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AN IN VITRO ASSAY REVEALS THE ANTI-AGING PROPERTIES OF TEMULAWAK EXTRACT (*Curcuma xanthorrhiza* L.)

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ABSTRACT

Aging is the gradual loss of a tissue's capacity to heal and maintain normal or physiological form and function. Elastase, hyaluronidase, and tyrosinase are the enzymes that contribute to the process of skin aging. The anti-aging effect is connected to the inhibition of these enzymes' activities. Numerous medicinal plants with active metabolites have been extensively utilized to treat aging. The active compounds of temulawak (*Curcuma xanthorrhiza* L.), such as phenolics, curcuminoids, and xanthorrhizol, have promising properties that may be used as anti-aging agents. This study aimed to determine whether temulawak extract, a potential option for cosmeceuticals, has anti-aging properties that can inhibit the enzymes elastase, hyaluronidase, and tyrosinase. A stock solution was made by dissolving 20 mg of temulawak extract in 1 mL of 100% dimethyl sulfoxide (DMSO). The stock solution was then diluted to produce working solutions with concentrations ranging from 31.25 to 1000 µg/mL. An in vitro assay was carried out in three replications to examine the anti-aging activity of the temulawak extract. The in vitro assay investigated the inhibition of the enzyme elastase, hyaluronidase, and tyrosinase at seven different concentrations, with the following ranges: 2.08–66.67 µg/mL for the anti-elastase, 5.21–166.67 µg/mL for the anti-hyaluronidase, and 3.125–100 µg/mL for the anti-tyrosinase. IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, N.Y., USA) was used to perform the statistical analysis, with a significance level of $p<0.05$. Temulawak extract exhibited the highest inhibition rates, reaching 82.72%, 89.41%, and 94.17% for the anti-tyrosinase, anti-elastase, and anti-hyaluronidase activities, respectively. The median inhibitory concentrations (IC_{50}) were 10.66, 70.39, and 55.87 µg/mL for the elastase, hyaluronidase, and tyrosinase activities, respectively. This study revealed that temulawak extract has strong anti-aging properties as it effectively inhibits the activities of elastase, tyrosinase, and hyaluronidase. In conclusion, temulawak extract can be considered a promising candidate for cosmeceutical applications.

Keywords: Anti-aging cosmeceutical; anti-elastase; anti-hyaluronidase; *Curcuma xanthorrhiza*; healthy lifestyle

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Highlights:
1. This study investigated the potential of temulawak extract as a cost-effective option for cosmeceutical applications in the treatment of aging.
2. Temulawak extract was found to have the ability to inhibit elastase, hyaluronidase, and tyrosinase activities, thus making it a suitable option for cosmeceutical applications.

INTRODUCTION

Age-related illnesses and geriatric syndromes, such as obstructive lung conditions, musculoskeletal issues, different types of cancer, neurological conditions, and skin conditions, are brought on by the gradual decline in physiological function that accompanies the aging process. The continuous reduction of the structural integrity and physiological function of the skin is caused by a variety of internal and external factors that affect skin aging (Popoola et al. 2015, Costa et al. 2022). All people experience natural processes that lead to aging, which are caused by intrinsic factors. Clinical alterations associated with intrinsic aging include decreased skin barrier function, increased vascularization of the skin layer, and reduced epidermal cell turnover, which result in skin

atrophy. Skin protection, absorption, excretion, secretion, thermoregulation, and sensory perception are consequently compromised. The ultraviolet (UV) spectrum has an impact on the occurrence of extrinsic skin aging. Wrinkles, hypopigmentation, hyperpigmentation, rough skin, loss of skin color, dryness, and melanoma are clinical symptoms resulting from external factors (Rihhadatulaisy & Putriana 2020).

Following exposure to photoaging stimuli, the skin accumulates reactive oxygen species (ROS), which can indirectly activate dermal enzymes such as elastase. The dermal enzyme activation effectively breaks down and degrades elastin (Popoola et al. 2015, Chatatikun & Chiabchalard 2017). Therefore, elastase synthesis promotes premature aging of the skin. Such aging is characterized by the presence of distinct symptoms such as deep furrows, freckles, sallowness, laxity, wrinkles or severe atrophy, and leathery texture (Ding et al. 2018). Hyaluronic acid, commonly known as hyaluronan (HA), is a glucose-based polymer found in the body's tissues and fluids. However, it is predominantly concentrated in the epidermal layer and dermal compartment of the skin. HA primarily aids in skin renewal, provides moisture, thickens fluids, and lessens extracellular fluid permeability (Jiratchayamaethasakul et al. 2020).

Because of the superior water-holding capacity of HA, areas rich in this substance have enhanced emollience, smoothness, and youthfulness, as well as reduced wrinkles. In contrast to hyaluronidase, which is normally produced as we age, HA regrettably and naturally diminishes. The skin ages due to the loss of strength, flexibility, and moisture caused by the HA-destructing enzyme hyaluronidase (Ndlovu et al. 2013, Jegasothy et al. 2014). One of the methods to combat wrinkles is by preserving the HA contents under the skin to prolong skin moisture. Tyrosinase, a melanogenic enzyme, plays a crucial role because it can limit the rapid rate of melanin-based coloration. Hence, a typical strategy for pigmentation disorder treatment and whitening for aesthetic purposes is the suppression or inhibition of tyrosinase activity (Chatatikun & Chiabchalard 2017, Kang et al. 2018).

The application of cosmetic products helps to enhance the natural beauty and appeal of the skin while protecting it against harmful effects from both external and internal factors. By minimizing skin disorders, cosmetic products not only enhance the surface appearance of the skin but also extend the longevity of healthy skin. Skin care products maintain the suppleness of the skin by reducing type I collagen and providing photoprotection, among other benefits, while simultaneously nourishing its health, texture, and integrity. The use of synthetic or natural substances in skin care formulations contributes to the traits of the resulting cosmetic products, including their abilities to reduce the visibility of free radicals on the skin and manage their properties over an extended period of time. Cosmetics are one of the best alternatives for treating various skin conditions, such as hyperpigmentation, wrinkles, rough skin texture, and skin aging. The evaluation of the efficacy of chemicals present in skin care products encounters limitations that restrict the development of novel skin care formulations. A range of in vivo models are employed to assess the safety and effectiveness of cosmetic formulations, often involving the participation of human volunteers. The in vivo evaluation of cosmetic products on human volunteers presents a number of drawbacks, including high costs, lengthy duration, and potential risks to the human clinical subjects. Additionally, it can be exceedingly challenging to obtain human ethical clearance. As a result, there is growing interest in conducting in vitro procedures due to their reduced reliance on human volunteers, improved budgetary efficiency, and time-saving benefits. Using an in vitro model to assess the efficacy of skin care formulations can also potentially reduce the price of the final products (Sahu et al. 2013).

Dermatologists currently have a variety of options for anti-aging treatments, each with its own advantages and disadvantages. Customers commonly seek non-invasive cosmetics and skincare products that are both safe and effective due to concerns over their health and well-being (Ahmed et al. 2020). Plant species offer an abundant supply of raw materials, which enables the production of standardized herbal products. This production process requires scientific assessments to determine the efficacy, safety, and quality control of the materials. These materials may be beneficial in the management and prevention of various disorders, particularly those associated with skin aging. Temulawak, scientifically known as *Curcuma xanthorrhiza* L., is one of the plants that possesses anti-aging properties. Temulawak is composed of the active ingredients curcuminoid and xanthorrhizol. Curcumin, a natural dietary polyphenol, has exhibited diverse biological and pharmacological effects, including anti-aging benefits. Meanwhile, a potential anti-aging compound known as xanthorrhizol has shown its ability to dramatically lower metalloproteinase-1 (MMP-1) expression and boost type 1 procollagen production (Vaiserman et al. 2020, Irfan et al. 2021). Therefore, this study aimed to ascertain whether temulawak extract, a potential candidate for a cosmeceutical ingredient, has anti-aging properties that can inhibit the enzymes elastase, hyaluronidase, and tyrosinase.

MATERIALS AND METHODS

The reagents and chemicals used for the elastase inhibitory assay were n-sucanyl-ala-ala-ala-p nitroanilide (Sigma-Aldrich S4760-25MG, Burlington, USA), elastase from porcine cell culture (HiMedia TC311-10MG, Maharashtra, India), tris (BioRad Cat. #1610716, Hercules, USA), sodium chloride (Merck 1.06404.1000, Darmstadt, Germany), distilled water, dimethyl sulfoxide (Merck 1.02952.1000, Darmstadt, Germany), and hydrochloric acid solution (Merck 1.00317.1000, Darmstadt, Germany).

In the hyaluronidase inhibitory assay, the reagents and chemicals used were sodium phosphate monobasic (1.06346.1000, Merck, Darmstadt, Germany), hyaluronic acid sodium salt (H5542-50MG, Sigma-Aldrich, Burlington, USA), cell-culture-tested grade hyaluronidase (TC331-25MG, HiMedia , Maharashtra, India), sodium chloride (1.06404.1000, Merck, Darmstadt, Germany), aquades, bovine serum albumin (A2153-100G, Sigma-Aldrich, Burlington, USA), sodium acetate (1.06268.1000, Merck, Darmstadt, Germany), acetic acid made in Indonesia by CV. Agung Menara Abadi (6°55'28.5"S 107°42'05.2"E), hydrochloric acid solution (1.00317.1000, Merck, Darmstadt, Germany), and sodium hydroxide (1.055.872.500, Merck, Darmstadt, Germany).

The reagents and chemicals utilized in the tyrosinase inhibitory assay were potassium dihydrogen phosphate (1.048.730.250, Merck, Darmstadt, Germany), dipotassium hydrogen phosphate (1.051.041.000, Merck, Darmstadt, Germany), mushroom tyrosinase (T3824-50KU, Sigma-Aldrich, Burlington, USA), l-3,4-dihydroxy-phenylalanine (L-DOPA) (D9628-25G, Sigma-Aldrich, Burlington, USA), potassium hydroxide (P5958-500G, Sigma-Aldrich, Burlington, USA), and distilled water.

Temulawak extract used in this study was produced by PT Fast (Depok, Indonesia) in compliance with Good Manufacturing Practices (GMP) with batch number 00110201069. The sample was initially prepared by adding 70% ethanol to dried temulawak, followed by the addition of lactose (Widowati et al. 2023). The stock solution was made by dissolving 20 mg of the extract in 1 mL of 100% dimethyl sulfoxide (DMSO) solvent. The stock solution was then diluted to achieve various concentrations of working solution, i.e., 31.25, 62.5, 125, 250, 500, and 1000 µg/mL.

By referring to the previous study conducted by Widowati et al. (2018), the evaluation of the inhibitory activity of elastase was conducted using a modified method. Elastase derived from porcine pancreas, with a concentration of 0.5 mU/mL in cold distilled water, was mixed with 135 µL of Tris buffer (100 mM, pH 8). The mixture was then combined with 10 µL of the samples, ranging in concentration from 2.08 to 66.67 µg/mL. The resulting mixture was pre-incubated for 15 minutes at 25 °C. After pre-incubating the mixture, 10 µL of the n-sucanyl-ala-ala-ala-p-nitroanilide substrate (2 mg/mL in Tris buffer) was added. Afterwards, the mixture was incubated at 25 °C for 15 minutes before measuring the absorbance at a wavelength of 410 nm using a microplate spectrophotometer (Multiskan™ GO microplate spectrophotometer, Thermo Fisher Scientific, USA). The proportion of elastase inhibitory activity was calculated using the following formula: inhibitory activity % = (A control - A sample)/ A control × 100. In this context, the A control represented the inhibitory activity observed in the group that did not receive temulawak extract, whereas the A sample referred to the inhibitory activity of temulawak extract.

According to the methods outlined in previous studies by Tu & Tawata (2015) and Widowati et al. (2018), the hyaluronidase inhibitory activity was assessed using an established technique from Sigma-Aldrich. Concisely, 25 µL of the samples (5.21–166.67 µg/mL) were pre-incubated at 37 °C for 10 minutes by mixing them with 3 µL of hyaluronidase (0.02 mg/mL in 20 mM phosphate buffer, pH 7, containing 77 mM sodium chloride and 0.01% bovine serum albumin) and 12 µL of phosphate buffer (300 mM, pH 5.35). After the pre-incubation, 10 µL of hyaluronic acid substrate (0.03% concentration in 300 mM phosphate buffer, pH 5.35) was then incubated at 37 °C for 45 minutes. The reaction was stopped by adding 100 µL of acidic albumin acid (24 mM sodium acetate, 79 mM acetate acid, and 0.1% bovine serum albumin). After 10 minutes at room temperature, the mixture was analyzed for absorbance at 600 nm. The following formula was used to determine the proportion of hyaluronidase inhibitory activity: inhibitory activity % = (A control - A sample)/ A control × 100. The A control signified the inhibitory activity in the group that was not given temulawak extract, while the A sample represented the inhibitory activity of temulawak extract.

Prior studies conducted by Tu & Tawata (2015) and Siregar et al. (2019) provided a reference for conducting a tyrosinase inhibitory assay. A modified procedure was employed to assess the inhibitory activity of tyrosinase. Briefly, 20 µL of the samples were mixed with 140 µL of potassium phosphate buffer (20 mM, pH 6.8) and 20 µL of mushroom tyrosinase (from a volume of 125 U/mL dissolved in potassium phosphate). Afterwards, the incubation was carried out for 15 minutes at room temperature. Following the incubation, 20 µL of L-DOPA (1.5 mM) was added. The resulting mixture was

incubated once more for 10 minutes at room temperature. The absorbance was measured using a spectrophotometer at 470 nm. The formula used for calculating the proportion of tyrosinase inhibitory activity was as follows: inhibitory activity % = (A control - A sample)/ A control × 100. The A control indicated the inhibitory activity of the group with no administration of temulawak extract. Conversely, the A sample represented the inhibitory activity of temulawak extract.

The statistical analysis was performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, N.Y., USA). The results were presented as mean±standard deviation. The normality and homogeneity of the data were examined using the Shapiro-Wilk test and the Levene test, respectively. If the data did not exhibit a normal distribution, the Kruskal-Wallis test and Mann-Whitney test were employed to examine differences across the groups. If the data showed a normal distribution, a one-way analysis of variance (ANOVA) was conducted, followed by Tukey's honest significant difference (HSD) post-hoc test. Statistically significant differences were defined as those with $p \leq 0.05$ (Vetter 2017).

RESULTS

The temulawak extract was tested for its inhibitory effect against elastase, hyaluronidase, and tyrosinase. Figure 1 depicts the inhibitory effects of temulawak extract on elastase, hyaluronidase, and tyrosinase, presented as a percentage.

The temulawak extract was diluted in 10% DMSO to produce different ranges of concentrations to be used in three experiments. The first experiment used various concentrations of 2.08, 4.17, 8.33, 16.67, 33.33, and 66.67 µg/mL (Figure 1a). In the second experiment, the concentrations used were 5.21 µg/mL, 10.42, 20.83, 41.67, 83.33, and 166.67 µg/mL (Figure 1b). Lastly, the third experiment tested the following concentrations: 2.08, 4.17, 8.33, 16.67, 33.33, and 6667 µg/mL (Figure 1c). The results of these experiments indicated the differences in inhibitory activity of temulawak extract against elastase, hyaluronidase, and tyrosinase, respectively. The data collected from the experiments were presented as mean±standard deviation. The Mann-Whitney test was used in the first experiment, while Tukey's HSD post-hoc test was used in the second and third experiments to determine the statistical differences. Various letters in the diagrams (i.e., a, b, c, d, e, and f) signified the significant differences in inhibitory activity at different concentrations ($p<0.05$).

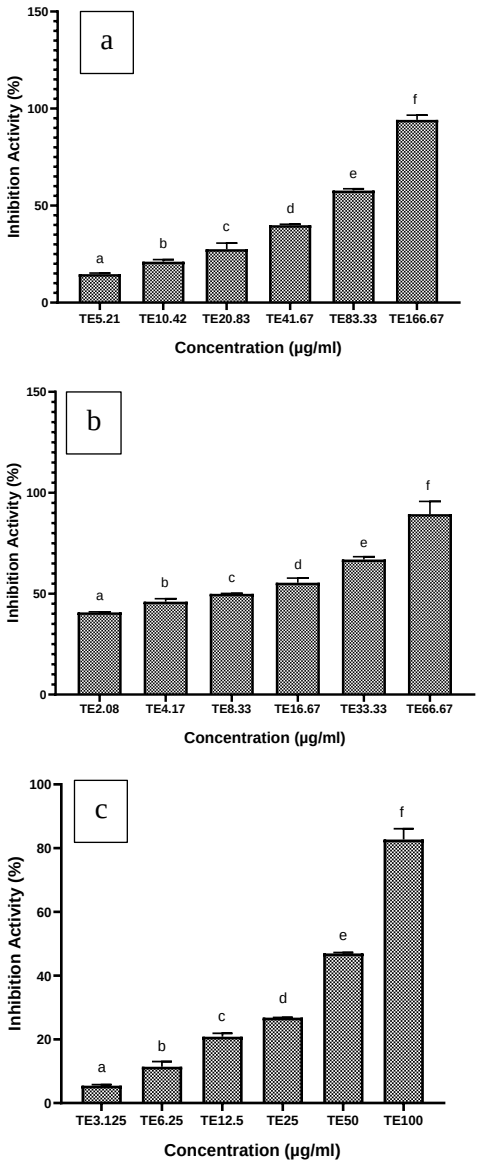

Figure 1. Different temulawak extract concentrations showed different levels of inhibitory effect on (a) elastase, (b) hyaluronidase, and (c) tyrosinase. Different superscripts (a, b, c, d, e, and f) show the significant difference among the treatments group ($p<0.05$).

The results demonstrated that there were inhibitions of elastase, hyaluronidase, and tyrosinase by the temulawak extract. The level of inhibition rose proportionally with the increasing concentration. Furthermore, the anti-aging inhibition was assessed by determining the median inhibitory concentrations (IC_{50}) of the samples, as exhibited in Table 1. The inhibition assays were carried out in triplicate. The coefficient of determination (R^2) indicated how well our data fit the regression model. The IC_{50} value of each sample was determined using linear regression. The temulawak extract showed the highest IC_{50} in inhibiting hyaluronidase and the lowest IC_{50} in

inhibiting elastase. Accordingly, the temulawak extract was the least potent hyaluronidase inhibitor and the most potent elastase inhibitor.

Table 1. The median inhibitory concentrations (IC_{50}) of temulawak extract.

Inhibition assays	R^2	Linear regression	IC_{50}
Elastase	0.99	y = 0.7163x + 42.394	10.62
Hyaluronidase	0.99	y = 0.4761x + 16.486	70.39
Tyrosinase	0.99	y = 0.7668x + 7.2013	55.81

Notes: R^2=coefficient of determination; IC_{50}=half-maximal inhibitory concentrations (µg/mL).

DISCUSSION

Chronic exposure to exogenous sources of ROS has been associated with melanogenesis, the formation of wrinkles, and the deterioration of the antioxidant system in the skin. Tyrosine, hyaluronic acid, and elastin are elements of the extracellular matrix (ECM) that are believed to be significantly affected by aging factors due to their susceptibility to oxidative damage caused by free radicals throughout the aging process. Excessive UV exposure leads to cellular damage, the production of ROS, inflammation, and angiogenesis, which in turn cause numerous skin alterations such as pigmentation, erythema, laxity, wrinkles, and skin cancer (Nurrochmad et al. 2018). Due to their medicinal capabilities, including the ability to slow down the aging of the skin, plants have been widely utilized to treat a variety of ailments. In order to identify the class of molecules that potentially have anti-aging properties, phytochemical examination of plants is necessary. A recent study showed that temulawak extract contains certain beneficial substances such as phenols, tannins, curcuminoids, and xanthorrhizol (Rahmat et al. 2021).

This study demonstrated that temulawak extract exhibited the strongest elastase inhibitory activity at a concentration of 66.67 µg/mL (89.41%), with an IC_{50} value of 10.66 µg/mL. Temulawak extract has been shown to possess very active anti-elastase properties, with an IC_{50} value below 50 µg/mL (Tu & Tawata 2015, Dewi et al. 2020). Elastase is a protease belonging to the chymotrypsin family. It is principally in charge of degrading elastin, which is essential for preserving skin suppleness. Elastin is a fibrose protein that accounts for 2–4% of the ECM and aids in maintaining skin hydration. Moreover, elastase accelerates the progression of inflammation, delays the healing of wounds, and serves as the primary enzyme that affects all of the main proteins in the connective tissue matrix (Azmi et al. 2014). The discovery of inhibitors for the elastase enzymes can help prevent the loss of skin elasticity and slow the aging process. The findings of this study suggest that the phenolic and curcumin components in temulawak extract may have anti-aging abilities through elastase inhibitory activity.

The proteolytic enzymes hyaluronidase and elastase, which are found in the dermis, are responsible for breaking down hyaluronan and elastin of the extracellular matrix, respectively. The visible indications of aging, such as wrinkles and sagging of the skin, are largely caused by the loss of elastin. This study demonstrated that temulawak extract exhibited remarkable anti-hyaluronidase activity at a concentration of 166.67 µg/mL (94.17%), with an IC_{50} value of 70.39 µg/mL. Previous studies have found that temulawak extract shows active anti-hyaluronidase properties with an IC_{50} value between 50 and 100 µg/mL (Tu & Tawata 2015, Dewi et al. 2020). The enzyme hyaluronidase aids in the breakdown of HA. As a glycosaminoglycan polymer that functions in the tissues, HA is a critical substance in the aging process. It acts as a molecule that enhances skin hydration by maintaining a consistent water balance. In addition, HA serves as a component of the ECM, which contributes to the preservation of the skin's suppleness. The breakdown of HA is catalyzed by the enzyme hyaluronidase, which breaks down the N-acetyl-D-glucosamine and D-glucuronic acid residues of HA through the hydrolysis of 1,4-hexosaminidic linkages (Azmi et al. 2014, Jusri et al. 2019). HA can be broken down enzymatically as well as non-enzymatically by free radicals in the presence of decreasing chemicals such as iron ions, ascorbic acid, copper ions, and thiol. The findings of this study indicated that temulawak extract possesses potent hyaluronidase inhibitory properties. This may be due to the abundance of tannin in temulawak extract. An in vitro study has shown the ability of tannin-rich plants to prevent the release of hyaluronidase from activated neutrophils (Kolakul & Sripanidkulchai 2017).

In this study, temulawak extract demonstrated potent anti-tyrosinase properties at a concentration of 100 µg/mL (82.72%), with an IC_{50} value of 55.87 µg/mL. The IC_{50} value for temulawak extract, which possesses potent anti-tyrosinase properties, has been found to range from 50 to 100 µg/mL, as demonstrated in earlier studies (Tu & Tawata 2015, Dewi et al. 2020). The tyrosinase enzyme stimulates pigmentation processes. This copper-containing enzyme is responsible for catalyzing the synthesis of melanin. L-DOPA, which activates the melanogenic pathway, is the substrate of tyrosinase. Tyrosinase transforms L-DOPA into dopaquinone, which interacts with cysteine to produce melanin, particularly brown melanin. Tyrosinase inhibitors are widely used in skin whitening research to effectively suppress pigmentation processes (Pillaiyar et al. 2017, Varghese et al. 2021). The results of this study revealed that temulawak extract

was identified as a substantial tyrosinase inhibitor. This could be attributed to the structural similarity between the hydroxyl group in the composition of temulawak extract phenolic compounds and the substrate of tyrosinase (L-DOPA) (Uchida et al. 2014). The hydroxyl group of phenolic substances is connected to the effectiveness of tyrosinase inhibitors. This group directly inhibits enzymatic activity by forming hydrogen bonds in the active areas of enzymes, thereby inducing steric hindrance and conformational alterations (Sun et al. 2017).

Strength and limitations

This study provides insight into the anti-aging testing of temulawak extract, including inhibition of elastase, hyaluronidase, and tyrosinase. It found that temulawak extract has strong anti-aging activity, which had not been widely explored in other studies. However, this study had limitations, although an in vitro study is an important first step before an in vivo study. Therefore, further in vivo research is necessary to validate the assertion regarding the anti-aging properties of temulawak.

CONCLUSION

Temulawak extract has anti-aging properties that can effectively inhibit the enzymes elastase, hyaluronidase, and tyrosinase. Thus, temulawak may be applied as a cosmetic ingredient formulated to impede the aging process.

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Conflict of interest

None.

Ethical consideration

The declaration of ethical exemption for this study was issued by the Research Ethics Committee, Faculty of Medicine, Maranatha Christian University, Bandung, Indonesia, with reference No. 001/SRT/KEP/XI/2023 dated 13/11/2023.

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Author contribution

TLW and WW contributed to the conception and design, the critical revision of the article for important intellectual content, and the final approval of the article. ASM contributed to the analysis and interpretation of the data, the drafting of the article, statistical expertise, and administrative, technical, or logistic support. RR contributed to the analysis and interpretation of the data, the drafting of the article, and the collection and assembly of the data.

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