, chromosomal lesions, and damage to mitochondrial DNA (mtDNA or mDNA). The processes cause disruptions in gene transmission and expression and react with DNA chains, leading to mitochondrial DNA damage. This DNA damage disrupts the gene regulatory system, disrupting protein regulation and expression. Mutations in the R48C gene can damage the stability of A-crystallin, which is associated with lens opacity (Hsueh et al., 2022; Wu et al., 2022). The formation of free radicals is characterized by the formation of malondialdehyde (MDA), which is the end product of lipid peroxidation in the body (Feriyani et al., 2021).

A cataract cannot be corrected with glasses, contact lenses, or refractive surgery (Made et al., 2020; Fernanda and Hayati, 2020). The primary treatment for cataracts is surgery, which involves replacing the cloudy lens with an artificial lens implant. However, surgery can be daunting for many patients and can be costly.

On the other hand, natural remedies are one of the alternative treatment options preferred by communities worldwide. The use of plant secondary metabolites is a fast alternative pathway to solving medical problems, including their evaluation as a COVID antivirus (Dutta et al., 2021; Khairan et al., 2021b; Maulydia et al., 2022; Mousavi et al., 2021; Tallei et al., 2020a), anti-inflammatory (Tallei et al., 2022b), antibacterial (Khairan et al., 2019; Khairan and Septiya, 2021), antifertility (Seriana et al., 2021), termiticidal and nematocidal activities (Khairan et al., 2021b), and anticholesterol effects (Rachmawati et al., 2022). Secondary metabolites with a hydroxyl group, phenolics, and flavonoids can act as effective electron donors in free radical scavenging (Bendary et al., 2013). Further, the secondary metabolites are thought to be responsible for the induction of endogenous antioxidant molecules catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Côté et al., 2010).

In Indonesia, especially in Aceh Province, people believe that *Hippobroma longiflora* (L.) G.Don leaves, family *Campanulaceae*, known as kitolod, can cure eye diseases such as cataracts. This plant originates from the West Indies and is very common in Indonesia. Based on the local ethnomedicinal practices, it has been proven to be effective in treating asthma, bronchitis, sore throat, wounds, cancer, eye medicine, antitumor medicine, and anti-inflammatory medicine. In the scientific literature, *H. longifora* leaf extract has been witnessed to exert antibacterial activity against *Streptococcus mutans* and *Enterococcus faecalis* (Fazil et al., 2017). More importantly, its potent antioxidant activities have been reported (Uddin et al., 2020). Due to its ethnomedicinal use as eye medicine, the plant has high potential as an anti-cataract. No report has been published on the anticataract effectiveness of *H. longiflora* leaf, especially those grown in Aceh. Without scientific evidence, it is compelling to investigate the anticataract activities using *ex vivo* experiments. We also intended to observe the effects of this plant against oxidative stress regulating proteins related to cataracts.

MATERIAL AND METHODS

Plant collection and extraction

The sample of *Hippobroma longiflora* leaves was collected in the Illie area, Ulee Kareng District, Banda Aceh City, Aceh Province, Indonesia (5° 54′ 57.02′′ latitude and 95° 35′ 16.52′′ longitude). Plant determination was carried out at the National Research and Innovation Agency (BRIN), Bogor, West Java Province, Indonesia (voucher no.: B-747/V/DI.05.07/12/2021).

The leaf samples were taken and washed before being air-dried for seven days. The samples were ground and macerated using 70% ethanol solvent for 48 hours. The resulting extract was evaporated using a Rotary Evaporator (Buchi Rotavapor® R-300, Flawil, Switzerland) to obtain the concentrated extract of *H. longiflora* leaves. It was stored at 4°C until further use.

Animals

Goats (≥2 years old) were raised in local farms, and the researchers did not control the treatment of the animals. No particular protocols were required for the treatment since we collected the lense from slaughtered animals that were sacrificed for human consumption. This is in line with international ethical guidelines for animal experiments that exclude the *ex vivo* model (Pasupuleti et al., 2016). Ethical clearance was obtained prior to the study from the Veterinary Ethics Committee, Universitas Syiah Kuala, Indonesia (No. 165/KEPH/IX/2022).

Cataract induction and treatment (*ex vivo*)

The preparation stage of the *ex vivo* model involved the extraction of goat lenses using the method described previously (Durgapal et al., 2021). Fresh goat eyeballs were collected from the local slaughterhouse, immediately transported to the laboratory, and stored at a temperature of 0-4°C. After that, the lenses were carefully extracted using the extracapsular extraction technique and incubated in artificial aqueous humor (sodium chloride: 140 mM; potassium chloride: 5 mM; magnesium chloride: 2 mM; sodium bicarbonate: 0.5 mM; sodium dihydrogen phosphate: 0.5 mM; calcium chloride: 0.4 mM; and glucose: 5.5 mM) at room temperature with a media pH level of 7.8. Streptomycin 100 µg/mL and penicillin 100 IU/mL were added to the media to prevent microbial growth. Lenses were divided into normal control (not receiving any treatment), cataract or negative control (receiving cataract induction with H_2O_2 10 mM), quercetin or positive control (receiving quercetin, 500 µg/mL) (Mestry and Juvekar, 2017). The other three groups were assigned to treatment groups, where the label of each group corresponds to the given extract concentration, namely 100, 200, and 300 µg/mL.

Estimation of malondialdehyde (MDA) level

The level of lipid peroxidation was determined using the procedure previously described by Ohkawa et al. (1979), in which the principle of this method was that MDA, the end product of lipid peroxidation, reacted with thiobarbituric acid to form a pink chromogen. The lenticular material was homogenized in 1.0 mL of 0.15 M potassium chloride for this test. In a reaction tube, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid (pH 3.5), and 1.5 mL of 0.81% thiobarbituric acid solution were added sequentially. 0.2 mL of lenticular homogenate was added to the reaction mixture and then heated in boiling water for 60 minutes. After cooling to room temperature, 5 mL of butanol/pyridine solution (15:1 v/v) was added and centrifuged at 5.000 rpm for 15 minutes. The upper organic layer was aspirated, and the pink color intensity produced was read at 532 nm using tetramethoxypropane as an external standard. The lipid peroxide level was expressed as nanomoles of MDA formed per gram of tissue.

Superoxide dismutase (SOD) activity assay

A total of 0.3 mL of chloroform and 0.5 mL of ethanol were added to 0.5 mL of homogenate and vigorously mixed for 1 minute (Ohkawa et al., 1979). The mixture was then centrifuged at 18 g for 60 minutes, and the supernatant was used for SOD testing. A SOD test reagent of 2.50 mL was added to each tube, followed by 0.5 mL of diluted standard solution (0-279 ng/tube) or supernatant fluid to each tube. The double-distilled water 0.5 mL was added to an empty tube to make the final volume of the reaction system 3.0 mL. The rack containing the tubes was placed in a water bath maintained at a temperature of 25°C. The xanthine oxidase solution of 50 µL. was added to each tube at 30-second intervals. After a 20-minute incubation period, the reaction was stopped by adding 1 mL of 0.8 mmol/L $CuCl_2$ solution per tube. The absorbance (Abs) was measured at a wavelength of 560 nm. The percentage of inhibition was calculated using the following equation [1].

$$\text{Inhibition (\%)} = \frac{\text{Blank Abs} - \text{Sample Abs}}{\text{Blank Abs}} \times 100 \quad [1]$$

The activity of Cu,Zn-SOD was calculated by comparing it with the standard curve of the protein (Cu,Zn-SOD) (Ozmen et al., 2002).

Catalase (CAT) activity assay

The CAT test was conducted according to the method by Ozmen et al. (2002), in which 0.2 mL of homogenate was incubated in 1 mL of substrate at 37°C for 60 seconds. The enzymatic reaction was stopped by adding 1.0 mL of 32.4 mM/L ammonium molybdate, and the yellow-colored molybdate complex and hydrogen peroxide were measured at a wavelength of 405 nm against blank 3. Blank 1 contained 1.0 mL of substrate, 1.0 mL of molybdate, and 0.2 mL of the sample. Blank 2 contained 1.0 mL of substrate, 1.0 mL of molybdate, and 0.2 mL of buffer. Blank 3 contained 1.0 mL of buffer, 1.0 mL of molybdate, and 0.2 mL of buffer. The catalase activity was calculated using the following equation [2].

$$\text{Inhibition (\%)} = \frac{\text{Abs blank 1} - \text{Sample Abs}}{\text{Abs blank 2} - \text{Abs blank 3}} \times 271 \quad [2]$$

Where Abs: absorbance.

Glutathione peroxidase (GPx) activity assay

The GPx test began by preparing a homogenate solution of the sample prepared earlier. Then, 2-50 µL of the sample was added to a 96-well plate, and the volume was made up to 50 µL with assay buffer. A positive control GPx was prepared using 100 µL of assay buffer (Ozmen et al., 2002)

Statistical analysis

MDA and SOD, CAT, and GPx activity levels were analyzed statistically to determine their meaningful differences. Firstly, the Shapiro-Wilk test was performed to identify whether the data distribution was normal. After that, one-way ANOVA was employed, followed by Tukey post hoc. Statistical significance

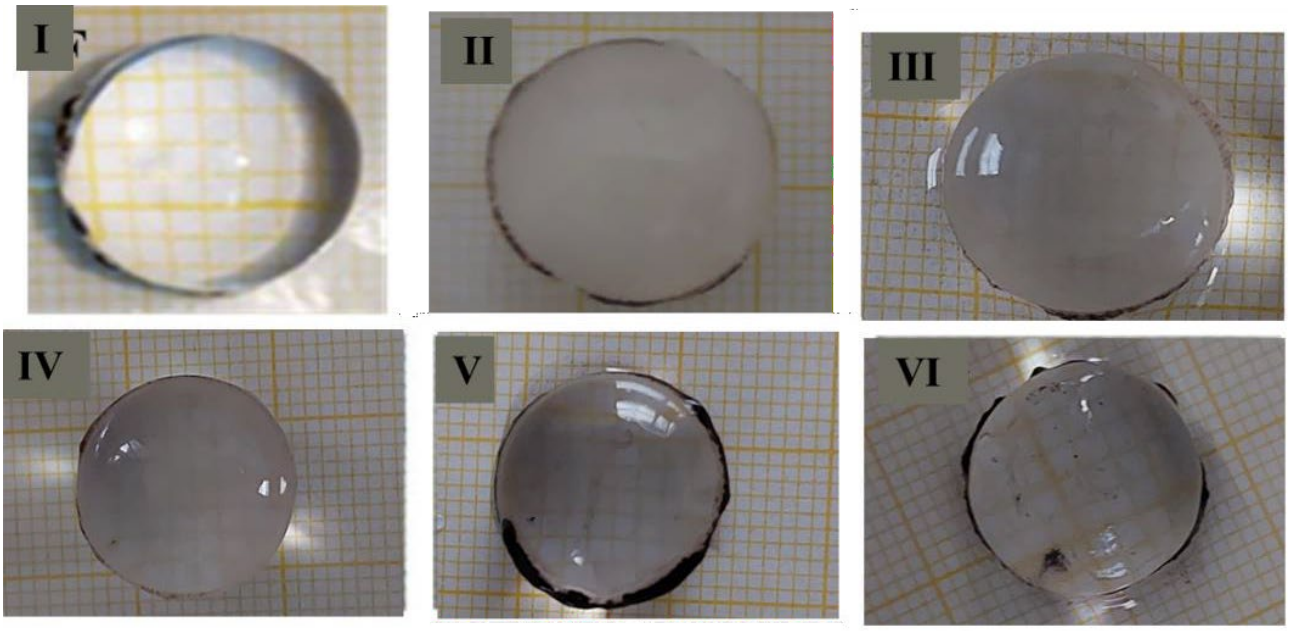

Figure 1. Photograph images depicting the reduced cataract opacity after the treatment with *H. longiflora* extract. Normal lens **(I)**, H_2O_2-induced cataract lens (10 mM) **(II)**, cataract lenses treated with 100 µg/mL **(III)**, 200 µg/mL **(IV)**, and 300 µg/mL **(V)** *H. longiflora* extract, and cataract lenses treated with quercetin (500 µg/mL) **(VI)**.

was considered achieved if $p<0.05$. This analysis was done on GraphPad Prism 9.2.0 (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Lenses opacity

Macroscopic morphology and opacity levels of goat lenses have been presented in Figure 1. All goat lens samples were placed on a grid paper and evaluated for opacity levels by observing the clarity of the lines. Morphology of the goat eye shows that normal lenses were clearly visible. Cataract lenses had the highest opacity level where the squares pattern could not be seen through, indicating the successful induction. Better squares pattern visibility could be observed in lenses treated with the ethanolic extract of *H. longiflora*. Those treated with *H. longiflora* 300 µg/mL demonstrated the clearest squares pattern visibility compared to cataract lenses treated with that of 100, 200, or 300 µg/mL. Similarly, previous studies have reported the improved lense clarity following the administration of plant extracts such as *Syzygium cumini*, *Aegle marmelos* (Hajarnavis and Bulakh, 2020), and *Moringa oliefera* (Kurmi et al., 2014). However, the improved opacity is more observable in the lens treated with quercetin (Fig. 1).

MDA levels

The ability of this oxidative stress can be measured with the malondialdehyde (MDA) biomarker, which correlates with cloudiness in the lenses (Bhatia et al., 2006). A number of goat lens test groups that have been treated with the addition of peroxide H_2O_2 and ethanolic *H. longiflora* extract had their MDA levels determined. The results of MDA level measurements have been presented in Fig. 2. The MDA level increased significantly in the cataract lens but improved significantly ($p<0.01$) once the quercetin or *H. longiflora* extracts were introduced. Interestingly, the MDA level reduction was dependent on the extract concentration. MDA is known to be a dominant breakdown product resulting from lipid peroxide. In human cortical cataracts as well as nuclear cataracts, MDA has been found to increase 3.5 times baseline and identified as a 2-thiobarbituric acid reactive substance (Bhuyan and Bhuyan 1984). A high MDA value indicates detrimental ocular oxidative stress, eventually manifested as cataract (Kaur et al., 2012). Reduced level of MDA in the lens indicates the attenuation of ocular oxidative stress (Xiang et al., 2012; Nazaruk and Borzym-Kluczyk, 2015). In this present study, the highest reduction of MDA reached 34.2%. The reduction of MDA is relatively lower compared to previous research using *Punica granatum* (Mestry and Juvekar, 2017). However, our results were relatively higher in reducing MDA compared to a previous study using *Syzygium cumini* seeds and *Aegle marmelos* leaves (Hajarnavis and Bulakh, 2020). Taken altogether, *H. longiflora* leaf extract plays a role in improving cataracts by reducing oxidative stress, which is characterized by decreased MDA levels.

Endogenous antioxidants

Other parameters tested were endogenous antioxidants, namely catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). The activity of SOD, CAT, and GPx was measured in goat lenses that had been treated, where the results have been presented in Fig. 3. All of these endogenous antioxidants were significantly lower in the cataract group ($p<0.01$). The introduction of quercetin (positive control) reversed this effect significantly ($p<0.05$), even somewhat higher than the normal lens. Interestingly, further increments of CAT, SOD, and GPx activities were observed in groups treated with *H. longiflora* extract with a concentration as low as 100 µg/mL. In the case of CAT and GPx, the elevation of their activities was observed to be dependent on the extract concentration. On the contrary, the increase of the extract concentration to 300 µg/mL reduced the SOD activity significantly ($p<0.01$), even lower than that of the cataract lens. This is worthy of further investigation to reveal the net medicinal benefits of each extract concentration.

Endogenous antioxidants scavenge reactive oxygen species or other free radicals that are formed excessively (Behrouzi et al., 2015; Izyumov et al., 2010). The present study is in line with previous reports that found an increase in SOD, CAT, and GPx activities following the addition of plant extracts (*Syzygium cumini* and *Aegle marmelos*), attributed to the improved cataract in goat lens (Hajarnavis and Bulakh, 2020).

CONCLUSION

The leaf extract of *H. longiflora* positively reduces oxidative stress, consequently enhancing the activity of SOD, CAT, and GPx enzymes. Compared to other extract concentrations, 300 µg/mL of *H. longiflora* leaf extract offered the most significant anticataract effect, as demonstrated by decreased levels of MDA and enhanced activity of SOD, CAT, and GPx enzymes. It has also been established visually that *H. longiflora* leaf extract at a concentration of 300 µg/mL produces the most precise cataract lens compared to other concentrations.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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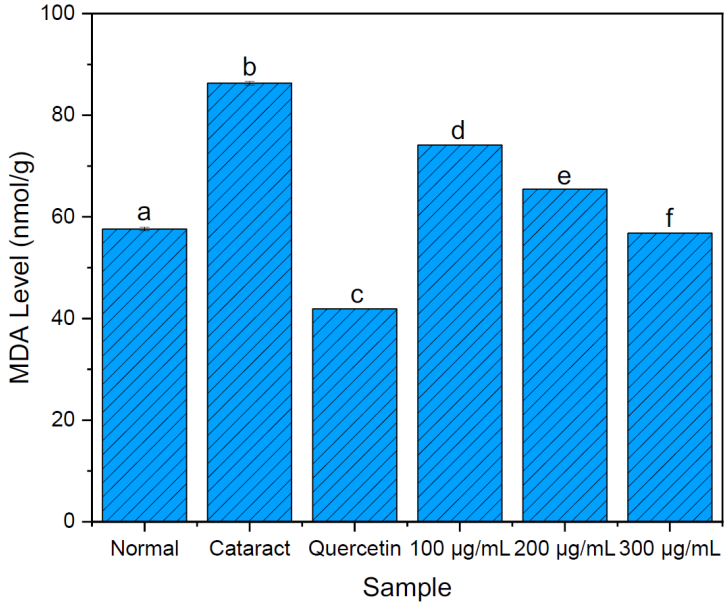

Figure 2. Effect of ethanolic *H. longiflora* extract on MDA levels in cataract goat eye lenses.

Data represent mean ± SD (n = 3). Difference in alphabet indicates the statistical significance at $p<0.05$ based on Tukey post hoc.

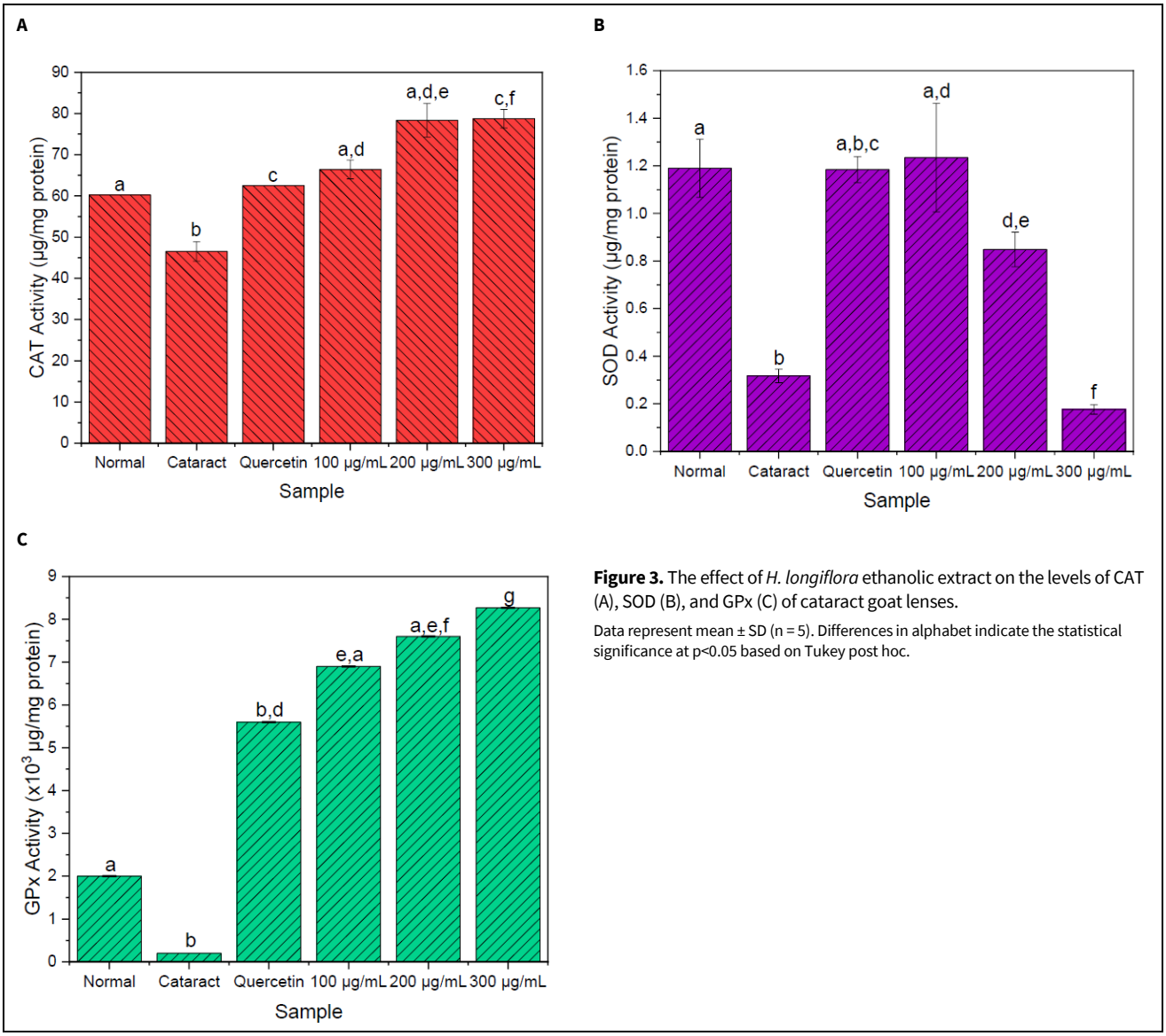

Figure 3. The effect of *H. longiflora* ethanolic extract on the levels of CAT (A), SOD (B), and GPx (C) of cataract goat lenses.

Data represent mean ± SD (n = 5). Differences in alphabet indicate the statistical significance at $p<0.05$ based on Tukey post hoc.

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AUTHOR CONTRIBUTION:

Contribution	Imelda E	Khairan K	Lubis RR	Kemala P	Zulfiani U	Rahayu S	Idroes GM	Adev SM	Maulydia NB	Idroes R
Concepts or ideas	x	x	x							
Design	x	x	x							
Definition of intellectual content	x									
Literature search	x			x	x					
Experimental studies	x			x	x	x	x	x		
Data acquisition	x									
Data analysis	x	x	x	x	x	x	x	x	x	x
Statistical analysis										
Manuscript preparation	x									
Manuscript editing		x	x	x	x					
Manuscript review	x	x	x	x	x	x	x	x	x	x

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