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Research Article

In Vitro Evaluation of Anti-Inflammatory Potential of Papaya (*Carica papaya* L.) Peel Extract and Fractions

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ABSTRACT

Inflammation is a protective response of the body to tissue injury or infection; however, excessive inflammation may lead to tissue damage, thus requiring intervention with anti-inflammatory agents. This study aimed to evaluate the potential of papaya (*Carica papaya* L.) peel as a source of anti-inflammatory compounds by analyzing its secondary metabolites and assessing its biological activity. Extraction was performed using maceration with 96% ethanol, followed by fractionation and phytochemical screening to identify the bioactive constituents. The anti-inflammatory activity was evaluated in vitro using the Bovine Serum Albumin (BSA) protein denaturation inhibition method. The results revealed that the ethyl acetate fraction exhibited the strongest anti-inflammatory activity, with an IC_{50} value of 86.47 ppm, which was lower than that of the crude extract and other fractions. An IC_{50} below 100 ppm indicates a strong potential of the ethyl acetate fraction as an anti-inflammatory agent. These findings suggest that papaya peel, commonly considered waste, could serve as a promising natural source of anti-inflammatory compounds.

Keywords: Anti-inflammatory activity, BSA protein denaturation, *Carica papaya* L, In vitro assay

1. INTRODUCTION

Inflammation is a protective biological response to tissue injury or infection. However, when it occurs excessively and becomes chronic, it can lead to tissue damage and contribute to the development of various degenerative diseases, including rheumatoid arthritis, inflammatory bowel disease, and other autoimmune disorders. Globally, the burden of inflammatory diseases continues to rise. Data from the Global Burden of Disease (GBD) 2019 indicate that the prevalence of rheumatoid arthritis reached approximately 208–224 cases per 100,000 population, affecting more than 17 million individuals worldwide, and has shown an increasing trend since 1990. The age-standardized prevalence rate increased from 207.46 to 224.25 per 100,000 population, with an estimated annual percentage change (EAPC) of 0.37%, while the incidence rate also rose from 12.21 to 13 per 100,000 population [1,2]. In addition, the incidence of inflammatory bowel disease has increased significantly, particularly in

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developing countries undergoing lifestyle changes and rapid urbanization [3,4]. These conditions not only reduce patients' quality of life but also impose a substantial economic burden on global healthcare systems.

Conventional anti-inflammatory drugs, particularly non-steroidal anti-inflammatory drugs (NSAIDs), are widely used and effective in controlling inflammation. However, their long-term use is associated with serious adverse effects, including gastrointestinal bleeding and increased cardiovascular risk [5,6]. Therefore, the development of safer alternative anti-inflammatory agents has become a major focus in modern pharmaceutical research, especially those derived from natural sources. Numerous studies have reported that plant-derived bioactive compounds, such as flavonoids and phenolics, exhibit anti-inflammatory activity through mechanisms including inhibition of pro-inflammatory cytokines, modulation of inflammatory signaling pathways, and protein stabilization [7–10].

One promising natural source is papaya peel (*Carica papaya* L.), which is often underutilized and commonly regarded as agro-industrial waste. In fact, papaya peel contains various secondary metabolites, including flavonoids, tannins, saponins, and phenolic compounds, which are known to possess antioxidant and anti-inflammatory properties [11,12]. The utilization of papaya peel waste not only provides added economic value but also supports the concept of zero waste and sustainability in the food and pharmaceutical industries.

The evaluation of anti-inflammatory activity can be conducted in vitro using protein denaturation inhibition methods, one of which employs the Bovine Serum Albumin (BSA) model. This method is based on the ability of compounds to prevent protein denaturation induced by thermal stress, which is one of the mechanisms occurring during inflammation [13,14]. Phenolic and flavonoid compounds are known to interact with proteins through hydrogen bonding and hydrophobic interactions, thereby stabilizing protein structure and inhibiting denaturation processes.

Several in vitro methods are available for evaluating anti-inflammatory activity, including the HRBC (human red blood cell) membrane stabilization assay, proteinase inhibition assay, and COX enzyme inhibition assay. However, the BSA protein denaturation inhibition method was selected in this study due to several advantages it offers over these alternatives. The BSA method directly reflects a pathological mechanism relevant to inflammation, as denatured proteins are known to act as autoantigens that trigger immunological responses associated

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with inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus [15]. Furthermore, BSA shares significant structural and functional homology with human serum albumin, making it a physiologically relevant model for assessing protein-stabilizing activity of bioactive compounds [16]. Compared to in vivo methods such as carrageenan-induced paw edema in rodents, the BSA assay offers an ethical, animal-free alternative suitable for high-throughput preliminary screening, particularly when numerous fractions or compounds require evaluation in the early stages of drug discovery [15]. The method is also technically simple, cost-effective, and readily quantifiable using standard UV–Vis spectrophotometry, which ensures reproducibility and accessibility across laboratories [17].

Based on this background, this study aims to evaluate the potential of papaya peel as a source of anti-inflammatory agents through extraction, fractionation based on solvent polarity, and biological activity assessment using the BSA protein denaturation inhibition method. This approach is expected to identify the most active fraction and provide a scientific basis for the development of natural-based anti-inflammatory agents that are safer and more sustainable.

2. EXPERIMENTAL SECTION

2.1. Materials

Fresh papaya (*Carica papaya* L.) peels were used as the plant material. Ethanol (96%), n-hexane, and ethyl acetate (Merck) were used as extraction and fractionation solvents. Distilled water (aquadest) was prepared in the laboratory. Reagents used for buffer preparation and anti-inflammatory assays included sodium chloride (NaCl) and tris base (Sigma-Aldrich), as well as glacial acetic acid (Merck). Bovine Serum Albumin (BSA) and diclofenac sodium (Sigma-Aldrich). Phytochemical screening reagents included magnesium powder (Mg) (Merck), concentrated hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) (Merck), Dragendorff's reagent (Sigma-Aldrich), ferric chloride ($FeCl_3$) (Merck), and Liebermann–Burchard reagent (Merck). All chemicals used were of analytical grade.

2.2. Instrumentation

The instruments used in this study included an analytical balance (Ohaus), drying oven (Memmert), blender (Philips), and standard laboratory glassware (Pyrex). Fractionation was performed using a separatory funnel (Pyrex). Concentration of extracts was carried out using

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a rotary evaporator (Buchi R-300) equipped with a water bath (Memmert). Absorbance measurements were performed using a UV–Vis spectrophotometer (Shimadzu UV-1800).

2.3. Procedure

2.3.1. Sample Preparation and Moisture Content Determination

Approximately 2 kg of fresh ripe papaya (*Carica papaya* L.) peels were thoroughly washed under running aqueous to remove adhering impurities, then dried under direct sunlight for 3–5 days to reduce moisture content. The dried samples were subsequently cut into small pieces and ground using a blender to obtain simplicia powder. Moisture content was determined by weighing 2 g of the simplicia powder into a pre-weighed porcelain crucible, followed by drying in an oven at 105–120°C for 30 minutes until a constant weight was achieved. The sample was then cooled in a desiccator to room temperature and reweighed. The percentage of moisture content was calculated using the following equation:

$$\%\text{Moisture content} = \frac{b - (c - a)}{b} \times 100\%$$

where a is the mass of the empty crucible (g), b is the initial mass of the sample (g), and c is the mass of the crucible plus sample after drying (g). This method was conducted in accordance with standard procedures for moisture analysis of natural materials [18,19]

2.3.2. Extraction of Papaya Fruit Peel

A total of 200 g of papaya peel powder was extracted using the maceration method with 600 mL of 96% ethanol for 3 × 24 hours at room temperature with periodic stirring. The filtrate was subsequently filtered and concentrated using a rotary evaporator at 40–50°C to obtain a viscous extract [20]. Maceration was selected due to its effectiveness in extracting phenolic and flavonoid compounds from natural materials. The extraction yield was calculated using the following equation:

$$\%\text{Yield} = \frac{Extract\ weight\ (g)}{Simplisia\ weight\ (g)} \times 100\%$$

2.3.3. Fractionation of Papaya Fruit Peel Extract

Twenty grams of the viscous ethanol extract were suspended in 500 mL of distilled water and transferred into a separatory funnel. Liquid–liquid partitioning was first carried out using n-hexane at a 1:1 (v/v) rasio. The mixture was shaken thoroughly and allowed to stand until two distinct phases were formed. The n-hexane layer (non-polar fraction) was collected, and the partitioning process was repeated several times until the n-hexane phase became

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clear. The collected n-hexane fractions were combined and concentrated under reduced pressure using a rotary evaporator to obtain the n-hexane fraction. Subsequently, the remaining aqueous phase was re-partitioned with ethyl acetate using the same procedure to obtain the semi-polar fraction. All resulting fractions were then concentrated under reduced pressure using a rotary evaporator to yield the respective concentrated extracts [21].

2.3.4. Phytochemical Test

Phytochemical screening was conducted qualitatively to identify the presence of secondary metabolites, including flavonoids, alkaloids, saponins, tannins, phenolics, triterpenoids, and steroids, using standard methods [22]. Flavonoids were detected using the Shinoda test (Mg–HCl), indicated by the formation of a red coloration. Alkaloids were identified using Dragendorff's reagent, which produced a brown precipitate. The presence of saponins was confirmed by the formation of stable and persistent foam. Tannins and phenolic compounds were detected using $FeCl_3$ reagent, indicated by the appearance of a dark blue or greenish-black coloration. Triterpenoids and steroids were identified using the Liebermann–Burchard test, where the formation of red or purple coloration indicated triterpenoids, while green or blue coloration indicated steroids.

2.3.5. In Vitro Anti-Inflammatory Activity Assay

The in vitro anti-inflammatory activity was evaluated using the protein denaturation inhibition method. Tris Buffer Saline (TBS) was prepared by dissolving 8.7 g of NaCl and 1.21 g of tris base in 900 mL of distilled water, followed by pH adjustment to 6.2–6.5 using glacial acetic acid, and dilution to a final volume of 1000 mL. A 0.2% Bovine Serum Albumin (BSA) solution was prepared by dissolving 0.2 g of BSA in TBS up to 100 mL.

The maximum wavelength (λ_max) of the BSA solution was determined by scanning in the range of 200–400 nm using a UV–Vis spectrophotometer. The maximum wavelength (λmax) of the BSA solution was determined by scanning in the range of 200–400 nm using a UV–Vis spectrophotometer. Sample solutions of the ethanol extract and water fraction were prepared at concentrations of 100, 200, 300, 400, and 500 ppm. The n-hexane fraction was prepared at concentrations of 50, 100, 150, 200, and 250 ppm, while the ethyl acetate fraction was prepared at concentrations of 30, 50, 70, 90, and 110 ppm due to its higher anti-inflammatory activity. Sodium diclofenac was used as a positive control at concentrations of

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10, 20, 30, 40, and 50 ppm, whereas the reaction medium without sample or sodium diclofenac served as the negative control.

The anti-inflammatory assay was conducted by preparing a reaction mixture consisting of 0.5 mL of sample solution, 2 mL of 0.2% BSA, and 1.5 mL of TBS, followed by the addition of distilled water to a total volume of 5 mL. The mixture was incubated at 37°C for 30 minutes, then heated at 70–72°C for 10 minutes to induce protein denaturation, and subsequently cooled. The samples were centrifuged at 2000 rpm for 10 minutes, and absorbance was measured at the predetermined maximum wavelength (approximately 287 nm). The percentage inhibition of protein denaturation was calculated using the following equation:

$$\%\ \text{Inhibition} = \frac{Absorbance\ sample - Absorbance\ negative\ control}{Absorbance\ positive\ control - Absorbance\ negarive\ control} \times 100\%$$

The IC_{50} values were determined using linear regression analysis based on the equation y = ax + b, where x represents the sample concentration and y represents the percentage inhibition of protein denaturation. This method is widely used to evaluate anti-inflammatory activity through protein stabilization mechanisms [23].

3. RESULTS AND DISCUSSION

3.1. Sample Preparation

Papaya (*Carica papaya* L.) peel samples were obtained from fully ripe fruits, indicated by a yellow–orange coloration, reflecting optimal maturity and stable secondary metabolite content. Sample preparation involved washing, drying, and grinding into powder to enhance extraction efficiency. The moisture content was determined to be 7.5%, meeting the standard requirement for simplicia (<10%) [24,25]. This low moisture level is essential for maintaining sample stability by inhibiting enzymatic activity and microbial growth, while also improving extraction efficiency by reducing interference from residual water. Therefore, the prepared simplicia was considered of good quality and suitable for further extraction and analysis.

3.2. Extraction and Fractionation

The extraction process in this study was carried out using the maceration technique with 96% ethanol as the solvent. This method was selected due to its ability to dissolve a wide range of bioactive compounds with varying polarity without the application of high temperatures, thereby preserving the stability of secondary metabolites [26–28]. Based on the corrected extraction yield calculation, the maceration process yielded a viscous extract

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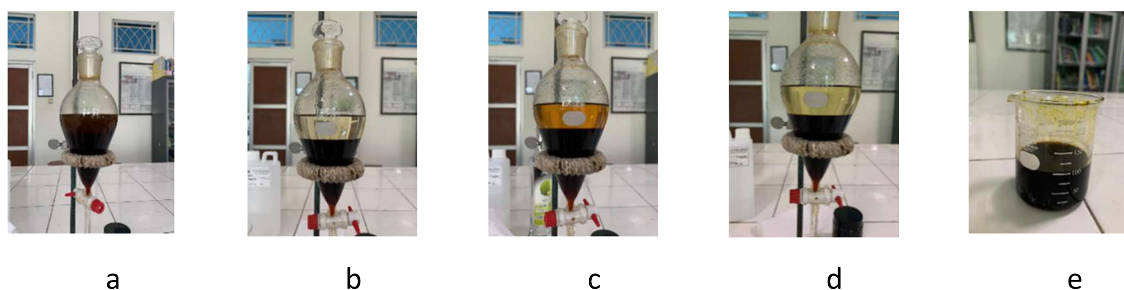
with a percentage yield of 30.785%. This relatively high yield indicates that ethanol is an effective solvent for extracting bioactive constituents from papaya peel, including phenolic compounds, flavonoids, and other secondary metabolites. Furthermore, the high yield suggests that most soluble components were successfully extracted, which is influenced by factors such as particle size of the simplicia, extraction time, and the compatibility between solvent polarity and target compounds.



a b c d e

Figure 1. (a). First hexane fractionation; (b). Final hexane fractionation; (c). First ethyl acetate fractionation; (d). Final ethyl acetate fractionation; (e). Aqueous fraction

Subsequently, liquid–liquid fractionation was performed to separate compounds based on differences in polarity. The process began by dissolving the ethanol extract in aqueous, followed by partitioning with n-hexane as a non-polar solvent. Based on the process documentation (Figures A and B), two distinct phases were formed: the upper phase (n-hexane) and the lower phase (aqueous layer), with the extract color gradually fading in the organic phase. The fractionation process was repeated until the n-hexane phase became clear, indicating that non-polar compounds had been maximally extracted.

The aqueous phase was then further fractionated using ethyl acetate as a semi-polar solvent. At this stage (Figures C and D), semi-polar compounds were separated from the aqueous phase into the ethyl acetate phase. The progressively lighter color observed in the ethyl acetate layer indicated the gradual transfer of semi-polar compounds into the solvent. This process was also repeated until the ethyl acetate phase became clear, signifying that the extraction had reached optimal conditions.

The remaining fraction (Figure E) constituted the aqueous fraction, which predominantly contains polar compounds. Overall, this sequential fractionation process successfully separated compounds according to their polarity, with non-polar compounds concentrated in the n-hexane fraction, semi-polar compounds in the ethyl acetate fraction, and polar compounds in the aqueous fraction. Repeating the extraction process until the solvent

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became clear served as a critical indicator that the separation had been carried out to completion, resulting in fractions with more specific chemical compositions. This is particularly important in enhancing the selectivity of biological activity analysis, as it minimizes potential interactions among compounds that may influence the observed bioactivity [29–31].

3.3. Phytochemical Screening

The phytochemical screening data revealed distinct distributions of secondary metabolites across the different fractions, which directly reflects the effectiveness of the fractionation process based on solvent polarity differences. The fundamental principle underlying this process is "like dissolves like," whereby compounds with a certain polarity are more readily dissolved in solvents with similar polarity.

Table 1. *Phytochemical Screening*

Secondary metabolite	Ethanol extract	Hexane fraction	Ethyl acetate fraction	Aqueous fraction
Flavonoid	+	-	-	+
Alkaloid	+	-	-	+
Saponin	-	-	-	-
Tannin	+	-	-	+
Phenolic	+	-	+	+
Triterpenoid	-	-	-	-
Steroid	+	+	-	-

The ethanol extract, as the initial crude extract, exhibited the most complex metabolite profile, containing flavonoids, alkaloids, tannins, phenolics, and steroids. Although 96% ethanol can extract compounds with varying polarity, the phytochemical screening results showed that the water fraction retained a metabolite profile closely resembling that of the ethanol extract. This finding indicates that the major secondary metabolites present in papaya peel are predominantly polar compounds, which were preferentially partitioned into the aqueous fraction during fractionation. Therefore, the biological activity observed in the extract is likely associated with these polar constituents, particularly phenolics, flavonoids, and tannins [32,33]

During the fractionation stage, the use of n-hexane as a non-polar solvent resulted in a fraction containing only steroids. Steroid compounds are generally non-polar due to their predominantly cyclic hydrocarbon structures, making them more soluble in non-polar solvents such as n-hexane [34]. The absence of flavonoids, alkaloids, tannins, and phenolics in

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this fraction indicates that these compounds possess higher polarity and therefore do not partition into the n-hexane phase.

The ethyl acetate fraction, which is semi-polar, showed the presence of phenolic compounds. This observation is consistent with the chemical characteristics of phenolics, which typically contain hydroxyl (-OH) groups contributing to polarity, alongside aromatic rings that impart semi-polar properties. Therefore, ethyl acetate serves as an optimal solvent for extracting phenolic compounds compared to highly polar or highly non-polar solvents [35,36].

Meanwhile, the aqueous fraction (polar) contained flavonoids, alkaloids, tannins, and phenolics. These compounds generally possess multiple polar functional groups such as hydroxyl (-OH), amine ($-NH_2$), or carbonyl groups, which enhance their solubility in polar solvents like aqueous. Flavonoids and tannins, in particular, are well-known polyphenolic compounds with high polarity, leading to their predominant presence in the aqueous fraction. Alkaloids also tend to be polar, especially in their salt forms, making them more stable in the aqueous phase.

The absence of saponins and triterpenoids in all fractions may be attributed to their low concentrations or the limited sensitivity of the phytochemical screening methods employed. Additionally, saponins, being amphiphilic in nature, may require more specific extraction techniques for optimal detection.

Overall, these results demonstrate that the fractionation process successfully separated compounds according to their polarity: non-polar compounds (steroids) were concentrated in the n-hexane fraction, semi-polar compounds (phenolics) in the ethyl acetate fraction, and polar compounds (flavonoids, alkaloids, tannins, phenolics) in the aqueous fraction. This separation is crucial as it enhances the specificity of biological activity, reduces antagonistic interactions among compounds, and facilitates the identification of the most promising fractions as potential bioactive agents.

3.4. Anti-inflammatory activity

The relatively low activity of the ethanol extract can be explained by its complex phytochemical composition, which includes flavonoids, alkaloids, tannins, phenolics, and steroids. The coexistence of multiple compounds within a single matrix may lead to antagonistic interactions [37], thereby reducing the effectiveness of active constituents in

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stabilizing BSA protein. Additionally, the presence of non-contributory compounds may hinder the accessibility of active molecules to the protein binding sites.

The aqueous fraction exhibited improved activity compared to the ethanol extract, with an IC_{50} value of 293.46 ppm. Phytochemical analysis indicates that this fraction contains polar compounds such as flavonoids, alkaloids, tannins, and phenolics. These compounds are known to exert anti-inflammatory effects through antioxidant mechanisms and hydrogen bond formation with proteins [38,39]. However, due to its relatively complex and highly polar composition, competitive interactions among compounds for binding to BSA may occur, resulting in suboptimal activity.

Table 2. Anti-inflammatory activity

Sample	IC_{50}
Diclofenac sodium	33.894 ± 4.366
Ethyl Acetate Fraction	69.854 ± 1.192
Hexane Fraction	129.569 ± 4.076
Water fraction	259.093 ± 6.002
Ethanol Extract	327.788 ± 3.864

The n-hexane fraction exhibited stronger anti-inflammatory activity (IC_{50} = 129.569 ± 4.076 ppm) than the water fraction and ethanol extract, although its activity remained lower than that of the ethyl acetate fraction and the positive control, sodium diclofenac. Despite containing only steroid compounds according to the phytochemical screening results, this finding suggests that steroids contribute to anti-inflammatory activity through hydrophobic interactions with non-polar regions of proteins. The non-polar nature of steroids facilitates their interaction with hydrophobic domains of BSA, thereby enhancing protein stability. Furthermore, the relatively simple phytochemical composition of the n-hexane fraction may promote more specific interactions with the target protein, with reduced interference from other constituents. [40,41]. The non-polar nature of steroids facilitates their interaction with hydrophobic domains of BSA, thereby enhancing protein stability. Moreover, the simpler composition of this fraction allows for more specific interactions with minimal interference from other compounds.

The ethyl acetate fraction exhibited the strongest anti-inflammatory activity (IC_{50} 69.854 ± 1.192 ppm), which is consistent with the phytochemical results indicating the dominance of phenolic compounds. Phenolics are well known for their strong ability to stabilize proteins through hydrogen bonding between hydroxyl (-OH) groups and amino acid residues in BSA, as

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well as π–π interactions with aromatic residues [42,43]. Furthermore, the semi-polar nature of phenolic compounds enables interactions with both polar and non-polar regions of the protein, thereby enhancing their effectiveness in inhibiting protein denaturation. This explains why the ethyl acetate fraction demonstrated superior anti-inflammatory activity compared to other fractions.

In comparison, sodium diclofenac exhibited the highest activity as it is a pure compound that acts specifically by inhibiting cyclooxygenase (COX) enzymes, thereby directly suppressing the production of inflammatory mediators. Unlike plant extracts and fractions, which are complex mixtures, diclofenac has a more targeted mechanism of action and greater potency.

Overall, the relationship between phytochemical composition and anti-inflammatory activity indicates that solvent polarity in the fractionation process plays a crucial role in determining both the types of compounds isolated and their biological activity. The semi-polar ethyl acetate fraction, rich in phenolic compounds, demonstrated the highest activity, followed by the non-polar n-hexane fraction and the polar aqueous fraction, while the crude extract exhibited the lowest activity due to its complex composition. These findings confirm that fractionation is a critical step in enhancing the selectivity and effectiveness of bioactive compounds as potential anti-inflammatory agents.

4. CONCLUSION

This study demonstrates that papaya (*Carica papaya* L.) peel is a promising source of anti-inflammatory bioactive compounds, as evidenced by the presence of flavonoids, alkaloids, tannins, phenolics, and steroids in the ethanol extract. Fractionation based on solvent polarity enhanced the selectivity and activity of the compounds, with the ethyl acetate fraction showing the strongest anti-inflammatory effect (IC_{50} 69.854 ± 1.192 ppm), indicating the important role of semi-polar phenolic compounds. These findings confirm that fractionation is essential for improving the effectiveness of natural extracts and highlight the potential of papaya peel, often considered waste, as a sustainable source for developing natural anti-inflammatory agents.

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AUTHOR CONTRIBUTIONS

Fathiya carried out the experimental work, while Mutista was responsible for data analysis and manuscript preparation. Both authors contributed to the writing and revision of the manuscript, and have reviewed and approved the final version.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this work.

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