Asia-Pacific Journal of Science and Technology :Volume :xx Issue :xx Article ID :APST-xx-xx-xx Research Article

APST

Asia-Pacific Journal of Science and Technology

https//:www.tci-thaijo.org/index.php/APST/index

Published by Research and Innovation Department,
Khon Kaen University, Thailand

Potential of Royal jelly as antioxidant and antibacterial on *Candida albicans*, *Streptococcus mutans*, and *Porphyromonas gingivalis* for toothpaste formulation

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Received 29 August 2024
Revised 14 October 2025
Accepted 28 November 2025

Abstract

Inadequate care can cause oral health issues, leading to harmful bacteria–fungi interactions. Natural compounds, such as royal jelly, which is rich in polyphenols and flavonoids, may help reduce the risk of such problems. This study investigated the antioxidant and antibacterial potential of royal jelly, particularly its 10-hydroxy-2-decenoic acid (10-HDA) component, in preventing oral diseases. Royal jelly was evaluated through antioxidant assays, including 2,2-diphenyl-1-picrylhydrazyl (DPPH); 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS); nitric oxide (NO); and hydrogen peroxide (H_2O_2) scavenging tests, as well as antimicrobial assays using minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and Kirby–Bauer disc diffusion methods. The antioxidant activity values were 107.67 ± 0.12 µg/mL DPPH, 234.63 ± 0.41 µg/mL (ABTS), 158.61 ± 0.25 µg/mL NO, and 210.24 ± 0.23 µg/mL H_2O_2. Disc diffusion tests showed inhibition against *C. albicans* (57.79%), *P. gingivalis* (56.64%), and *S. mutans* (53.00%). These findings indicate that royal jelly is a promising candidate for toothpaste formulation due to its antibacterial and antioxidant properties.

Keywords: Antioxidant activities, Antibacterial activities, *Apis mellifera*, Royal jelly, Tooth health

1. Introduction

The human mouth provides numerous ecological niches and a rich supply of nutrients, making it an ideal environment for natural microbial biofilm growth. Bacterial biofilms are generally described as structured communities of microorganisms that cooperate and adhere to surfaces, although definitions vary. The main differences among classifications relate to the degree of structural organization within the community and whether bacterial adherence to a surface is required [1].

Streptococcus mutans is a gram-positive microorganism that resides predominantly in the human oral cavity and is a key cariogenic organism due to its ability to produce large amounts of glucans and acids. *Candida albicans*, a common oral yeast, further enhances *S. mutans'* ability to adhere to oral surfaces, forming a cooperative interaction that strengthens biofilm development. The secretion of glucosyltransferase B (GtfB) by *S. mutans*, which binds to mannans on the surface of *C. albicans*, is a central mechanism. This promotes glucan synthesis and forms a scaffold that stabilizes mixed-species biofilms, making them resistant to salivary clearance. The glucan-rich matrix also protects both organisms from host defenses and antimicrobial agents [2]. These interactions highlight why the co-existence of *S. mutans* and *C. albicans* significantly contributes to the formation of pathogenic oral biofilms.

Royal jelly (RJ) is a thick, creamy secretion produced by the hypopharyngeal and mandibular glands of worker bees. According to Pasupuleti [3], RJ serves as the queen bee's exclusive diet. RJ contains water, proteins,

fructose, glucose, amino acids, and high concentrations of 10-hydroxy-2-decenoic acid (10-HDA) [4,5]. Extensive research has examined its biochemical composition and physiological effects, and recent studies have highlighted its antioxidant, probiotic [6], and antibacterial properties [7,8].

RJ is widely recognized as a natural substance produced by honeybees and used as a functional food and nutraceutical. It is rich in nutrients and bioactive compounds, including major royal jelly proteins (MRJPs), which contribute to its biological functions and therapeutic potential. RJ offers higher nutritional value and greater bioactive compound content than honey. It also exhibits anti-inflammatory, antioxidant, and anticancer properties that are not typically associated with honey [9].

Previous studies have reported the antioxidant potential of RJ through free radical scavenging and its antibacterial activity. Mokaya et al. [10] demonstrated significant free radical scavenging activity (170 ± 60 mg/mL), suggesting the potential use of African RJ as a nutraceutical substance. Other investigations by Bagameri et al. [11] and Uthaibutra et al. [12] focused primarily on its antibacterial effects against skin and intestinal microbiota–associated bacteria. However, few studies have assessed its activity against major oral pathogens such as *S. mutans*, *C. albicans*, and *P. gingivalis*. Additionally, there is limited evidence integrating both its antioxidant and antibacterial properties within a single study framework, despite the relevance of oxidative stress and microbial imbalance in oral disease pathogenesis.

This study investigated the antioxidant and antibacterial potential of RJ as a therapeutic agent for oral health. The antioxidant activity was assessed using free radical scavenging assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH); 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); nitric oxide (NO); and hydrogen peroxide (H_2O_2) assays. Antibacterial activity was evaluated through inhibition zone measurements and inhibition percentage against *C. albicans*, *S. mutans*, and *P. gingivalis*. The novelty of this research lies in the combined assessment of RJ's antioxidant and antibacterial activities in the context of oral health, the targeted evaluation of key cariogenic and periodontopathogenic microorganisms, and the comprehensive examination using four distinct antioxidant assays, as an approach that, to our knowledge, has been rarely reported. These findings may support the development of functional oral care formulations incorporating RJ as a bioactive component, such as toothpaste.

2. Materials and methods

2.1 Preparation of Royal jelly

The RJ was extracted using a solvent of 70% ethanol with the addition of maltodextrin as an excipient to produce dry extracts. Maltodextrin was incorporated to enhance the physical characteristics of the resulting powder, facilitate improved stability, and optimize hygroscopicity, which are essential for storage and usability [13]. The extract was then dissolved in DMSO to various concentrations [14]. The RJ concentrations used for the antibacterial test consisted of: 3.13%, 6.25%, 12.5%, 25%, 50%, 75%, and 100%. The concentrations used for the antioxidant test consisted of: 3.13, 6.25, 12.5, 25, 50, and 100 µg/mL. All concentrations were repeated three times.

2.2 2,2-Diphenyl-1-Picrylhydrazil (DPPH) Assay

The 96-well microplate was filled with 50 µL sample with different concentrations before adding DPPH 200 µL from Sigma Aldrich D9132 (0.077 mmol/L in methanol). The blank consisted of methanol 250 µL (without the sample and without DPPH), while the control consisted of 250 µL of DPPH solution without the sample. The mixture was resuspended and incubated with no light at room temperature for 30 min. The absorbance was measured using a microplate reader (Multiskan^{TM} GO Microplate Spectrophotometer from Thermo Scientific, Waltham, MA, USA) was utilized to measured the absorbance at 517 nm [14]. The DPPH scavenging activity (%) using equation (1):

$$\text{Scavenging activity (\%)} = \frac{(AC-AS)}{AC}\ x\ 100 \quad (1)$$

AC: Negative control absorbance
AS: Sample absorbance

2.3 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Assay

The ABTS test (Sigma Aldrich A1888) was used to measure the antioxidant activity. A solution of 4.9 mM potassium persulfate and 14 mM ABTS (Merck 1.05091.0250) was mixed to get an $ABTS^{\bullet+}$ solution. The mixture was incubated in a room temperature for 16h with no light before being diluted with 5.5 mM PBS (Biowest, X0520-500 (pH 7.4) and measured with microplate reader at 745 nm. Royal jelly sample with various

concentrations (312.5, 625, 1250, 2500, 5000, and 10000 µg/mL) was added (2µL) onto the plate that already filled with 198 µL $ABTS^{\bullet+}$ solution. DMSO was used as the blank (200 µL) while ABTS solution without the sample was used as the control. Sample was incubated for 6 min at 37°C, then measured at 745 nm with microplate reader [15]. The ABTS reduction activity (%) was calculated using the following equation (2):

$$\text{Scavenging activity (\%)} = \frac{(AC-AS)}{AC}\ x\ 100 \quad (2)$$

AC: Negative control absorbance
AS: Sample absorbance

2.4 Nitric Oxide (NO) Scavenging Assay

Samples were added as much as 10 µL into the plate before adding the sodium nitroprusside (Merck, 1065410100) solution into both sample and control wells for 40 µL. DMSO (10% in ddH_2O) was added to the blank (140 µL) and control wells (10 µL) before incubating for 2 h at room temperature in the dark. Griess reagent (1% sulfanilamide (Merck, 1117990100), 2% H_3PO_4 from Merck (100573) and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) (Merck, 1062370025)) was added to the sample and control wells as much as 100 µL, and the absorbance was measured with a microplate reader at 516 nm [16]. The NO level was calculated using the following equation (3):

$$\text{Scavenging activity (\%)} = \frac{(AC-AS)}{AC}\ x\ 100 \quad (3)$$

AC: Negative control absorbance
AS: Sample absorbance

2.5 Hydrogen Peroxide (H_2O_2) Scavenging Assay

The hydrogen peroxide (H_2O_2) scavenging assay conducted to measure the activity of RJ against radical peroxide using a colorimetric assay. Samples were added to a 96-well plate as much as 60 µL. Ferrous ammonium sulfate (12 µL) was added to the control and sample wells. DMSO (Sigma Aldrich, 1029521000) was also added to the blank (90 µL) and control (63 µL). Subsequently, 5 mM H_2O_2 (Merck 1.08597.1000) was administered (3 µL) to the sample well and incubated for 5 min at a room temperature with no light. Phenanthroline (Merck, 1.07223.0010) was administered to the plate before the mixture was incubated for another 10 min at room temperature. Measurements were performed using a spectrophotometer at 510 nm [16]. The H_2O_2 scavenging activity (%) was calculated using the following equation (4):

$$\text{Scavenging activity (\%)} = \frac{(AC-AS)}{AC}\ x\ 100 \quad (4)$$

AC: Negative control absorbance
AS: Sample absorbance

2.6 Antibacterial activity RJ with Kirby-Bauer disk diffusion test

Antibacterial activities of RJ were evaluated against *C. albicans*, *P. gingivalis*, and *S. mutans* using the Kirby-Bauer disk diffusion. The bacterial suspension in NaCl solution was inoculated onto the Mueller Hinton Agar media surface. Various concentrations of the tested samples were prepared in DMSO at 100%, 75%, 50%, 25%, 12.5%, 6.25%, and 3.13%. Paper discs that have been prepared in sufficient quantities with each concentration of extracts. Then, it was transferred onto a 6 mm blank paper disk before it transferred into the agar media. Chlorhexidine (Minosep) was used as the positive, whereas DMSO was used as negative control. The inhibition zones were observed by measuring the clear zone around the paper discs after 24h incubation at 37°C [16].

2.7 Antibacterial activity of RJ by MIC and MBC methods

The next antimicrobial test was conducted with the determination of MIC levels by broth microdilution method. Before the determination of MIC and MBC levels, each bacterium and fungus was made into an inoculum of *direct colony suspension method.* Inoculum was obtained by inoculating colonies of *C. albicans*, *S. mutans, and P. gingivalis* that had been cultured for 24 h on MHA medium, into Mueller Hinton Broth (MHB) (Himedia, M391). The turbidity was adjusted to McFarland 0.5 standard solution (1-5×10^8 CFU/mL). The MIC levels were determined in 96 well plates filled with 100 µL of each RJ concentration of 100%, 75%, 50%, 25%, 12.5%, 6.25%,

and 3.13% supplemented with 100 µL of each bacterium. DMSO 10% was set as the negative control and chlorhexidine 0.2% was set as the positive control. The plate was then incubated at 37ºC for 24 h. After incubation, the turbidity of the plate was measured using spectrophotometry (Multiskan GO Thermo Scientific 51119300) at a 500~600 nm wavelength. After absorbance, the MBC levels were determined by taking as much as 100 µL which was then carried out at stratified dilutions of 10^2-10^5 in each well of the MIC results. A total of 50 µL of dilution results were grown on MHA using the pour plate technique. The plate was placed in an incubator at 37ºC for 24 h. The following day, the quantity of colonies of bacteria was determined using a colony counter known as the Funke Gerber 8500. The lowest extract concentration was identified as the minimum bactericidal concentration (MBC) that resulted in a 99% inhibitory effect [17].

2.8 Statistical Analysis

Data results are represented as mean values with standard deviation (SD) to avoid negative values. Statistical analysis was performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA) with one-way analysis of variance (ANOVA), followed by Independent T test and Tukey's post hoc test. A *p* value of <0.05 was considered statistically significant.

3. Results

3.1 DPPH Scavenging Assay

Figure 1 shows the percentage of RJ DPPH scavenging activity. The data suggest that higher RJ concentrations correspond to increased trapping of DPPH free radicals. The experiment reveals that RJ exhibits DPPH scavenging activity, achieving an IC_{50} of 107.67 ± 0.12 µg/mL (Table 1).

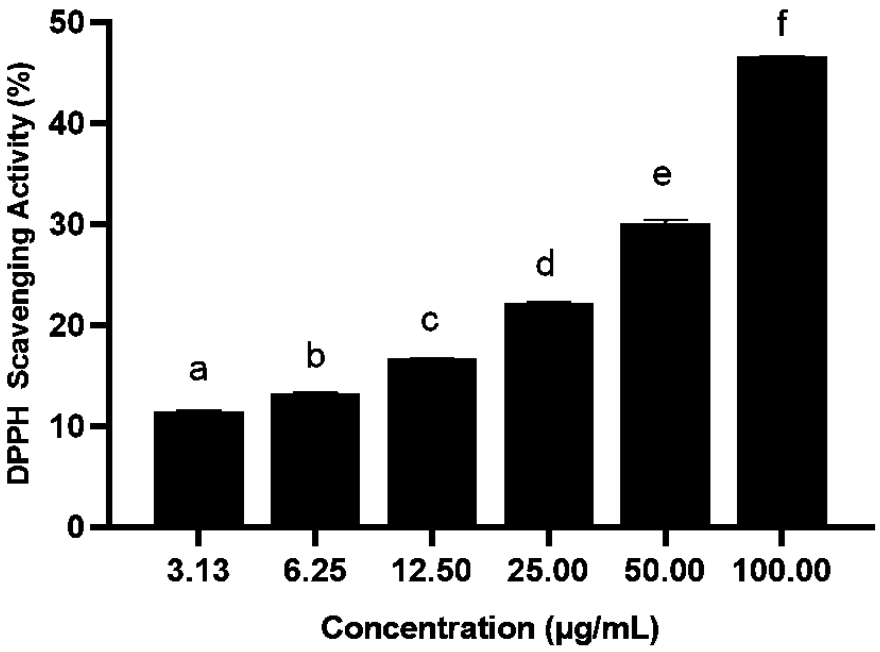

Figure 1 DPPH scavenging activity percentage of royal jelly extract.
*The data were presented as mean ± SD. The numbers of 3.13, 6.25, 12.5, 25, 50, 100 µg/mL are the various concentrations of RJ. The assay was performed in triplicate for each concentrations. The different letters indicate significant differences among RJ concentration (p<0.05).

3.2 ABTS Scavenging Assay

Figure 2 illustrates the reduction activity of ABTS, and the results indicate a direct correlation between sample concentration and reduction activity. Specifically, the highest sample concentration demonstrates the greatest reduction activity towards ABTS salt, with an IC_{50} value of 234.63 ± 0.41 µg/mL, as detailed in Table 1.

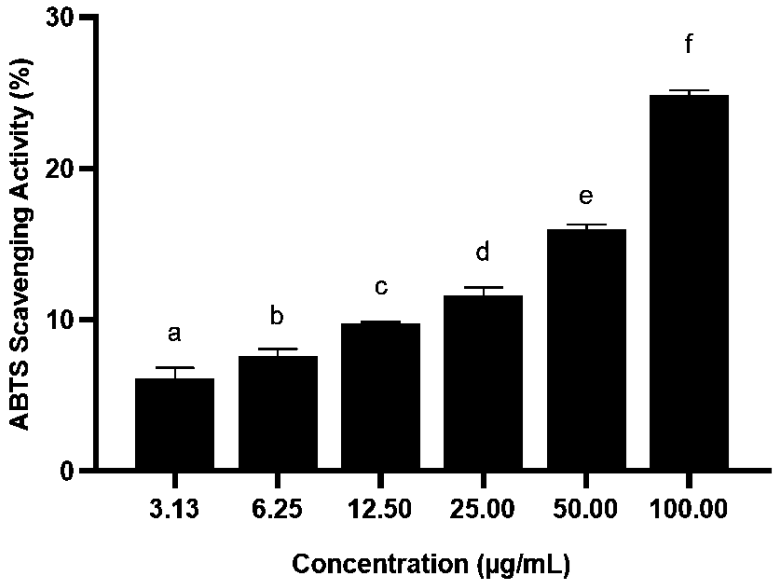

Figure 2 ABTS scavenging activity percentage of royal jelly extract.
*The data were presented as mean ± SD. The numbers of 3.13, 6.25, 12.5, 25, 50, 100 µg/mL are the various concentrations of RJ. The assay was performed in triplicate for each concentration. The different letters indicate significant differences among RJ concentrations (p<0.05).

3.3 NO Scavenging Assay

Nitric oxide is a volatile species that readily reacts with O_2, forming stable products such as nitrates and nitrites through intermediates such as NO_2, N_2O_4, and N_3O_4. Nitrous acid decreases in the presence of antioxidant compounds. The antioxidant activity of RJ, expressed as a percentage in Figure 3, demonstrates IC_{50} value of 158.61 ± 0.25 µg/mL, as shown in Table 1.

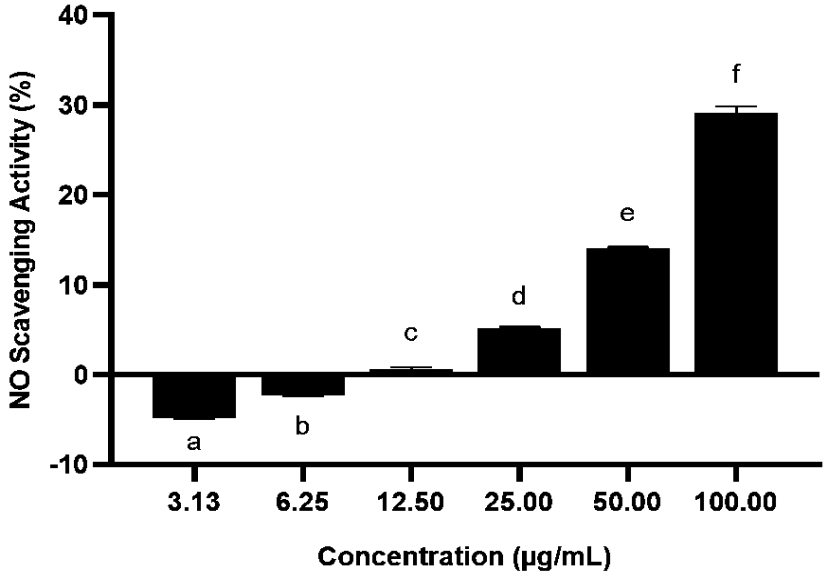

Figure 3 NO scavenging activity percentage of royal jelly extract.
*The data were presented as mean ± SD. The numbers of 3.13, 6.25, 12.5, 25, 50, 100 µg/mL are the various concentrations of RJ. The assay was performed in triplicate for each concentration. The different letters indicate significant differences among RJ concentrations (p<0.05).

3.4 H_2O_2 Scavenging Assay

Figure 4 revealed that RJ has the highest scavenging activity at a concentration of 100 µg/mL, with IC_{50} = 210.24 ± 0.23 µg/mL (Table 1).

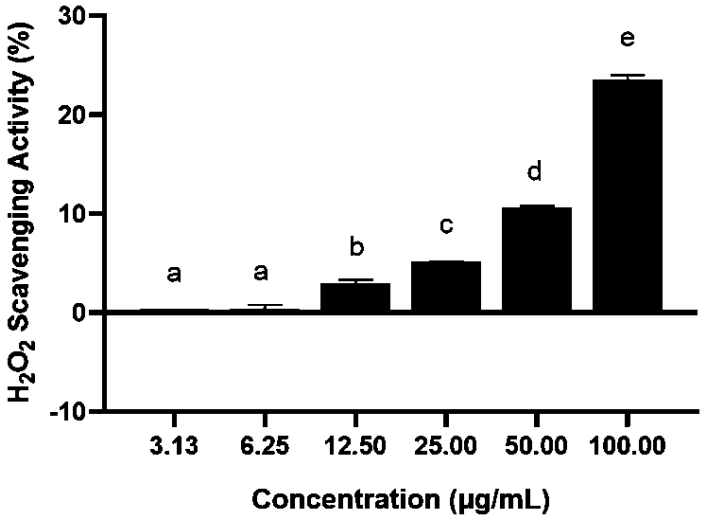

Figure 4 H_2O_2 scavenging activity percentage of royal jelly extract.
*The data were presented as mean ± SD. The numbers of 3.13, 6.25, 12.5, 25, 50, 100 µg/mL are the various concentrations of RJ. The assay was performed in triplicate for each concentration. The different letters indicate significant differences among RJ concentrations (p<0.05).

Table 1 IC_{50} value of DPPH, ABTS, NO, and H_2O_2 radical scavenging in RJ.

Assay	Linear equation	R^2	IC_{50} (µg/mL)
DPPH	y = 0.3555x + 11.725	0.99	107.67 ± 0.12
ABTS	y = 0.185x + 6.5928	0.99	234.63 ± 0.41
NO	y = 0.3419x – 4.2292	0.99	158.61 ± 0.25
H_2O_2	y = 0.2416x – 0.7949	0.99	210.24 ± 0.23

*The IC_{50} values were presented based on the average value of triplicate experiments.

3.5 Effect RJ toward bacterial mouth disease with Disc diffusion test Kirby-Bauer

Figure 5 illustrates that the inhibitory effect on each microbe depends on the concentration of RJ. The clear zone observed around the disc soaked with the sample indicates a reduction in the presence of live microbes. In the fungal microorganism, *C. albicans* was able to start being inhibited at a concentration of 12.5% with a diameter width of (0.47 mm). *S. mutans* 25% (0.67 mm), and *P. gingivalis* became the most resistant with 50% inhibition (2.50 mm). Higher concentrations contributed to increased inhibition of *C. albicans*, *P. gingivalis,* and *S. mutans.*

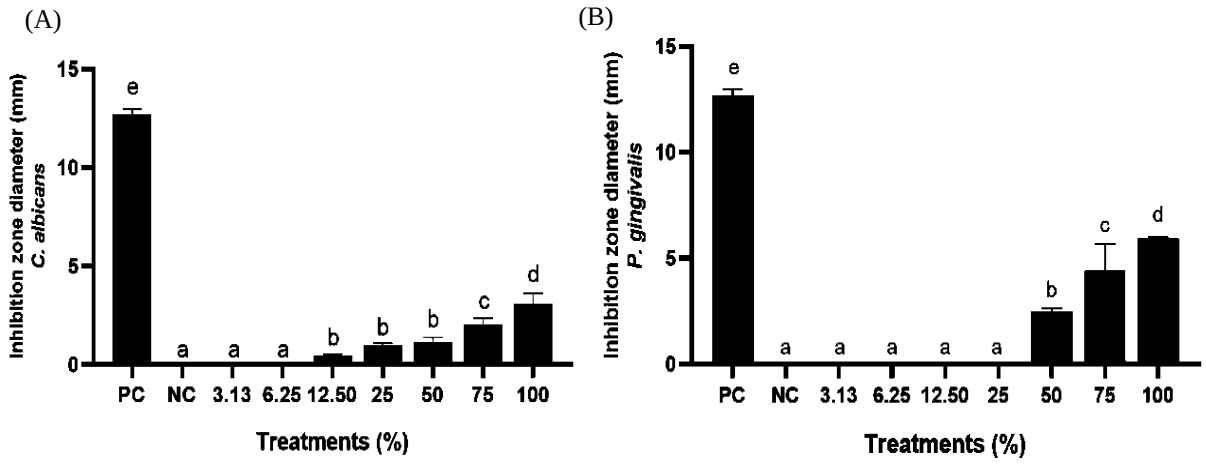

Figure 5 Effect of various samples of RJ concentrations on the antibacterial inhibition zone of (A) *C. albicans*, (B) *P. gingivalis*, and (C) *S. mutans*.
*Note: PC (Positive control); NC (Negative control); RJ 3.13, 6.25, 12.5, 25, 50, 75, 100% these are all RJ treatments. Data are presented as mean ± SD, on the average value of triplicate experiments. Different letters indicate significant differences between treatments based on the Independent Sample T-Test (p<0.05).

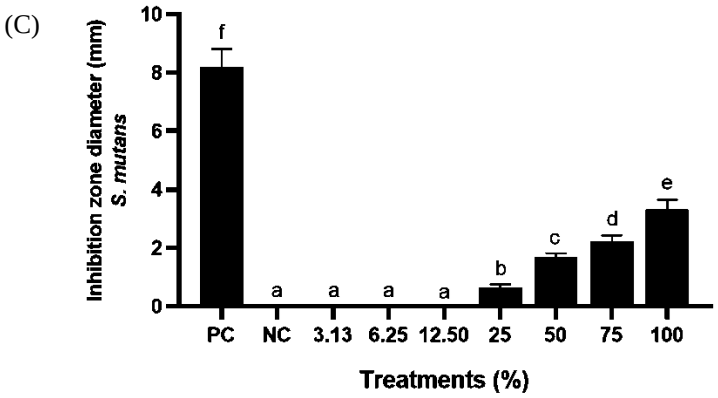

Figure 5 (Cont.) Effect of various samples of RJ concentrations on the antibacterial inhibition zone of (A) *C. albicans*, (B) *P. gingivalis*, and (C) *S. mutans*.
*Note: PC (Positive control); NC (Negative control); RJ 3.13, 6.25, 12.5, 25, 50, 75, 100% these are all RJ treatments. Data are presented as mean ± SD, on the average value of triplicate experiments. Different letters indicate significant differences between treatments based on the Independent Sample T-Test (p<0.05).

3.6 Effect of RJ on S. mutans, P. gingivalis, and C. albicans by MIC and MBC methods

Antibacterial evaluation by MIC and MBC methods is presented in Figure 6(A)-(F). The MIC and MBC methods revealed the effect of RJ concentration on bacterial and fungal microorganisms. The MIC values of *C. albicans* (57.79%), *P. gingivalis* (56.64%), and *S. mutans* (53.00%) were 12.5% (Figure 6(B), 6(D), 6(F)). The MBC value of RJ was at 100% concentration which was significantly different (p<0.05) and in line with the colony counts.

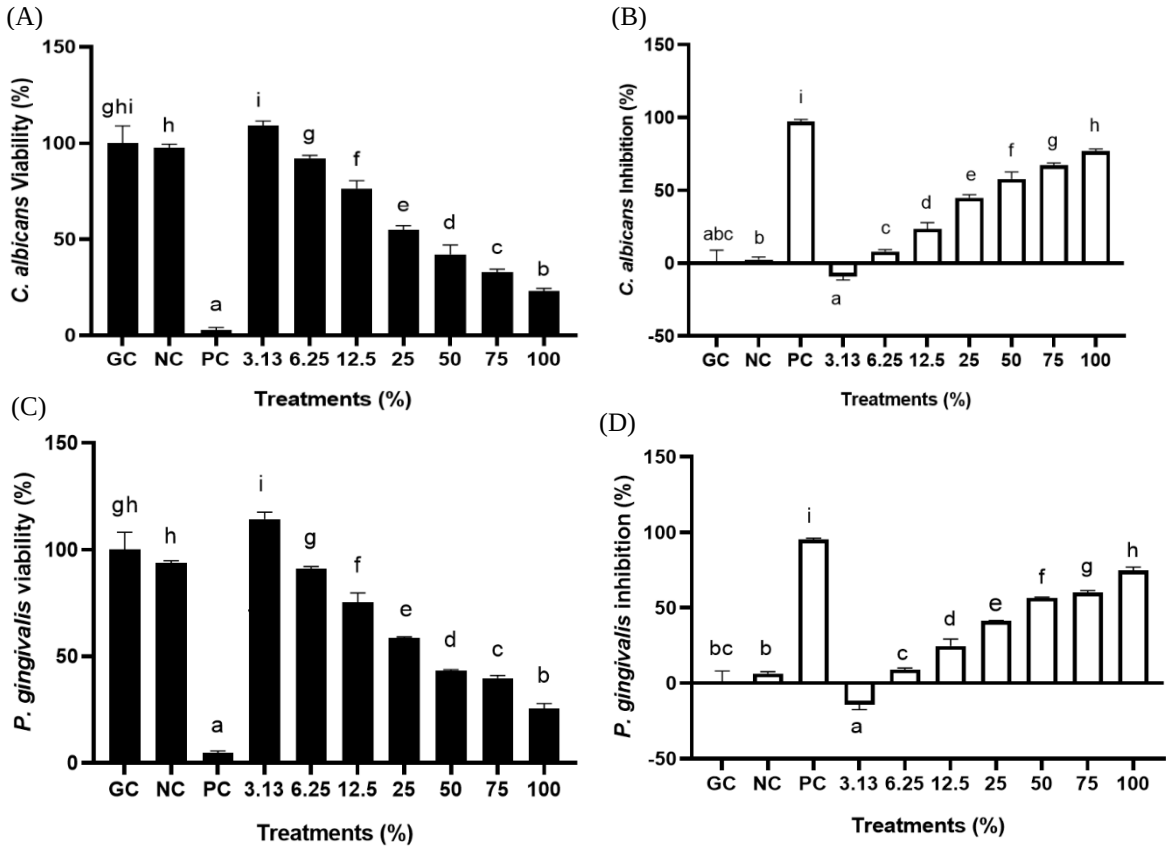

Figure 6 Effects of different RJ concentrations on the viability and inhibition of *C. albicans* (A–B), *P. gingivalis* (C–D), and *S. mutans* (E–F).
*Note: growth control (GC); negative control (NC); positive control (PC; Chlorhexidine 0.2%); 3.13, 6.25, 12.5, 25, 50, 75, 100% (various concentration of RJ. *Data are presented as mean ± SD. Different letters show significant difference among treatments based on Independent T test (Figure 6(A)-(F)) (p<0.05).

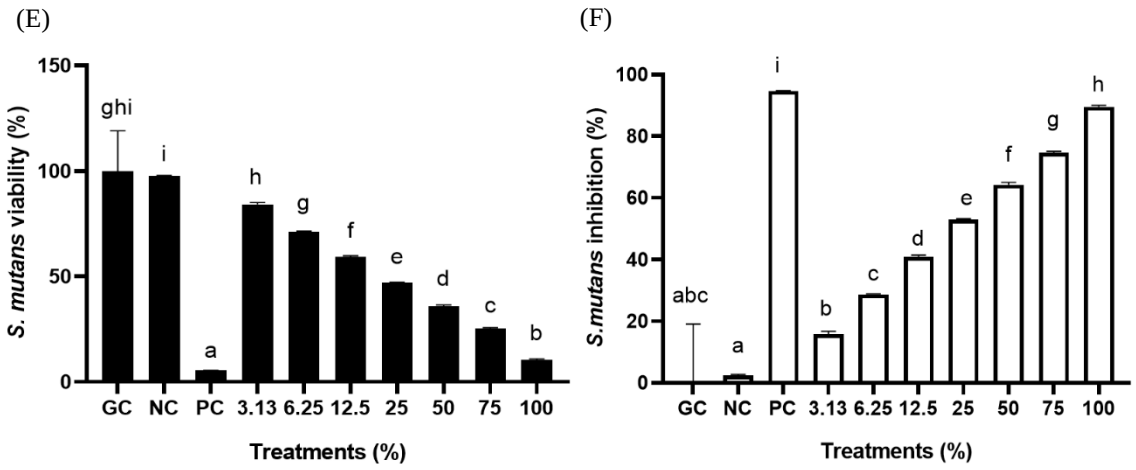

Figure 6 (Cont.) Effects of different RJ concentrations on the viability and inhibition of *C. albicans* (A–B), *P. gingivalis* (C–D), and *S. mutans* (E–F).
*Note: growth control (GC); negative control (NC); positive control (PC; Chlorhexidine 0.2%); 3.13, 6.25, 12.5, 25, 50, 75, 100% (various concentration of RJ. *Data are presented as mean ± SD. Different letters show significant difference among treatments based on Independent T test (Figure 6(A)-(F)) (p<0.05).

Table 2 Effect of RJ on the average colony counts of *C. albicans, P. gingivalis*, and *S. mutans*.

Sample	CFU/mL		
	C. albicans	*P. gingivalis*	*S. mutans*
GC	TNTC	TNTC	TNTC
NC	TNTC	TNTC	TNTC
PC	0	0	0
RJ 3.13%	TNTC	TNTC	TNTC
RJ 6.25%	TNTC	TNTC	TNTC
RJ 12.5%	219,67x 10^4	237,67 x 10^4	211 x 10^4
RJ 25%	106,67 x 10^4	151,00 x 10^4	140.67 x 10^4
RJ 50%	68,33 x 10^4	125,33 x 10^4	71.67 x 10^4
RJ 75%	31,00 x 10^4	36,33 x 10^4	30 x 10^4
RJ 100%	7,00 x 10^4	9,33 x 10^4	0

*Note: GC: growth control; NC: negative control (DMSO); PC: positive control (chlorhexidine 0.2%); RJ (Royal Jelly); TNTC: Too Numerous To Count >250 colonies.

4. Discussions

Maintaining oral health can be supported by using toothpaste that can reduce plaque, caries, and freshen the breath [18]. Royal jelly, which is high in water, carbohydrates, proteins, lipids, B vitamins, and minerals [19], may be used as an ingredient in toothpaste. The antibacterial and antioxidant qualities of royal jelly were assessed through antimicrobial and antioxidant assays.

The antioxidant capacity of a substance was measured using the DPPH assay method, assessing its capability to scavenge the stable DPPH radical exhibiting a rich violet hue. The assay relies on antioxidants that reduce the DPPH radical to its colorless form, and the extent of this reduction gauges the antioxidant capacity of the substance. Widely used in biochemistry, food science, and agricultural research, the DPPH assay evaluates various substances, including plant extracts, food products, and antioxidants [20]. In the experiment, RJ demonstrates DPPH scavenging activity, achieving an IC_{50} of 107.67 ± 0.12 µg/mL (Figure 1). Mechanisms of the inhibition of DPPH (antioxidant) free radicals by the extract have been explored in several research journals. Based on these studies, it has been found that plant extracts can contribute hydrogen or electrons to the DPPH radical, resulting in its neutralization and transformation of its vibrant violet hue to a more subdued yellow or colorless shade. This is connected to their ability to decrease power capacity and their dependence on concentration [21,22]. The DPPH assay is well-known for assessing the capacity of compounds to scavenge free radicals, providing valuable evidence about the antioxidant capabilities of extracts [23].

An established approach for determining a substance's antioxidant capability is the ABTS test. Plant extracts, food items, and antioxidants may have their antioxidant potential assessed using the ABTS test, which finds use in biochemistry, food science, and agricultural research. It operates on the principle that antioxidants can decrease the blue ABTS radical cation to its colorless form, and the degree of reduction indicates the substance's antioxidant capacity [24]. In this context, the highest sample concentration exhibits the most significant reduction activity towards ABTS radical, exhibiting an IC_{50} of 234.63 ± 0.41 µg/mL (Figure 2). Research journals, including studies on *Ficus religiosa* extracts' antioxidant activity and a review article on the genesis and development of the ABTS method, discuss the mechanism of extract-mediated inhibition of ABTS (antioxidant) free radicals. These sources emphasize concentration-dependent hydrogen donation and electron transfer mechanisms involved in ABTS radical scavenging activity of antioxidants. Overall, these findings underscore the ABTS assay's reliability for evaluating the antioxidant activity of various substances [25].

In line with this, royal jelly comprises phenolic chemicals, fatty acids, and minor peptides (2–4 amino acids in length) that exhibit strong antioxidant activity, particularly due to tyrosine residues that effectively scavenge hydroxyl radicals, hydrogen peroxide, and DPPH radicals. Furthermore, compounds such as 10-hydroxy-2-decenoic acid (10-HDA), a significant component of RJ, have been shown to enhance the activity of important antioxidant enzymes, contributing to the overall reduction of oxidative damage in tissues, including the gums and mucosal membranes of the mouth. The mechanisms include the upregulation of protective enzymes including glutathione peroxidase and superoxide dismutase, suggesting that RJ may enhance tissue resilience against oxidative insults. Such antioxidant properties are relevant for oral health, as oxidative stress contributes to periodontal tissue damage and gingival inflammation, suggesting that RJ-derived antioxidants could have potential applications in toothpaste formulations for protecting oral tissues against oxidative injury [26-28].

A key signaling molecule in many physiological and pathological processes, NO influences neurotransmission, immunological responses, and vascular control. The function of NO in oxidative stress, inflammation, and cardiovascular health can be better understood by measuring NO levels. The results of the study highlighted the need for NO assays to evaluate the antioxidant capacity of compounds, as NO is involved in oxidative damage and degenerative processes. The potential of plant extracts to reduce oxidative stress and related health problems is demonstrated by their capacity to scavenge NO radicals [29]. The RJ in Figure 3 has a detrimental effect on NO radicals, as evidenced by its IC_{50} value of 158.61 ± 0.25 µg/mL. Several studies have investigated the mechanisms underlying extract-mediated inhibition of NO free radicals. Some examples involve the study of the impact of *Croton Linearis* Jacq. leaves on NO production in LPS-stimulated macrophages and the ability to scavenge free radicals have been stuided [30]. Other research reports that RJ is capable to increase cGMP levels as well as NO production in isolated aortic rings, suggesting that RJ has potential as an antihypertensive agent associated with increased production of NO. Furthermore, RJ comprises a muscarinic receptor agonist, possibly acetylcholine-like substances, and induces vasodilation through the calcium channels and NO/cGMP pathway [31]. These findings indicate that the inhibition of NO free radicals in plant extracts is related to free radical scavenging and its potential to reduce oxidative stress and inflammation [30].

Hydrogen peroxide (H_2O_2), which is used as a disinfectant and produced naturally in the body, is important in various analyses. Testing scavenging activity against H_2O_2 evaluates a sample's ability to neutralize it, often due to phenolic compounds. Figure 4 shows that RJ exhibited the highest scavenging activity at 100 µg/mL, with an IC_{50} of 210.24 ± 0.23 µg/mL [32].

According to the results of antimicrobial evaluation using the method of disk diffusion test, each microorganism has inhibition values at different concentrations (Figure 5(A)-(C)). Antibacterial activity using the disk diffusion method on *S. mutans* is the same as in study, which at a 25% concentration demonstrated the ability to inhibit *E. faecalis* which is a bacteria that contributes to the formation of biofilms. In other examinations using MIC and MBC methods, RJ obtained significant results with an average prevalence of antibacterial activity as seen in Figure 6A-F. Many studies have conducted the same test on various bacteria, namely *B. cereus* 12.5 mg/mL [33], *B. subtilis* 7.8–500.0 µg/mL [34], *E. faecalis* 50.0–90.0 w/w [33], *E. coli* 60.0–100.0 w/w [35], and *S. aureus* 15.6–500.0 µg/ml [34]. The reported results of this research showed that RJ was capable to inhibit the growth of the three microorganisms, which is in line with the number of colonies that grew, as shown in table 2. In the report of Khosla [36] RJ showed activity against the periodontal pathogens that were tested and showed strong antimicrobial effectiveness for anaerobic bacteria located in subgingival plaque.

Its efficacy appears modest when compared to synthetic antimicrobials or conventional antibiotics. Previous investigations reported MIC and MBC values of RJ around 5–10 mg/mL against *S. aureus* and *E. faecalis* [37]. This difference highlights that, while RJ has demonstrable antibacterial effects, it is not as potent as many synthetic compounds or antibiotics when used as a standalone agent. Nevertheless, the relatively high MIC/MBC values do not diminish the potential of RJ in specific applications. As a natural product with low toxicity, biocompatibility, and reduced risk of resistance development, RJ could be particularly valuable in topical or localized formulations, such as toothpastes, mouth rinses, or endodontic medicaments, where higher concentrations are achievable. Moreover, its role may be more promising as part of a combination strategy. Studies have shown additive or synergistic interactions when RJ is combined with other natural products such as honey [38,39] or when used

alongside antimicrobial agents, suggesting that it could enhance efficacy while potentially lowering the required dose of conventional antimicrobials.

RJ is recognized for its rich composition of proteins, including antimicrobial peptides (AMPs) including royalisin, jelleins, and major royal jelly proteins (MRJPs), which exhibit significant antimicrobial activity against various pathogens, particularly those relevant to oral health, including *C. albicans, P. gingivalis*, and *S. mutans*. Royalisin, highlighted as an important AMP in RJ, has demonstrated effective bactericidal activity against several strains of Gram-positive bacteria, such as *S. mutans* and *P. gingivalis*, which are known contributors to oral infections. Studies indicate that royalisin has a low MIC, underscoring its potency in inhibiting the growth of these pathogens. This antimicrobial action is particularly relevant given the increasing prevalence of antimicrobial resistance, positioning RJ as a potential natural alternative to conventional antibiotics. RJ's activity extends beyond specific pathogens; it also impacts biofilm structures, where *S. mutans* and *P. gingivalis* thrive [40-42]. In addition to its antibacterial properties, royal jelly demonstrates antifungal effects, notably against *C. albicans*, a significant pathogen associated with oral thrush and other opportunistic infections. The antifungal mechanism of action attributed to RJ involves disrupting the fungal cell membrane integrity, leading to cell death, which is crucial for managing oral fungal infections [43]. The high sugar content and two main fatty acids, namely trans-10-hydroxydecenoic acid (10-HDA) and trans-10-hydroxyoctadecanoic acid (10-HOA) in RJ can bind bacterial molecules which then inhibit bacterial metabolism and reduce the ability of bacteria to communicate and colonize [44].

This study provides valuable insights into the antioxidant potential of RJ under laboratory conditions; however, several limitations should be considered when translating the findings into real toothpaste formulations. First, the use of ethanol extraction, maltodextrin encapsulation, and dissolution in DMSO was primarily intended to enable in vitro testing and does not fully represent the physical conditions, stability, or interactions that would occur in a commercial dentifrice environment. Therefore, the antioxidant effects observed in this study may not directly correspond to those achievable in consumer-ready toothpaste formulations. In addition, the stability of RJ bioactive constituents, including small peptides and phenolic compounds, could be influenced by formulation factors such as pH, surfactants, humectants, and storage conditions. A loss of stability under such conditions might reduce the antioxidant activity of RJ in oral care applications. Future investigations should therefore explore the incorporation of RJ into more relevant formulation vehicles, such as aqueous- or glycerol-based systems, which are widely used in herbal and functional toothpaste formulations. Previous studies have demonstrated the application of glycerol as a humectant in herbal toothpaste bases [45], as well as the successful optimization of functional toothpaste containing plant extracts in commercially compatible systems [46]. Evaluating RJ under these conditions will be essential for bridging laboratory evidence with practical product development.

From a methodological perspective, although all statistical analyses in this study demonstrated significant differences with p-values < 0.05, detailed statistical parameters such as degrees of freedom and effect sizes were not included in the current reporting. This limits the depth of statistical interpretation and highlights an area for refinement in future studies, where more comprehensive statistical outputs will be incorporated to strengthen the robustness of the findings.

5. Conclusions

Royal jelly demonstrated strong antioxidant activity, as evidenced by DPPH, ABTS, NO, and H_2O_2 scavenging assays with values of 107.67 ± 0.12 µg/mL, 234.63 ± 0.41 µg/mL, 158.61 ± 0.25 µg/mL, and 210.24 ± 0.23 µg/mL, respectively, confirming its capacity to neutralize free radicals. In addition, disc diffusion assays revealed notable inhibitory effects against *C. albicans* (57.79%), *P. gingivalis* (56.64%), and *S. mutans* (53.00%), which are key pathogens associated with oral and dental diseases. Collectively, these findings suggest that royal jelly, owing to its combined antioxidant and antimicrobial properties, holds strong potential as an active ingredient in toothpaste formulations.

6. Acknowledgements

All authors would like to take this opportunity to thank the Ministry of Education and Culture Republic of Indonesia for Research Grant 2023-2024 for supporting this research. We thank to Biomolecular and Biomedicine Research Center, Aretha Medika Utama, Indonesia for their technical assistants.

7. Conflicts of interest

There are no potential conflicts of interest to declare.

8. Author contributions

Euis Reni Yuslianti contributed to the conceptualization, study design, supervision, methodology development, validation, and final approval of the manuscript. Agus Susanto contributed to methodology refinement, oversight of specific experimental procedures, data interpretation, and manuscript review and editing. Afifah Bambang Sutjiatmo performed data curation and laboratory analysis, contributed to validation, and assisted in manuscript editing. Wahyu Widowati contributed to formal analysis, provided expert input on data interpretation, and assisted in manuscript review. Vini Ayuni provided resources, supported laboratory activities, and assisted in data curation. Dhanar Septyawan Hadiprasetyo contributed to data visualization and minor data processing tasks.

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