Clitoria Ternatea Ethanol Extract Antibacterial and Anti-Inflammatory Ability Towards Peri-Implantitis Biomarkers: An in Vitro and in Silico Study

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Abstract

Clitoria ternatea may possess antimicrobial, antioxidant, and anti-inflammatory activities that are expected to provide benefits for dental peri-implantitis treatment.

Objective to investigate C. ternatea ethanolic extract (CTEE) antibacterial activity toward peri-implantitis bacteria in vitro and C. ternatea's anthocyanin anti-inflammatory, antimicrobial, and antioxidant activity in silico.

CTEE was examined with phytochemical tests, antioxidant activity tests, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) tests to analyze antibacterial activity toward dental peri-implantitis bacteria such as Prophyromonas gingivalis (Pi), Aggregatibacter actinomycetemcomitans (Aa), Provotella intermedia (Pi), and Fusobacteria nucleatum (Fn). Molecular docking of CEET's anthocyanin to tumor necrosis factor-α (TNF-α), nuclear factor kappa beta (NFkB), receptor activator nuclear kappa (RANK) and its ligand (RANKL), interleukin-6 (IL-6), osteoprotegrin (OPG), osteocalcin, nuclear factor associated T-cells 1 (NFATc1), tartate resistant acid phosphatase (TRAP), peptidoglycan, flagellin, dectin, heat shock protein-10 (HSP-10) and -70 (HSP-70) was performed. The Shapiro-Wilk test, Levene's test, and one-way analysis of variance (ANOVA) test continued with a post-hoc test ($p<0.50$) to analyze the data.

CTEE contains active compounds including flavonoids, saponins, quinones, alkaloids, tannins, terpenoids, and steroids. CTEE has lower antioxidant activity than vitamin C. CTEE at 50% concentration has antibacterial activity that inhibits Aa, Pg, Pi, and Fn growth. Anthocyanins of CTEE show anti-inflammatory, antimicrobial, and antioxidant activity in silico.

CTEE has antibacterial activity towards Aa, Pg, Pi, and Fn at 50% concentration and potential antioxidant ability in vitro. CTEE's anthocyanin has anti-inflammatory, antimicrobial, and antioxidant activity in silico.

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Introduction

Dental implant is one of the main options in replacing missing teeth[1]. From large-scale studies that have been published, it is said that the use of implants has a high long-term survival rate of up to 98%[2]. Dental implant was considered survived if they were still in the oral cavity without peri-implant mucositis, peri-implantitis or severe bone loss[3]. However, a recent report on the prevalence of dental peri-implantitis in dental implant patient stated that dental peri-implantitis occurred in 5% of patients after 8.3 years of implant use[4]. Dental peri-implantitis is a pathological condition of the hard and soft tissues surrounding the implant accompanied by bone resorption, increased

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pocket formation and purulence[5,6]. Microbial plaque accumulation is considered to be the most important factor in the pathogenesis of dental peri-implantitis[7]. Dental peri-implantitis is associated with several bacterial species, including Porphyromonas gingivalis (Pg) and Prevotella intermedius (Pi)[8].

Treatment of dental peri-implantitis through nonsurgical methods generally involves mechanical debridement of the implant surface using a curette, ultrasonic device, or laser and can be combined with local antibiotics or antiseptics such as chlorhexidine[9]. In addition, anti-inflammatory agents are also often used in the form of non-steroidal anti-inflammatory drugs (NSAIDs), such as mefenamic acid, diclofenac sodium, ketorolac, and ibuprofen[10]. A study showed that the antibiotic prescription had some side effects, such as changes in blood pressure, respiratory depression, diarrhea, and urticaria[11].NSAIDs as anti-inflammatory agents also have side effects, such as cardiovascular disease, impaired kidney function, and gastrointestinal bleeding[10]. Administration of chlorhexidine as a mouthwash is also known to have some side effects and discomfort, such as discoloration of the teeth and tongue, dry mouth, white patches on the lips, a sore throat, and allergies in some users. Therefore, it is necessary to explore herbal compounds as alternatives to minimize the side effect[12].

One of the most popular herbal medicines in Indonesia lately is Clitoria ternatea, widely known by the public as the butterfly pea flower. C. ternatea provides health benefits and is usually used as an ingredient in traditional medicine. All parts of this plant, from the roots to the flowers, are believed to have functional benefits and have the effect of treating and strengthening the performance of organs that are useful for the human body. C. ternatea has many pharmacological effects, including antioxidant, anti-cancer, anti-inflammatory, antipyretic, antidiabetic, antimicrobial, analgesic, and many other pharmacological effects[13]. According to several studies, C. ternatea consists of various bioactive compounds, including flavonoids, anthocyanins, triterpenoids, flavonol glycosides, quercetin glycosides, kaempferol glycosides, myrisetin glycosides, alkaloids, tannins, resins, steroids, saponins, and phenols[14-16]. Flavonoids are known as antioxidant agents by counteracting free radicals and also as anti-inflammatory agents by inhibiting the activity of cyclooxygenase and lipoxygenase enzymes, thereby shortening the inflammatory reaction[17,18]. As antibacterial agents, alkaloids are able to cause cell death by interfering with the peptidoglycan constituent of bacterial cells so as not to form a cell wall layer[19]. The various pharmacological effects of C. ternatea are expected to provide benefits in minimizing the occurrence of dental peri-implantitis, and it may be an appropriate therapy in cases of dental peri-implantitis. Furthermore, the aim of this study is to investigate C. ternatea ethanolic extract's (CTEE) antibacterial activity toward peri-implantitis bacteria in vitro and C. ternatea's anthocyanin anti-inflammatory, antimicrobial, and antioxidant activity in silico.

Materials and methods

This study is a true experimental laboratory analytical study with a post-test-only control group design. The sample for this study was Prophyromonas gingivalis (Pi), Aggregatibacter actinomycetemcomitans (Aa), Provotella intermedia (Pi), and Fusobacteria nucleatum (Fn). The samples were selected using a blind random technique. This study was approved and obtained an ethical qualification number (253/HRECC.FODM/V/2022) from the Komisi Kelaikan Etik Penelitian dan Kesehatan (KKEPK) Faculty of Dental Medicine, Universitas Airlangga. C. ternatea was used as the test material and was obtained from the Center for Research and Development of Medicinal Plants and Traditional Medicines in Sidoarjo, East Java.

The extraction process of C. ternatea flower was carried out at Widya Mandala Catholic University by drying the plucked C. ternatea flower in an oven at 40 °C and obtaining dried C. ternatea flower simplicia. C. ternatea flower simplicia (3.226 g) was grinded with a dry blender to obtain a powder, which was then dissolved in 500 mL of 80% ethanol in a glass beaker. Extraction was carried out by emitting ultrasonic waves on a mixture of C. ternatea flowers and solvent with an ultrasonic power output of 60% for 15 minutes. The immersion depth of the ultrasonic probe is 2 cm with 5 repetitions. The extraction results were filtered using Whatman No.40 filter paper (8 micrometers) or a Buchner funnel with the help of

a vacuum filter. The filtrate from the extraction was then concentrated or reduced in water content using a rotary evaporator at a temperature of 40°C. The extraction results are stored in a container lined with aluminum foil to prevent degradation prior to testing[20,21].

Phytochemical tests of CTEE were carried out at the Widya Mandala Catholic University, Surabaya, East Java, Indonesia, by providing certain reagents to see the presence of bioactive compounds in the CTEE. Forty mg of the CTEE was added to 100 ml of hot water, boiled for 5 minutes, and then filtered. Five ml of filtrate were prepared, and then 0.05 mg of magnesium powder and 1 ml of concentrated HCl were added, and shaken. The + (positive) result is indicated by a change that occurs in the solution to a red, yellow, or orange color[22]. CTEE was dissolved in 1% HCl, and the solution was divided into four different tubes. Tube 1 was added with 0.5 mL of dilute acid as a comparison; tube 2 was given 3 drops of Dragendorff's reagent, tube 3 was given 3 drops of Mayer's reagent; and tube 4 was given 3 drops of Wagner's reagent. The + (positive) reaction was indicated by the presence of an orange precipitate in tube 2, a yellowish white precipitate in tube 3, and a brown precipitate in tube 4[21].

Forty mg of CTEE was given in 10 ml of water and shaken for 60 seconds. I added two drops of 1% HCL. If the foam that has been formed remains in a stable condition for 7 minutes, then the extract shows a positive result (+) containing saponins. A hundred mg of CTEE was weighed and dissolved in 10 ml of water. Take 2 ml of dissolved CTEE and add 3 drops of concentrated HCl and 1 drop of concentrated H_2SO_4. The + (positive) result is indicated by the formation of purple and red colors. Forty mg of CTEE was dissolved in 4 ml of water, and 2 ml of the dissolved extract was taken, and then 1 ml of 10% FeCl3 was added. The reaction (+) is indicated by the formation of a greenish-black color or a dark blue color. Fifty mg of CTEE were given in 10 mL of water, heated for 5 minutes, and then filtered. A total of 3 mL of the solution was put in 2 test tubes. Tube 1 was used as a blank, and Tube 2 was given a few drops of NaOH solution. The + (positive) reaction is indicated by the formation of a red solution in the tube. One mL of CTEE was given 0.5 mL of anhydrous acetic acid and then dripped with 2 mL of concentrated H_2SO_4through the tube wall. The + (positive) reaction is indicated by the appearance of a bluish-green color[21].

A total of 10 mg of 2-diphenyl-1-picrylhydrazyl (DPPH) powder was dissolved in methanol p.a. in a 100-ml volumetric flask, so that a concentration of 100 g/ml was obtained. 35 ml of the 100-ppm solution was then put in a 100-ml volumetric flask, and methanol p.a. was added to the mixture. The DPPH solution with a concentration of 35 g/ml was pipetted in several parts and then put in a cuvette. Observe using a UV-Vis spectrophotometer with a wavelength of 600 nm[21].

A total of 2.5 ml of CTEE with concentrations of 20, 40, 60, 80, 100, and 120 ppm was pipetted and put in each test tube, and then a solution of DPPH at 35 g/ml was added at 2.5 mL in each tube reaction. The solutions were shaken until homogeneous and incubated for 30 minutes. Then the absorbance was measured using a UV-Vis spectrophotometer[22]. Determination of the EC_{50} value and the creation of a calibration curve. The absorbance results that have been obtained from each concentration can be used to calculate the percentage attenuation value with the formula:

$$\%\ \text{attenuation} = \frac{DPPH\ absorbance\text{-}\ sampel\ absorbance}{DPPH\ absorbance}\ x\ 100\%$$

The results of % attenuation at each concentration are then made into a regression curve, and the equation y = bx + a is obtained, where the extract concentration in ppm is the x axis (abscissa) and the % attenuation value is the y axis (ordinate). Then the EC_{50} value is calculated. The value of EC_{50} is a parameter used to determine the effective concentration to inhibit or reduce 50% of free radicals. Based on the linear regression equation, the lower EC_{50} value has higher antioxidant activity[22,23].

Before conducting the research, the tools used were sterilized by autoclaving at 121°C for 15 minutes at a pressure of 1.5 atm. The next step is to make bacterial samples. Aa, Pg, Fn, and Pi were cultured in Brain Heart Infusion Broth (BHIB) media. BHIB media consisted of a mixture of 3,8 grams of Brain Heart Infusion Broth Powder with 100 mL of sterile distilled water in an Erlenmeyer tube. Bacterial cultures of Aa, Pg, Fn, and Pi were carried out by inoculating 1 ose of pure culture of these bacteria into BHIB media and incubating for 24 hours at

37°C[24].

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were performed using the microdilution method. First, prepare the bacteria Aa, Pg, Fn, and Pi by suspending each bacterium in BHIB media with a standard of 0.5 McFarland ($1.5x10^8$ CFU/ml). CTEE preparations that have been made were serially diluted with concentrations of 100%, 75%, 50%, 25%, and 12.5% as the treatment group. In the treatment group, 0.01 ml of the tested bacterial suspension according to the 0.5 McFarland standard was put in a test tube containing BHIB media and CTEE at various concentrations. In addition, positive and negative controls were also made. The positive control contained BHIB media, test bacteria, and doxycycline as a reference drug, as much as 30mg, while the negative control contained BHIB media, aquadest, and bacteria without the CTEE. Then all tubes were incubated at 37°C for 24 hours. In the test tube that has the lowest concentration of CTEE and does not show any turbidity, the MIC of CTEE produces an inhibitory effect on the test bacteria.

A total of 0.1 mL of samples of Aa, Pg, Fn, and Pi in tubes were planted by scratching with the spread plate method on the surface of the medium in the form of nutrient agar and then incubated for 48 hours at 37°C. After incubation, the number of colonies can be counted by making a grid line on a petri dish, using a colony counter, and expressing it in CFU/ml. Counting the number of tested bacterial colonies can also be done using the turbidity method with a spectrophotometer at a wavelength of 600 nm. This method works by measuring the absorbance of the standard McFarland 0.5 solution and the test bacterial suspension, which is then used to obtain the density value of bacterial cells in culture. If no bacterial colony growth is found on the agar medium, the MBC or the lowest concentration of CTEE that produces a bactericidal effect on the test bacteria[25].

Processing and analyzing data using the statistical package for social science (SPSS) version 21 for Windows (IBM Corporation, Chicago, US) The data obtained from the research were statistically analyzed, namely the normality test using the *Shapiro Wilk* test. The normality test is conducted to determine the shape of the data distribution, whether it is in the form of a normal distribution or not, which will be used as one of the basic assumptions in the accuracy of the selection of statistical tests to be used. The homogeneity test was then carried out using *Levene's test* to determine whether the data distribution group had homogeneous variants, in the sense that the data taken came from populations with the same variance. After getting the results of a normal and homogeneous distribution of the bacteria being tested, a parametric test can be performed using the *one-way* analysis of variance (ANOVA) test. After testing with *one-way ANOVA*, it is necessary to do a Tukey honest significant difference (HSD) post-hoc analysis, which will be carried out to find out which groups are different and not different.

This study used CTEE-containing chemical compounds consisting of anthocyanin and ternatin, and information on the canonical 3D and SMILE structures of these two compounds was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The target proteins identified in this study were Tumor Necrosis factor-α (TNF-α), Nuclear Factor Kappa Beta (NF-κB), receptor activator nuclear kappa (RANK) and its ligand (RANKL), interleukin-6 (IL-6), osteoprotegrin (OPG), osteocalcin, nuclear factor associated T-cells 1 (NFATc1), tartate resistant acid phosphatase (TRAP), peptidoglycan, flagellin, dectin, heat shock protein-10 (HSP-10), and -70 (HSP-70). The RCSB PDB database (https://www.rcsb.org/) was used to gather structural information in 3D, visualization technique, PDB ID, resolution (), weight (kDa), sequence length (mer), and chain.

The Swiss ADME server (http://www.swissadme.ch/) and ProTox-II (https://tox-new.charite.de/protox_II/) were used to predict the absorption, distribution, metabolism, excretion, and toxicity (ADMET) of anthocyanins and ternatin compounds. In general, physicochemical characteristics, water solubility, and drug similarity are utilized to forecast the ability of query compounds to be good drug molecule candidates, and the degree of toxicity is assumed to be class IV or V.[26,27].

The query chemical activity of *C. ternatea* flower was predicted to interact to all target proteins in this investigation using molecular docking simulations. According to the research objectives, molecular docking may be used to identify the kind of activity of a ligand and the pattern of molecular interactions when it binds to the target protein. The PyRx 0.9.9

version software was used in this investigation to determine the binding ability of the C. ternatea floral compound to fifteen target proteins. The Discovery Studio 2016 version of the program was used to identify molecular interactions arising from the docking of the *C. ternatea* flower chemical with the target in this work. Docked molecular complexes have chemical bond interactions such as Van der Waals, hydrogen, hydrophobic, electrostatic, and pi. The established contact is a weak bond that plays a role in activating the target protein[28]. The 3D structure of the *C. ternatea* flower and fifteen target proteins were shown as clear surfaces, cartoons, and sticks and underwent color selection using PyMol 2.5 version software. The software uses Python programming and is used for structural selection or coloring of docked molecular complexes[29].

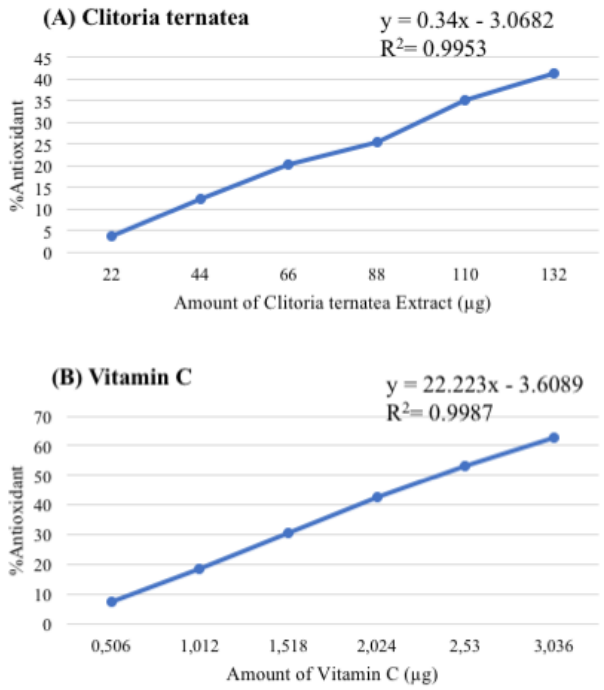

Figure 1. The result of antioxidant analysis on (A) CTEE compared to vitamin C (B) showed that CTEE possessed lower antioxidant ability compared to vitamin C.

Results

The CTEE contains quite a lot of active compounds and was positive for flavonoids, alkaloids, saponins, terpenoids, tannins, quinones, and steroids Antioxidant tests were carried out to determine the antioxidant compounds contained in CTEE. The antioxidant activity of CTEE was tested using a UV-Vis spectrophotometer with a wavelength of 600 nm. The amount of antioxidant activity is indicated by the EC50 value. Based on the relationship curve between the number of samples of CTEE and the percentage of antioxidants, the EC50 value is 156.0605 mg/mL. The curve of the relationship between the number of samples of *C*TEE and the percentage of antioxidants can be seen in Figure 1 (A). As a comparison, the antioxidant activity of vitamin C was also tested, and the EC_{50} value of vitamin C is 2.4123 mg/mL. The curve of the relationship between the number of vitamin C samples and the percentage of antioxidants can be seen in Figure 1 (B).

In this study, subcultures were carried out on nutrient agar media to see the number of colonies that formed. Figure 2 shows that the growth of bacterial colonies was not found at a concentration of 50% CTEE. The following are the results of the average antibacterial ability of CTEE against periodontopathogenic bacteria, namely Aa, Pi, Fn, and Pg, based on several different treatment groups **(**Figures 3A and 3B).

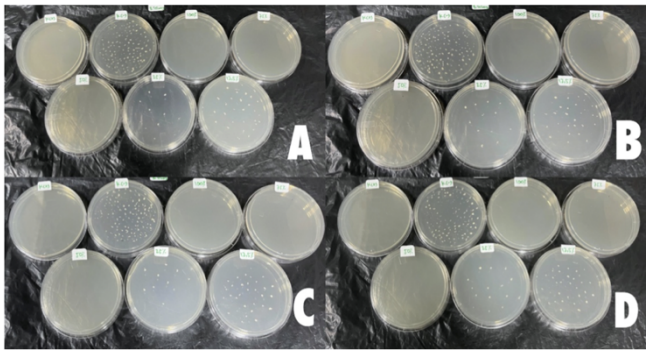

Figure 2. The antibacterial activity of CTEE toward bacteria colony of(A) Aa; (B) Pg; (C) Fn; (D) Pi bacterias in 100%, 75%, 50%, 25%, 12.5%. K group: without CTEE and K+: doxycycline as reference drug.

Statistical tests that have been carried out on the CTEE showed that the normality test for colony count data and spectrophotometer data in all treatment groups with concentrations of *C. ternatea* extract of 100%, 75%, 50%, 25%, 12.5%, positive control, and negative control on Aa, Pi, Fn, and Pg had a *p* value > 0.05, so it can be concluded that the research data are normally distributed. The homogeneity test that has been carried out shows that the results of the homogeneity test for colony count data in all

treatment groups with concentrations of CTEE were 100%, 75%, 50%, 25%, 12.5%, positive control, and negative control on Aa, Pi, Fn, and Pg with p values 0.05. It can be concluded that the research data has a variance that is not homogeneous. The homogeneity test that has been carried out shows that the results of the homogeneity test for spectrophotometer data in all treatment groups with concentrations of CTEE were 100%, 75%, 50%, 25%, and 12.5%, and positive control and negative control on Aa, Pi, and Fn had p>0.05, so it can be concluded that the research data has homogeneous variance. The results of the homogeneity test on *Pg* bacteria have a *p*<0.05, which means it can be concluded that the spectrophotometer data has an inhomogeneous variance.

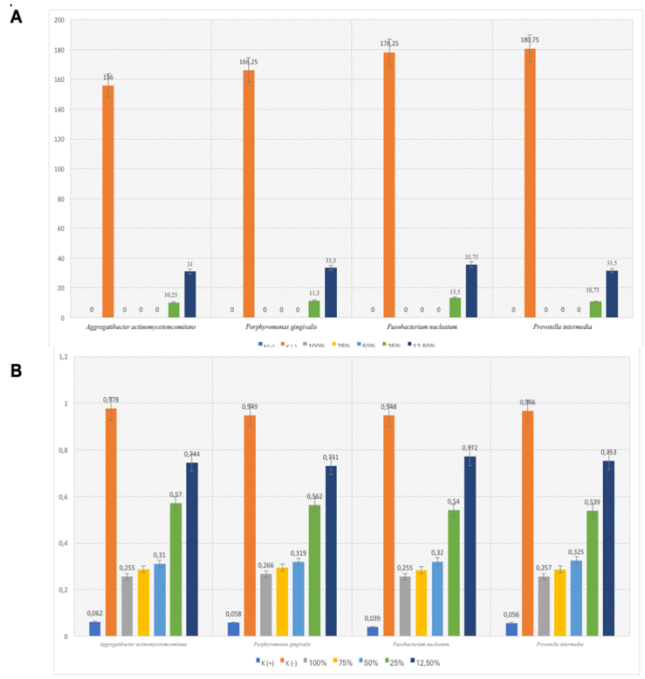

Figure 3. (A) The MBC of CTEE toward (A) Aa; (B) Pg; (C) Fn; (D) Pi bacterias in 100%, 75%, 50%, 25%, 12.5%. K group: without CTEE and K+: doxycycline as reference drug. (B) The MIC of CTEE toward (A) Aa; (B) Pg; (C) Fn; (D) Pi bacterias in 100%, 75%, 50%, 25%, 12.5%. K group: without CTEE and K+: doxycycline as reference drug.

The results of the normality test showed that the data on colony count in each concentration of CTEE were normally distributed in all test bacteria. However, all bacteria did not meet the assumption of homogeneity. Even so, the parametric test of the colony count data on all bacteria will still be analyzed using *one-way ANOVA* with the *post-hoc* test using *Dunnett's T3*. A one-way *ANOVA* statistical test was carried out to determine whether there were differences in colony count in each treatment group in the concentration of *C. ternatea* extract on the test bacteria. At a significant level of 5%, if the *p* > 0.05, it means that there is a significant difference between the concentration groups; on the contrary, if the *p* value is > 0.05, there is no significant difference between the concentration groups.

The result of the *one-way ANOVA* test comparing the colony count in each concentration group of CTEE. With the results of the *one-way ANOVA* of colony count data on the Aa, Pi, Fn, and Pg given the concentrations of CTEE at 100%, 75%, 50%, 25%, 12.5%, positive control, and negative control having a *p* <0.05, it can be concluded that there are significant differences in the number of colonies in each concentration group. Due to the significant difference, the *post hoc* test was then carried out using *Dunnett's T3* to determine the difference between each treatment group. Based on the *post-hoc* test, it can be concluded that the treatment that gave the best results in reducing the number of colonies on the bacteria Aa, Pi, Fn, and Pg was the concentration of CTEE at 100%, 75%, or 50% (Table 1).

The results of the normality test showed that the spectrophotometer data for each concentration of all bacteria were normally distributed. However, not all bacteria meet the assumption of homogeneity. For bacteria that meet the assumption of homogeneity, namely Aa, Pi, and Fn, the spectrophotometer parametric test data will be analyzed using one-way ANOVA with a post-hoc test using Tukey's. Pg do not meet the assumption of homogeneity, but the parametric test will still be analyzed using one-way ANOVA with a post-hoc test using Dunnett's T3. At a significant level of 5%, if the p value <0.05, it means that there is a significant difference between the concentration groups; on the contrary, if the p > 0.05, there is no significant difference between the concentration groups.

The result of the comparison of the spectrophotometer one-way ANOVA test for each concentration group of CTEEE (Table 1) The results of the one-way ANOVA test of spectrophotometer data on Aa, Pi, Fn, and Pg Given that the concentrations of CTEE at 100%,

75%, 50%, 25%, and 12.5%, positive control and negative control had *p* values <0.05, it can be concluded that there are significant spectrophotometer differences in each concentration group. Due to the significant difference, a *post hoc* test was then carried out to determine the difference between each treatment group. Bacteria with homogeneous variance used *Tukey's* test, while bacteria that were not homogeneous used *Dunnett's T3* test. Based on the results of the *post-hoc* test, it can be concluded that the treatment that gave the best results in reducing the number of colonies on the spectrophotometer data of the Aa, Pg, Pi, and Fn was the concentration of 100% *CTEE*. In Pi, the best concentrations of *CTEE* extract were 100% and 75%, with the lowest spectrophotometer results.

MBC of Periodontopathogenic bacteria	***P value***
A. actinomycetemcomitans	0.0001*
P. gingivalis	0.0001*
F. nucleatum	0.0001*
P. intermedia	0.0001*
MIC of Periodontopathogenic bacteria	***P value***
A. actinomycetemcomitans[a]	0,000*
P. gingivalis	0,000*
F. nucleatum	0,000*
P. intermedia	0,000*

Table 1. Colony Count *One-Way ANOVA* Test Results. (*) Significantly different at 5% significant level ($p < 0.05$).

Based on the ADMET prediction results of anthocyanins and Ternatin compounds, *C. ternatea* content can function as a good drug molecule because it meets several drug likeness parameters, and both compounds can be soluble, allowing them to pass through the selectively permeable cell layer if they have a target in the cytoplasmic environment. The toxicity level of class 5 implies that the toxicity level of the two compounds is low or non-toxic, allowing *C. ternatea* to operate as a suitable therapeutic molecule (Table 2).

Computer simulation of CTEE's molecular interaction with the target protein The RCSB PDB database (https://www.rcsb.org/) was used to acquire fifteen target proteins to be monitored (Table 3). According to the docking data, anthocyanin has a higher negative binding affinity value than ternatin (Table 4). Table 5 displays the 3D molecule complex obtained from docking simulation results. In this work, docked molecular complexes were seen using PyMol 2.5 software with structural selection and staining. Figures 4 and 5 illustrate a 2D depiction of the position and kinds of chemical bond interactions in the ligand-protein complex.

Compounds	**Physicochemical Properties**	**Water Solubility**	**Druglikeness**	**Toxicity**
Anthocyanin	Formula: $C_{15}H_{11}O$ Weight: 207.25 g/mol Heavy atoms: 16 Arom. heavy atoms: 16 Fraction Csp3: 0.00 Rotatable bonds: 1 H-bond acceptors: 1 H-bond donors: 0 Molar Refractivity: 66.06 TPSA: 13.14 Å²	Log S (ESOL): -4.01 Class: Moderately soluble Log S (Ali): -3.47 Class: Soluble Log S (SILICOS-IT): -5.32 Class: Moderately soluble	Lipinski: Yes Ghose: Yes Veber: Yes Egan: Yes Muegge: No Bioavailability: 0.55	Predicted LD50: 2500mg/kg Similarity: 71.67% Predicted Toxicity Class: 5 (Low Toxic)
Ternatin	Formula: $C_{19}H_{18}O_8$ Weight: 374.34 g/mol Heavy atoms: 27 Arom. heavy atoms: 16 Fraction Csp3: 0.21 Rotatable bonds: 5 H-bond acceptors: 8 H-bond donors: 2 Molar Refractivity: 97.93 TPSA: 107.59 Å²	Log S (ESOL): -4.24 Class: Moderately soluble Log S (Ali): -5.05 Class: Moderately soluble Log S (SILICOS-IT): -5.43 Class: Moderately soluble	Lipinski: Yes Ghose: Yes Veber: Yes Egan: Yes Muegge: Yes Bioavailability: 0.55	Predicted LD50: 5000mg/kg Similarity: 95.31% Predicted Toxicity Class: 5 (Low Toxic)

Table 2. ADMET analysis of Anthocyanin and Ternatin.

No	**Name**	**Visualization Method**	**Resolution (Å)**	**PDB ID**	**Weight (kDa)**	**Chain**	**Sequence Length (mer)**
1	TNF-α	X-RAY DIFFRACTION	2.60	1TNF	52.11	A/B/C	157
2	NF-κB	NMR	-	2DBF	10.62	A	100
3	RANKL-RANK	X-RAY DIFFRACTION	2.70	3URF	38.38	A	162
4	IL-6	NMR	-	1IL6	21.01	A	185
5	IL-10	X-RAY DIFFRACTION	2.00	1INR	18.67	A	160
6	RUNX2	X-RAY DIFFRACTION	4.25	6VGE	62.53	D	117
7	RANKL-OPG	X-RAY DIFFRACTION	2.70	3URF	38.38	A	162
8	Osteocalcin	X-RAY DIFFRACTION	2.00	1Q8H	5.85	A	49
9	NFATC1	NMR	-	1A66	27.33	A	178
10	TRAP	X-RAY DIFFRACTION	2.22	1WAR	35.48	A	310
11	Peptidoglycan	X-RAY DIFFRACTION	2.10	2OQO	23.77	A	200
12	Flagellin	X-RAY DIFFRACTION	2.00	2ZBI	60.96	A/B	292
13	Dectin	X-RAY DIFFRACTION	2.80	2CL8	32.92	A/B	139
14	Hsp70	X-RAY DIFFRACTION	1.84	1S3X	42.75	A	382
15	Hsp10	NMR	3.82	6MRD	27.33	C	178

Table 3. Protein sample preparation from database.

Protein	Autogrid						Binding Affinity (kcal/mol)	
	Center (Å)			Dimensions (Å)			Anthocyanin	Ternatin
	X	Y	Z	X	Y	Z		
TNF-α	27.916	29.166	32.098	125.319	112.104	88.609	-7.4	-6.8
NF-κB	22.627	28.275	34.222	103.045	113.495	101.619	-6.0	-5.9
RANKL-RANK	30.910	28.556	33.392	92.113	116.115	83.797	-6.6	-5.4
IL-6	7.939	-21.159	9.499	58.092	62.897	43.139	-6.3	-6.0
IL-10	18.002	18.980	5.752	58.882	38.528	85.481	-7.4	-6.2
RUNX2	-57.315	37.846	-14.640	64.939	33.703	47.175	-5.4	-5.1
RANKL-OPG	9.124	-4.025	21.970	95.207	98.636	77.806	-7.1	-6.9
Osteocalcin	9.184	21.566	22.871	47.411	32.476	27.635	-5.8	-5.7
NFATC1	14.193	-7.635	1.970	60.425	53.410	57.713	-8.1	-6.1
TRAP	69.711	-24.322	17.856	38.414	38.191	40.670	-6.6	-6.1
Peptidoglycan	35.826	36.515	21.932	60.634	42.966	45.926	-7.1	-6.6
Flagellin	-18.468	41.964	30.415	143.049	40.586	98.210	-7.0	-6.2
Dectin	45.707	18.305	47.691	55.338	39.093	31.835	-5.9	-5.6
Hsp70	17.579	26.808	23.066	81.800	54.749	57.266	-8.5	-8.0
Hsp10	-32.949	61.226	22.898	107.037	65.608	86.197	-7.6	-7.4

Table 4. The docking results of CTEE active compound to target protein.

Ligan-Protein	Chemical Interaction
Anthocyanin_TNF-α	**Van der Waals:** Trp114, Glu116, Ser99, Pro100, Cys101, Gln102, Glu104 **Pi:** Cys101, Pro100, Gln102
Anthocyanin_NF-κB	**Van der Waals:** His67, Arg59, Arg57, Gly55, Gly68, Gly69 **Pi:** Pro71, Phe56, Leu70, Lys80
Anthocyanin_RANKL-RANK	**Van der Waals:** Ala166, Tyr217, His167 **Pi:** Phe165, Phe311, Lys195
Anthocyanin_IL-6	**Hydrogen:** Gln175 **Van der Waals:** Arg30, Leu178, Ser37, Ile36, Arg40, Asp34 **Pi:** Leu33, Lys171
Anthocyanin_IL-10	**Van der Waals:** Met77 **Pi:** Ala80, Phe37, Val76, Phe30, Leu94, Tyr72, Leu26, Leu98
Anthocyanin_RUNX2	**Hydrogen:** Thr198, Ser196 **Van der Waals:** Asn160, Asp161, Gly159 **Pi:** His214, Lys195
Anthocyanin_RANKL-OPG	**Hydrogen:** Tyr217 **Van der Waals:** Phe165, His167, Tyr215, Tyr307 **Pi:** Phe311
Anthocyanin_Osteocalcin	**Hydrogen:** Cys29 **Van der Waals:** Tyr42, Tyr46, Asn26, Leu25, Asp30 **Pi:** Ala33, Phe38
Anthocyanin_NFATC1	**Van der Waals:** Lys102, His76, Tyr79, Ile123, Ile172, Phe151 **Pi:** Ile65, Ala121, Ile106, Ile118, Leu104, Leu63, Leu149
Anthocyanin_TRAP	**Hydrogen:** Thr52, Thr49 **Van der Waals:** Val21, Gly23, His34, His51 **Pi:** His33, Ile22, Ile55, Ala46
Anthocyanin_Peptidoglycan	**Van der Waals:** Gln61, Asn162, Gln163, Leu122, Leu126 **Pi:** Lys62, Tyr65, Leu159
Anthocyanin_Flagellin	**Van der Waals:** Asn121, Leu120, Asp171, Leu173, Gln113, Asn174, Ala411, Val379, Gly377, Gln176, Thr382, Asn393, Lys396, Asn121 **Pi:** Ala412, Thr117
Anthocyanin_Dectin	**Van der Waals:** His113, Ile152, Leu114, Asn205, Ser89, Ser88, Glu203, Asn118 **Pi:** Val116, Asp153
Anthocyanin_Hsp70	**Van der Waals:** Thr13, Gly12, Thr14, Gly202, Arg272, Thr37, Gly339, Asp366, Gly338, Val337 **Pi:** Asp199, Asp10, Val369, Tyr15
Anthocyanin_Hsp10	**Van der Waals:** Asp96, Asp16, Phe55, Asp57, Thr56, Phe55, Gln66, Pro65, Asp96 **Pi:** Met15, Val63

Table 5. Molecular interaction analysis results between Anthocyanin and target proteins.

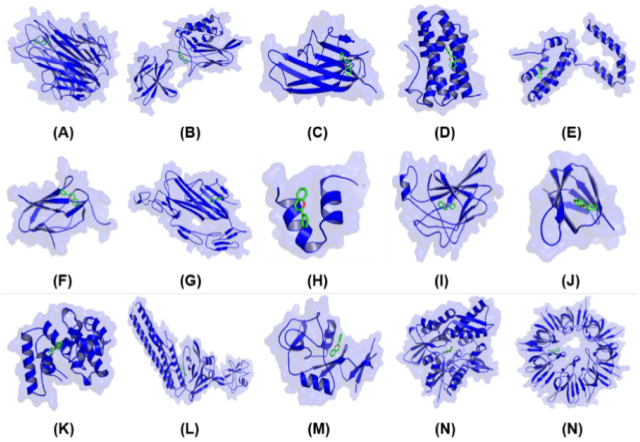

Figure 4. Molecular complex from docking simulation. (A) Anthocyanin_TNF-α; (B) Anthocyanin_NF-κB; (C) Anthocyanin_RANKL-RANK; (D) Anthocyanin_IL-6; (E) Anthocyanin_IL-10; (F) Anthocyanin_RUNX2; (G) Anthocyanin_RANKL-OPG; (H) Anthocyanin_Osteocalcin; (I) Anthocyanin_NFATC1; (J) Anthocyanin_TRAP; (K) Anthocyanin_Peptidoglycan; (L) Anthocyanin_Flagellin; (M) Anthocyanin_Dectin; (N) Anthocyanin_Hsp70; (O) Anthocyanin_Hsp10.

Figure 5. 2D visualization of the position and types of chemical bond interactions in the ligand-protein complex. Molecular complex from docking simulation. (A) Anthocyanin_TNF-α; (B) Anthocyanin_NF-κB; (C) Anthocyanin_RANKL-RANK; (D) Anthocyanin_IL-6; (E) Anthocyanin_IL-10; (F) Anthocyanin_RUNX2; (G) Anthocyanin_RANKL-OPG; (H) Anthocyanin_Osteocalcin; (I) Anthocyanin_NFATC1; (J) Anthocyanin_TRAP; (K) Anthocyanin_Peptidoglycan; (L) Anthocyanin_Flagellin; (M) Anthocyanin_Dectin; (N) Anthocyanin_Hsp70; (O) Anthocyanin_Hsp10.

Discussion

Phytochemical assays in CTEE were performed on each extract, with the protocol and reagents provided based on the content of the active chemicals to be examined. As a positive reaction, the findings of this phytochemical test are characterized by a color shift. The affirmative result in question is a color change after testing for flavonoids, alkaloids, saponins, terpenoids, tannins, quinones, and steroids. CTEE contains a variety of active chemicals, including flavonoids, alkaloids, saponins, terpenoids, tannins, quinones, and steroids. It causes CTEE to have antibacterial, anti-inflammatory, antioxidant, anti-cancer, antipyretic, antidiabetic, analgesic, and many other pharmacological effects[13,31]

C. ternatea is contain flavonoids, anthocyanins, triterpenoids, flavonol glycosides, quercetin glycosides, kaempferol glycosides, myrisetin glycosides, alkaloids, tannins, resins, steroids, saponins, and phenols.[31] *Streptococcus mutans, Staphylococcus aureus*, Pg, Escherichia coli, *Pseudomonas aeruginosa, Candida albicans*, and *Salmonella typhimurium* may all be inhibited by the bioactive chemicals found in *C. ternatea* [32]. The EC_{50} value, which is the effective concentration for blocking or decreasing 50% of the number of free radicals, indicates the level of antioxidant activity. Using a linear regression equation, the EC_{50} value may be calculated from the curve showing the connection between the number of samples and the percentage of antioxidants. According to the linear regression equation, the lower the EC_{50} value, the greater the antioxidant activity[22]. Based on the findings of CTEE and Vitamin C antioxidant activity tests, the EC_{50} value of *C. ternatea* extract was 156.0605 g/mL and the EC_{50} value of Vitamin C was 2.4123 g/mL. When compared to CTEE, vitamin C had a lower EC_{50} value. This shows that vitamin C has a higher antioxidant activity than *C. ternatea* extract. Vitamin C has very significant antioxidant activity since it is a reference medicine when compared to CTEE, which includes many other active chemicals that might interact with antioxidant activity[31]. According to other research, the CTEE has antioxidant qualities by counteracting free radical's due to the inclusion of active substances such as flavonoids. CTEE's antibacterial activity was evaluated at various concentrations, with two controls serving as controls. The positive control was administered doxycycline (30 g) as a reference medicine since it is a bacteriostatic semi synthetic antibiotic that is a tetracycline derivative with a broad range. Doxycycline is widely used as a treatment for infections caused by gram-positive and gram-negative bacteria[32].

The greatest colony counts were obtained for Aa, PG, Fn, and Pi when CTEE was not administered or used as a negative control. When CTEE concentrations of 50%, 75%, 100%, and a positive control (doxycycline 30 g) were used, the average colony count was lowest in the Aa, PG, Fn, and Pi. At a concentration of 50%, CTEE extract has an antibacterial action, inhibiting the development of periodontopathogenic bacteria. CTEE's active component plays a vital role in preventing bacterial growth. According to the findings of phytochemical testing, the active components found in CTEE extract are flavonoids, alkaloids, saponins, terpenoids, tannins, quinones, and steroids. Flavonoids have an antibacterial effect by limiting bacterial energy metabolism, which is required in the process of generating macromolecules such as DNA, RNA, and protein. According to one study, flavonoids can also impede membrane function and enter the lipid bilayer of bacteria, destroying the outer membrane's barrier function. This leads to membrane fusion and cell leakage[33].

The release of intracellular chemicals caused by bacterial cell membrane breakdown can impair bacterial energy metabolism. Bacterial macromolecular production requires energy; therefore, inhibiting metabolism can prevent bacterial molecules from developing and induce bacterial cell death. Antibacterial alkaloid chemicals can induce cell death by interfering with the peptidoglycan constituent components of bacterial cells, resulting in the lack of a cell wall layer. Quinones, as antibacterial agents, generate irreversible complex compounds with nucleophilic amino acid residues on transmembrane proteins present in cell wall polypeptides, plasma membranes, and enzymes on cell surfaces, which can disrupt bacterial cell life[34].

Tannins operate as antibacterial agents by blocking reverse transcriptase and DNA topoisomerase enzymes and inactivating enzymes and the activity of genetic material. This causes bacterial cell wall construction to be less than complete, resulting in bacterial lysis and cell

death. Saponin chemicals work as antibacterial agents by generating protein and enzyme leakage from the bacterial cell. Saponin damages the permeability of cell membranes and diffuses through the outer membrane and cell wall before binding to the cytoplasmic membrane and causing the cytoplasm to leak out of the cell because the cell membrane's stability is disrupted, resulting in bacterial cell death. Steroids that are also antimicrobial can cause lysosomal leakage and interact with cell phospholipid membranes that are permeable to lipophilic substances, reducing membrane integrity and changing cell membrane shape, resulting in cell fragility and lysis[35]. The statistical tests used to analyze the research data included the one-way ANOVA test[36]. In this work, a one-way ANOVA statistical test was used to assess whether there were differences in the number of colonies in each CTEE treatment group on the Aa, PG, Fn, and Pi. Normality and homogeneity tests must be performed before the one-way ANOVA test. The normality test was performed to assess whether or not the study data originated from a population with a normal distribution. The homogeneity test was used to examine if the data distribution group had homogenous variations, which means that the data were collected from populations with similar variance.

The binding mechanism of anthocyanins and ternatin with proteins was predicted using molecular docking simulations; this work employed PyRx 0.9.9 docking software. The simulation tries to evaluate a ligand's level of binding capacity in the protein domain based on the value of binding affinity in a stable ligand-protein complex and create negative energy[37].The grid in the docking simulation helps guide the ligand's binding to the target protein[38]. The capacity of a drug to bind to a receptor is measured by binding affinity. A lower binding affinity number indicates a stronger affinity between the receptor and the ligand. In contrast, higher binding affinity values result in lower receptor affinity[39]. The findings of molecular docking simulations demonstrate that anthocyanins have more activity than ternatin. This is evaluated using the binding affinity value created when it binds to all target proteins, and the results show that anthocyanin has a larger negative binding affinity value than ternatin. Anthocyanins have antibacterial potential by inhibiting peptidoglycan, flagellin, and dectin activity, and they can also be antioxidants by upregulating Hsp70 and Hsp10. The activity of anthocyanin compounds contained in *C. ternatea* allows it to be anti-inflammatory through inhibition of regulation or decrease in the activity of pro-inflammatory proteins such as TNF-α, NF-κB, RANKL-RANK, and IL-6, which can trigger upregulation of anti-inflammatory proteins such as IL-10. Anthocyanin activity can also increase osteoblast and osteoclast activity through RUNX2, RANKL-OPG, osteocalcin, NFATC1, and TRAP. The identification of molecular interactions and binding locations on the docked protein-ligand complex revealed that anthocyanin binding to all target proteins resulted in non-covalent bond interactions, including Van der Waals, pi, and hydrogen. Overall, weak binding interactions can lead to the creation of persistent ligand-protein complexes and induce activity responses such as enhancement and inhibition of target proteins. Hydrogen bonds, hydrophobicity, Van der Waals interactions, and pi all play a part in the creation of unique biological activities by interacting with weak bonds in the docking complex[38,40].

Conclusions

In conclusion, CTEE has antibacterial activity against Aa, Pg, Pi, and Fn at 50% concentration and potential antioxidant ability in vitro. CTEE's anthocyanin has anti-inflammatory, antimicrobial, and antioxidant activities, as documented in silico.

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Declaration of Interest

The authors have no conflicts of interest regarding this investigation.

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