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Investigation of Bioactivities of the Extract from *Bacillus subtilis* RGT2

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Abstract The antimicrobial, antioxidant, and anti-inflammatory properties of extract from *Bacillus subtilis* RGT2 was examined to assess the prospective of bacteria as a source of natural products with therapeutic potential. These properties were analyzed by performing a set of standard assays. The extract obtained with ethyl acetate showed variety of components: flavonoids, polyphenols, alkaloids, steroids, glycosides, and tannins, as they yielded promising results in all completed assays. The antioxidant activity of *Bacillus subtilis* RGT2 extract was evaluated using several methods (DPPH (1,1-diphenyl-2-picrylhydrazyl, EC_{50}=57.38±2.30 µg/mL); $ABTS^{\bullet+}$ (2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid, EC_{50}=57.01±0.50 µg/mL); FRAP (ferric reducing antioxidant power, EC_{50}=41.41±0.31 µg/mL), TAC (total antioxidant capacity, EC_{50}=94.80±3.16 µg/mL), RP (reducing power, EC_{50}=32.00±0.37 µg/mL), and the results showed good antiradical effects. Moreover, the antimicrobial evaluation showed a potent antibacterial activity against *Staphylococcus aureus* (MIC=160 µg/mL; MBC=1280 µg/mL. The extract from *Bacillus subtilis* RGT2 demonstrated anti-inflammatory activity in a dose dependent pattern with a half maximal inhibitory concentration (IC_{50}) value of 73.74±1.77 µg/mL. Results demonstrated that *Bacillus subtilis* RGT2 extract can represent an important natural source with high antimicrobial, antioxidant, and anti-inflammatory potential effects.

Keywords *Bacillus subtilis* RGT2, antibacterial, antioxidant, anti-inflammatory, *Staphylococcus aureus*

1. Introduction

The skin is a very important immune organ for human health. Unhealthy skin is easily subjected to the attack and invasion of many different strains of pathogenic bacteria in which *Staphylococcus aureus* is the cause of pimples, folliculitis and skin abscesses. Skin and soft-tissue infections caused by *Staphylococcus aureus* usually appear as small pimples and can progress to serious infections involving muscles or bones, which can spread to the lungs or heart valves [1]. *Staphylococcus aureus* infection provokes inflammatory reactions. After being infected with *Staphylococcus aureus*, the body will release proinflammatory cytokines and regulate the immune system. Cytokines are mainly inducible proteins produced when stimulating white blood cells and other cells by pathogens [2]. However, the inflammatory response to overproduction of proinflammatory cytokines can be dangerous, possibly even causing multi-organ dysfunction and death [3]. Overproduction of proinflammatory cytokines increases the levels of free radicals in the body [4]. Free radicals are substances normally produced by the human body as one of the defense mechanisms against harmful substances. When the rate of their production exceeds the antioxidant capacity of the body, oxidative stress occurs. Oxidative stress carries harmful effects to all the body

systems and is implicated in the pathogenesis of various diseases [5]. Because of the above reasons, the research and potential of a natural remedy capable of resistance to *Staphylococcus aureus*, anti-inflammatory and neutralizing free radicals in the human body is necessary.

Currently, the world has more and more concerns about endogenous bacteria in medicinal plants. Because, endogenous bacteria strains in medicinal plants not only help them to like to grow fast in host plants but also have the ability to produce natural antibacterial compounds. In addition, endogenous bacteria strains are capable of stimulating host plants to produce secondary metabolic compounds [6]. These secondary metabolic compounds not only possess antimicrobial activity, but also have anti-inflammatory ability and neutralize many free radicals. In nature there are many plants and trees that have antibiotics and have been used as medicine for a long time. Many studies to isolate endogenous bacteria in different medicinal plants have been performed. However, studies of endogenous bacteria with antibacterial, anti-inflammatory and antioxidant activities in *Houttuynia cordata* have not been performed. *Houttuynia cordata*, which is a very familiar vegetable in Vietnamese daily meals, has a bitter and mild taste. In Oriental and Western medicine, *Houttuynia cordata* is known for many uses of plant extracts that have antibacterial, anti-inflammatory and antioxidant properties [7]. Many studies use *Houttuynia cordata* to treat diseases based on the antibacterial, anti-inflammatory and antioxidant properties of *Houttuynia cordata*. However, the study of endogenous bacteria in *Houttuynia cordata* with antibacterial, anti-inflammatory and anti-oxidant activities has not caught much attention.

2. Materials and research methods

2.1 Material

The *Bacillus subtilis* RGT2 used in the study is the endogenous bacteria strain isolated from *Houttuynia cordata* collected in Kien Giang province, which was selected and identified in 2019 [8]. The strain of *Staphylococcus aureus* used in research was isolated from human furunculosis and stored in the laboratory of the Department of Microbiology, Can Tho University of Medicine and Pharmacy.

2.2 Extracts preparation from *Bacillus subtilis RGT2*:

The *Bacillus subtilis* RGT2 (40 mL; 10^8 bacteria cells/mL) was inoculated into 3960 mL of liquid potato dextrose medium, cultured for 24 hours on a 150 rpm horizontal shaker. Extraction extract from *Bacillus subtilis* RGT2 by extracting liquid-liquid with ethyl acetate solvent in the ratio 1:1. The ethyl acetate extract was obtained by filter and evaporation. Extract was stored and preserved at 4°C and used for further investigation.

2.3 Determination of chemical composition in the extract of *Bacillus subtilis RGT2*

The ethyl acetate extract of *Bacillus subtilis* RGT2 were qualitatively dertermined for the presence of phytochemical constituents such as alkaloids, flavonoids, steroids, tannins, saponins, glycosides according to the description of Nguyen Kim Phi Phung (2007) [9].

2.4 Quantification of the chemical fraction in the extract of *Bacillus subtilis* RGT2

2.4.1. Quantification of total polyphenols

The total phenolic content of plant extract was conducted by the Folin-Ciocalteu method [10]. The extract (250 µL) included 250 µL distilled water and 250 µL Folin-Ciocalteu reagent. After 8 minutes, 250 µL of 10% sodium carbonate was added and evently shaken. The absorbance of the reaction mixture was measured by spectrophotometer at 765 nm after incubation for 30 min at 40°C. The polyphenol content was determined to be equivalent to milligrams of gallic acid per gram of extract (mg GAE/g extract).

2.4.2 Determination of total flavonoid content

The total flavonoid content of *Bacillus subtilis* RGT2 extract were determined according to the method of Sultana *et* al (2007) [11]. and some revisions. A reaction mixture containing 200 µL extract in 200 µL deionized water, 200 µL of 5% $NaNO_2$ incubated for 5 min. Then, 40 µL of 10% $AlCl_3$ was added and incubated in 6 minutes. After that, 400 µL of 1M NaOH was added. The final volume was adjusted to 1000 µL with deionized water. After 15 min, the absorbance of the mixture was determined at 510 nm. The flavonoid content was determined to be equivalent to milligrams of quercetin per gram of extract (mg QE/g extract).

2.5. Investigation of antibacterial activity of *Bacillus subtilis* RGT2 extract

2.5.1. Methods for determining the diameter of the antibacterial ring

The antibacterial ability of the extract from *Bacillus subtilis RGT2* was determined based on the formation of an antibacterial ring around the small well of the extract. The *Staphylococcus aureus* bacteria solution with a density of 10^8 bacteria/mL was spread evenly on the surface of the PDA agar plate with a bacterial culture volume of 100 µL. Carry out punching holes and put 50 µL of extract solution into the agar well at concentrations of 80, 160, 320, 640 and 1280 µg/mL. The diameter of the antibacterial ring (excluding the agar well diameter of 9 mm) was measured with a ruler in mm after 24 h of incubation at 37°C [12].

2.5.2 Methods for determining the minimum inhibitory concentration (MIC, Minimum Inhibitor Concentration) and minimum bactericidal concentration (MBC, Minimum Bactericidal Concentration)

The minimum inhibitory concentration (MIC) of *Bacillus subtilis* RGT2 extract was determined by dilution on a 96-well plate based on the color change of resazurin reagent [12]. Each well was added to 200 µL of extracts at concentrations of 80, 160, 320, 640 and 1280 µg/mL. Add 10 µL of bacterial broth at a density of 108 bacterial cells/mL to each well. Add 40 µL of 0.01% resazurin reagent after 24 h of incubation at 37ºC. After 5 min of observing the color change, the well had a discoloration of the resazurin solution from blue to pink indicating bacterial growth in the well. The MIC concentration was the lowest in the tested concentration range of plant extracts that inhibited bacterial growth (concentration did not change the color of the resazurin reagent).

The minimum bactericidal concentration (MBC) of the extract from *Bacillus subtilis* RGT2 was determined by the method of droplet survival: drop 30 µL of test solution in the wells which did not change color of resazurin on the surface of the solid PDA medium. After 24h, monitoring the survival of the bacteria. The concentration of MBC is the lowest concentration in the concentration range of plant extracts that can kill all bacteria in the well, no colonies appear on PDA agar [12]

2.6. Investigation of antioxidant activity of *Bacillus subtilis* RGT2 extract

2.6.1. Total antioxidant method (total antioxidant capacity)

The total antioxidant activity of the extract of *Bacillus subtilis* RGT2 was evaluated as described by Prieto et al. (1999) [13]. The extract (300 µL) was combined with 900 µL of test solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction solution was incubated at 95°C for 90 min. Then, the spectral absorbance of the solution was measured at 695 nm.

2.6.2. Method to neutralize free radicals $ABTS^{•+}$

$ABTS^{•+}$ free radicals are generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate in the dark at 25°C. After 16 h of incubation, the mixture was diluted and the optical density measured at 734 nm was 0.70±0.05. Conduct the survey by reacting 10 µL of Bacillus subtilis RGT2 with 990 µL $ABTS^{•+}$ at room temperature for 6 minutes. Then, the reaction mixture was measured for absorbance at 734 nm [14].

2.6.3. Method to determine the reducing power (Reducing power, RP)

The reducing power of *Bacillus subtilis* RGT2 extract was performed according to the method of Oyaizu (1986) [15]. The reaction mixture consisted of 500 µL extract, 500 µL phosphate buffer (0.2 M, pH=6.6) and 500 µL K3Fe(CN)6 1%, respectively. After the reaction mixture was incubated at 50°C for 20 min, 500 µL of 10% CCl_3COOH was added and centrifuged at 3000 rpm for 10 min. The aliquot after centrifugation was withdrawn 500 µL into 500 µL water and 100 µL $FeCl_3$ 0.1%, shaken well. The spectral absorbance of the reaction mixture was measured at 700 nm.

2.6.4. Investigation of DPPH free radical neutralization efficiency (2, 2-Diphenyl-1-Picrylhydrazyl)

The antioxidant capacity of Bacillus subtilis RGT2 extract was determined by the modified DPPH free radical neutralization method of Sharma & Bhat (2009) [16]. The reaction mixture consisted of 40 µL DPPH (1000 µg/mL) and 960 µL extract. The reaction mixture was incubated in the dark at 30°C for 30 minutes. Then, the spectral absorbance of DPPH was measured at 517 nm.

2.6.5. Determination of the reducing-antioxidant power (Ferric reducing-antioxidant power, FRAP)

The reduction potential of *Bacillus subtilis* RGT2 extract was determined by determining the FRAP reduction capacity [17] with revision. The principle of this method is based on the reduction of the ferric-tripyridyltriazine

complex. The extract (10 µL) was reacted with FRAP solution (990 µL) for 30 min under dark conditions. Determine the absorbance at 593 nm.

The extract was investigated for its free radical scavenging activity of DPPH, ABTS, TAC, RP and FRAP at concentrations 0, 10, 20, 40, 60, 80 and 100 µL. Trolox essence was used as positive control for all of the above methods. In addition, the antioxidant activity in the solutions was evaluated through the EC50 value (the concentration at which 50% of the radicals were neutralized or reduced) as described by Linh & Trang (2019) [18]

2.7. Investigation of in vitro anti-inflammatory activity of *Bacillus subtilis* RGT2 extract

The anti-inflammatory activity of *Bacillus subtilis* RGT2 extract was investigated through inhibition of protein denaturation by the method of Shah et al. (2017) with correction [19]. The reaction mixture consisted of 150 µL of extracts at concentrations of 3.125; 6.25; 12.5; 25; 50 and 100 µg/mL with 150 µL of 5% bovine serum albumin (BSA) solution. Then, the mixture was incubated at 27oC for 15 minutes. Protein denaturation was induced by keeping the reaction mixture at 60°C for 10 min. After cooling, the absorbance was measured at 660 nm. Prednisolone was used as a positive control. The ability to inhibit protein denaturation was determined by the following formula: percentage inhibition (%)=100×(1-Vt/Vc). In which, Vt is the optical density of the sample containing the extract or standard, and Vc is the optical density of the sample containing the phosphate buffer.

2.8. Statistical analysis of data

The data were analyzed and statistically processed by Minitab 16 software. The graphs were drawn with Microsoft Excel 2016 software. The experiments were repeated 3 times.

3. Results and Discussion

3.1. Results of preparation, qualitative and quantitative chemical composition of extracts from *Bacillus subtilis* RGT2

From 4000 mL of *Bacillus subtilis* RGT2 culture solution, which was liquid extracted with 12L of ethyl acetate solvent through evaporation, 0.641 g of extract was obtained. The extract is obtained in a viscous state with a copper-yellow color and a characteristic aroma. The study conducted qualitatively the chemical composition of the extract based on a number of characteristic reactions showing the presence of flavonoids, polyphenols, alkaloids, steroids, glycosides and tannins. The saponin group was not present in the extract. Many studies show that polyphenols and flavonoids play important roles in antibacterial activities and are closely related to other biological activities [20]. Therefore, this study quantified the polyphenols and flavonoids present in the extract. The total polyphenol content with gallic acid standard substance in the concentration range from 2 to 10 µg/mL had the linear regression equation $y = 0.0918x + 0.0487$ ($R^2 = 0.9862$). Total flavonoid content with quercetin standard was in the concentration range from 20 to 120 µg/mL with linear regression equation $y = 0.0052x – 0.0089$ ($R^2 = 0.9958$). On the basis of these calibration curves, the content of polyphenols and flavonoids in the extract was determined to be 39.54±2.50 mg GAE/g extract and 330.03±11.55 mg QE/g extract, respectively.

3.2. Results of investigation of antibacterial activity of *Bacillus subtilis* RGT2 extract

3.2.1. Antibacterial ring diameter

The experiment was conducted to investigate the antibacterial ability of *Staphylococcus aureus* of extracts from *Bacillus subtilis* RGT2 at concentrations of 80, 160, 320, 640 and 1280 µg/mL. The antimicrobial activity of the extracts and antibiotics against *Staphylococcus aureus* is presented in Table 3 and illustrated in Figure 1.

Table 1: Antibacterial ring diameter of the extract

Samples	**Diameters (mm) of inhibition zones at different concentrations of extracts (µg/mL)**				
	80	160	320	640	1280
Extract	-	13.67^{d}±0.58	17.50^{c}±0.50	18.67^{b}±0.58	20.33^{a}±0.58
Vancomycin	11.00^{d}±1.00	15.33^{d}±0.58	18.00^{d}±1.00	20.67^{d}±0.58	22.85^{d}±0.79

Note: Values followed by the same letter in the same column are not significantly different at 5% level

The extract and the commercial antibiotic vancomycin both showed antibacterial activity proportional to the concentration, the diameter of the antibacterial rings increasing gradually from low to high concentration. However,

the extract from *Bacillus subtilis* RGT2 at a concentration of 80 µg/mL did not form a sterile ring. Probably, the cause is that the concentration of 80 µg/mL does not provide enough dose to inhibit the growth of pathogenic bacteria. When comparing the antibacterial activity of the extract to *Staphylococcus aureus* with vancomycin, the results showed that the antibacterial activity of the extract was much worse than the antibiotic vancomycin. The reason is that vancomycin antibiotic can form an antibacterial ring even at a concentration of 80 µg/mL with a diameter of 11.00±1.00 mm up to a concentration of 1280 µg/mL, the ring diameter increases to 22 ,85±0.79 mm, the difference is statistically significant between concentrations ($p<0.05$)

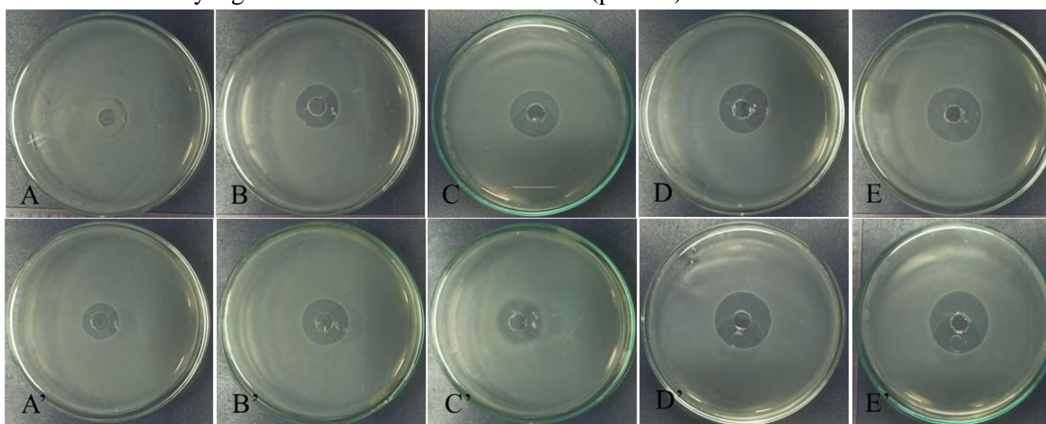

Figure 1: Antibacterial ring diameter of extract and antibiotic vancomycin

Note: A, B, C, D, E are extracts at concentrations 80, 160, 320, 640 and 1280 µg/mL, respectively; A', B', C', D', E' are vancomycin antibiotics at concentrations 80, 160, 320, 640 and 1280 µg/mL, respectively.

3.2.2. Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration was determined by dilution on a 96-well plate with the resazurin reagent. Live cells maintain a reducing environment in their cytoplasm and mitochondria, in which resazurin (blue) is reduced by dehydrogenase enzymes to form resorufin (red) (Figure 2A) [21]. Therefore, observing the color change of resazurin reagent can assess the status of bacterial cells. The extract at a concentration of 160 µg/mL made the solution in the well blue, which proved that starting at a concentration of 160 µg/mL *Staphylococcus aureus* was completely inhibited. From that, the study determined that the minimum inhibitory concentration (MIC) of the extract from *Bacillus subtilis* RGT2 against *Staphylococcus aureus* strain was 160 µg/mL.

Note: A, B, C, D, E are extracts at concentrations 80, 160, 320, 640 and 1280 µg/mL, respectively; A', B', C', D', E' are vancomycin antibiotics at concentrations 80, 160, 320, 640 and 1280 µg/mL, respectively.

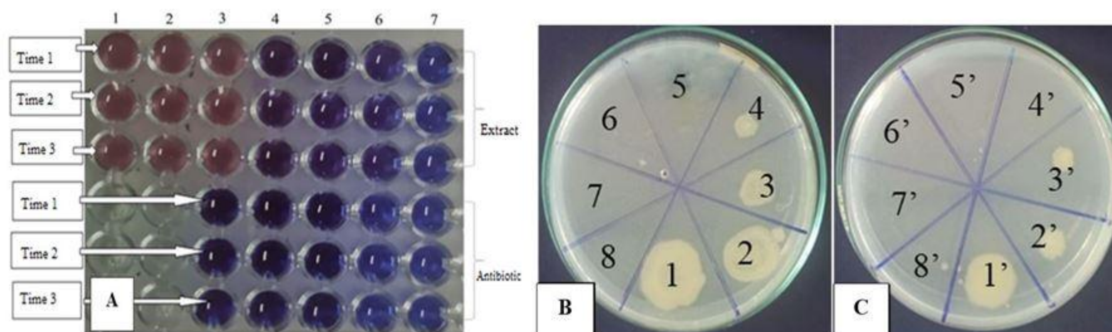

Figure 2: Determination of MIC and MBC values of extract and antibiotic vancomycin

Note Figure 2A: 1-The well contains only bacteria and resazurin; 2- Well with only bacteria, PDA medium, physiological saline and resazurin; 3, 4, 5, 6, 7-Well with only bacteria, extracts or antibiotics at concentrations 80, 160, 320, 640 and 1280 µg/mL and resazurin, respectively.

Note Figure 2B (Determining MBC value of the extract): 1, 2, 3, 4, 5, 6, 7, 8 are the extract concentrations from 0, 160, 320, 640, 1280, 2560, 5120 and 10240 µg/mL, respectively.

Note Figure 2C (Determining MBC value of antibiotics): 1', 2', 3', 4', 5', 6', 7', 8', respectively, are the high concentrations of extracts from 0.80, 160, 320, 640, 1280, 2560 and 5120 µg/mL.

Based on the results of the MIC value survey, the study continued to investigate the minimum bactericidal concentration of the extract by checking the regeneration of *Staphylococcus aureus* bacteria from a concentration of 160 (the first well is green), 320, 640, 1280, 2560, 5120 and 10240 µg/mL. Based on Figure 2B, it can be seen that *Staphylococcus aureus* bacteria can still grow on PDA agar at 640 µg/mL extract concentration, when increasing the extract concentration to 1280 µg/mL, no bacteria were detected. This proves that at the high extract concentration of 1280 µg/mL, *Staphylococcus aureus* bacteria were completely destroyed. From that, the study determined that the minimum bactericidal concentration of the extract extracted from *Bacillus subtilis* RGT2 was 1280 µg/mL. The antibiotic vancomycin was also investigated for the minimum inhibitory concentration and the minimum bactericidal concentration. The antibiotic vancomycin inactivated the activity of *Staphylococcus aureus* bacteria right from the concentration of 80 µg/mL, making the resazurin reagent remain blue (MIC=80 µg/mL). From the concentration of 80 µg/mL (the first well is green), the research has increased to the next concentrations of 160, 320, 640, 1280 and 2560 µg/mL to determine the minimum bactericidal concentration. As shown in Figure 2C, the antibiotic vancomycin completely killed *Staphylococcus aureus* from a concentration of 320 µg/mL (no colonies on PDA agar surface) (MBC=320 µg/mL). Compared with the antibacterial ring diameter determination method performed above, the 96-well plate microdilution method gave similar results. The cell membrane plays an important role in osmotic protective transport processes, cell biosynthesis, and membrane disruption can lead to bacterial death [22]. Polyphenols and flavonoids can increase membrane permeability and disrupt bacterial cell membranes [23, 24].

3.3. Investigation results of antioxidant activity of *Bacillus subtilis* RGT2 extract

The antioxidant activity of the extract was evaluated on the neutralization concentration or 50% reducing of free radicals (EC50-Half maximal effective concentration) and compare with that of trolox essence (Table 2).

Table 2: Neutralization concentrations or 50% reducing of the free radicals (EC_{50}) of the extract and trolox

Samples	Values EC_{50} (µg/mL)				
	$ABTS^{\bullet+}$	DPPH	RP	TAC	FRAP
Extract	57.01^a±0.50	57.38^a±2.30	32.00^a±0.37	94.80^a±3.16	41.41^a±0.31
Trolox	2.40^b±0.02	7.22^b±0.17	3.05^b±0.27	2.32^b±0.08	1.57^b±0.01

Note: Values followed by the same letter in the same column are not significantly different at 5% level.

Extracts from *Bacillus subtilis* RGT2 have diverse antioxidant capacity on many different free radicals with EC50 values ranging from 32.00±0.37 in RP method to 94.80±3, 16 in the TAC method. Thus, the extract in this study is evaluated to have strong reducing power. However, the antioxidant activity of the extracts by all methods was weaker than that of trolox.

Studies on the antioxidant activity of *Bacillus subtilis* RGT2 extracts have not been performed. Therefore, the antioxidant activity of the *Bacillus subtilis* RGT2 extract will be compared with some extracts from the *Houttuynia cordata* because the *Bacillus subtilis* RGT2 strain was isolated from the *Houttuynia cordata*. In 2018, the extract of Houttuynia cordata extracted from the solvents of ethanol, methanol and water gave EC50 values ranging from 0.05 to 2.19±0.03 mg/mL for DPPH, ABTS free radicals [25]. Thus, the extracts extracted from *Houttuynia cordata* had less antioxidant activity than those extracted from *Bacillus subtilis* RGT2 in our study. The human body's antioxidant capacity is closely related to disease resistance, antimicrobial resistance and anti-aging. The antioxidant activity of the extract from *Bacillus subtilis* RGT2 was evaluated against various free radical groups and by different methods. The extract possesses antioxidant activity due to secondary metabolites. Polyphenols and flavonoids protect cell membranes damaged by lipid peroxidation. Thus, polyphenols and flavonoids contribute as antioxidants in the prevention of many diseases caused by oxidative stress [26].

3.4. Results of investigation of anti-inflammatory activity of *Bacillus subtilis* RGT2 extract

Inflammation is a normal product of the host defense response to tissue damage caused by a variety of stimuli such as physical trauma, chemicals, and infectious agents. The natural antioxidants in medicinal plants are potential anti-inflammatory agents and have been attracting attention in recent years [27]. Protein denaturation is one of the proven causes of inflammation [28]. In the process of acne, inflammation is an inevitable response. The anti-inflammatory activity of the extract was compared with prednisolone based on the concentration of the extract or standard that inhibited 50% of denaturation (IC_{50}-the half maximal inhibitory concentration) (Table 3).

Table 3: Anti-inflammatory activity of extract and standard substance

Samples	IC_{50} value
Extract	$73.74^{a}\pm1.77$
Prednisolon	$15.79^{b}\pm0.55$

Note: Values followed by the same letter in the same column are not significantly different at 5% level.

The ability to inhibit bovine serum albumin denaturation of the extract (IC50=73.74±1.77 µg/mL) was 4.67 times lower than that of prednisolone (IC50=15.79±0.55 µg/mL). Inflammation is a complex biological response of body tissues to harmful stimuli, such as pathogen infection, cell destruction, tissue damage, and chemical irritation. Flavonoids act on the site of inflammation by influencing inflammatory cells, releasing ROS, RNS, and proinflammatory cytokines to clear out foreign pathogens including bacteria and repair injured tissues [29].

4. Conclusion

Research has demonstrated that the extract from *Bacillus subtilis* RGT2 has effective antioxidant, anti-inflammatory and antibacterial activities. Besides, the extract also contains many compounds with high biological activity. The extract from *Bacillus subtilis* RGT2 has a wide range of antioxidant activity, neutralizing and reducing many oxidizing compounds as well as inhibiting the growth of *Staphylococcus aureus.* The *Bacillus subtilis* RGT2 extract shows potential as a natural or pharmaceutical source of antioxidants with potential application to reduce oxidative stress in adjuvant treatment of infectious and inflammatory diseases.

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