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Original Article

In Vitro Antibacterial, Anticoagulant, and Antioxidant Screening of Aqueous Extracts of Blue Ternate (*Clitoria ternatea* L.) Flower

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Abstract

Background and Aim: Extracts of *Clitoria ternatea* flowers obtained through laboratory standard methods have been constantly reported to have various medicinal activities. However, no reports on such properties are available for this flower's extracts obtained through common domestic extraction procedures. This study aimed to determine and compare the aqueous extracts (leachate, decoction, tea, and homogenate) of *C. ternatea* flowers for their antibacterial, anticoagulant, and antioxidant activities.

Materials and Methods: Agar well diffusion and Lee-White test tube method were used for antibacterial and anticoagulant assays, respectively. Moreover, enzymatic and non-enzymatic antioxidant assays of the extracts were conducted through the floating disc assay, and DPPH scavenging activity, respectively.

Results: The results of the present study revealed that although the extracts exhibited antibacterial activities against *Salmonella enterica* and *Staphylococcus aureus*, they did not differ significantly at $p>0.05$. The extracts did not exhibit *in vitro* anticoagulant activity. Only the homogenate preparations showed catalase activity while all the extracts showed DPPH scavenging activity with the flower decoction as the highest % DPPH RSA harmonic mean of 56.24%, followed by the leachate (45.03%), and the tea (38.79%) and homogenate (37.74%) as the least.

Conclusion: *C. ternatea* flowers extracted through common domestic extraction methods were revealed to have antibacterial, procoagulant, and antioxidant properties. The extracts could also be subjected to phytochemical screening and could be studied for their other bioactive properties.

Keywords: Antibacterial agents, Anticoagulants, Antioxidants, Decoction, Homogenate, Leachate and Tea, Flower extracts

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Introduction

There has never been a greater need to screen medicinal plants for their effectiveness against various diseases. Common domestic preparations from medicinal plants are being exploited for their health benefits. One of the commonly used medicinal plants is the *Clitoria ternatea* Linn (1). The *C. ternatea* is a climbing legume, 90 to 160 cm long, having five leaflets and deep blue flowers. They are solitary and

have light yellow markings, adapting to a wide range of soil pH (5.5-8.9) and soil type, and surviving in both rainy and drought seasons. It is propagated through seeds that are normally sown from the beginning until the middle of the wet season. The plant is distributed in Asia, including the Philippines where it is commonly called Pukinggan (2). Meanwhile, the locals of Leyte call it the blue ternate. The phytochemical constituents of *C. ternatea* flowers include saponin, tannin, alkaloids, glycosides, phytosterols, and carbohydrates (3). Glycosides are phenolic compounds combined with sugar. The prominent phenolics in plants are the anthocyanins, pigments responsible for the colors red, blue, and purple that attracts pollinators (4). The medicinal effects of this plant include antimicrobial, anti-parasitic and insecticidal, anti-inflammatory and antipyretic, anticancer, antioxidant, antidiabetic, central nervous effects (anti-amnesic, antidepressant, and anxiolytic), antiulcer, hypo-lipidemic, antihistaminic and anti-asthmatic, immunomodulatory, diuretic and urolithiasis effect, wound healing effect, and hepatoprotective activity (5).

Blue ternate flowers are used in several preparations with testimonials claiming for its medicinal effect. Due to its antioxidants, consumption of blue tea is believed to help the body fight against toxic free-radicals (6). As a colorant in the famous blue rice, consumers believe that they can harness medicinal benefits of the *C. ternatea* flower namely, antioxidant, anti-inflammatory, antidiabetic and antimicrobial properties (7). Health benefits are also believed to be harnessed from the *C. ternatea* flowers when incorporated in smoothies. However, before this study, no scientific investigations had been conducted to confirm these claims. Neither the phytochemical nor the biological aspects of the aqueous extracts from the *C. ternatea* flowers have been previously investigated. While most of the studies involve crude methanolic and/or ethanolic extracts, this study focused on aqueous extractions through leachate, decoction, tea, and homogenate preparations of *C. ternatea* flowers because these are the most commonly used preparations of the folks for the blue ternate flowers. Specifically, this study aimed to determine and compare the antibacterial, anticoagulant, and antioxidant activities of *C. ternatea* flower aqueous extracts.

Materials and Methods

Flower Collection & Preparation

C. ternatea flowers were collected from Brgy. Dist. III, Babatngon, Leyte, Philippines. During the collection, the individual flowers were selected and destemmed. After collection, the flowers were washed with sterile distilled water. The flowers used for leachate and homogenate preparations were stored in sterile plastic bags at 4ºC before the experiments, which was not more than 3 days after harvest. On the other hand, the flowers for decoction were dried in an oven at 70ºC for forty-eight hours (8) while the flowers for tea preparation were only air-dried (9). The identity of the plant specimen was verified by Dr. Beatriz Belonias, a plant systematic biologist at the Department of Biological Sciences, Visayas State University.

Preparation of Flower Aqueous Extracts

The extracts were prepared following the established ratio of Patil *et al*. (10), 5 g fresh weight of *C. ternatea* flowers in 50 mL sterile distilled water. For leachate preparation, fresh flowers were chopped into approximately 5 mm slices and soaked in sterile distilled water at ambient room temperature (20.2-28.7C) for 24 h and were filtered using sterile cheesecloth.

For the flower decoction, the oven-dried *C. ternatea* flowers were ground and added with sterile distilled water afterwards. It was boiled for 5 min and left to stand for 20 min to cool and was filtered (8). To obtain the tea, boiling sterile distilled water was added to the pounded dried flowers placed in a strainer for 10 min, and then filtered. Finally, homogenate was prepared following the method described by Pereira and Brazon (11) with slight modification. *C. ternatea* flowers were immersed in sterile distilled water and homogenized in a blender for 3 min, and then filtered. All the extracts were stored in an Erlenmeyer's flask at 4 ºC in the dark. Each preparation was replicated three times.

Antibacterial Assay

Salmonella enterica (Gram negative) and *Staphylococcus aureus* (Positive +) from the culture collection of the College of Veterinary Medicine of Visayas State University were used as test organisms for the assay. The antibacterial activity of the *C.*

ternatea aqueous extracts was evaluated using the agar well diffusion method of Clause (12) with slight modifications. Ciprofloxacin, a broad-spectrum antibiotic, was used as the positive control while sterile distilled water served as the negative control.

Prior to the assay, liquid cultures of *S. enterica* and *S. aureus* were prepared. A 0.5 McFarland Standard was used to standardize the approximate number of bacteria in the suspension. When the concentrations of the test organisms were standardized, sterile Petri plates prepared with hardened nutrient agar were inoculated with the bacteria through swabbing using sterile cotton. Preparation of 7 mm sterile "wells" on the agar was then accomplished. Three hundred (300) µL of each aqueous extract and sterile distilled water (negative control) was delivered to their designated wells. Positive control, Ciprofloxacin (5µg), was incorporated in a paper disc and was hence applied in this manner. The plates were incubated at 37ºC without inverting. After 24 hours of incubation, the Zone of Inhibition was measured to quantify the antibacterial efficacy of the treatments.

Anticoagulant Assay

The anticoagulant activities of *C. ternatea* aqueous extracts were evaluated using the Lee-White test tube method as described by Ochei and Kolhatkar (13) on human blood type O+. The blood coming from three donors served as replicates. Three sub-replications were also employed. The donors underwent blood typing prior to the extraction. The donors were not undergoing any medication prior to the extraction. The extraction of blood from the antecubital veins through venipuncture was done by a registered medical technologist. A total of 54 mL of blood was extracted from each donor. Three (3) mL of this was used for each sub-replicate. For the positive control, CPDA-1, a known in vitro blood preservative, was used.

Enzymatic Antioxidant Assay

The catalase activities of the aqueous extracts were determined using the floating disc method with slight modifications (14). A sterile filter paper disc (Whatman no. 1) previously soaked in the flower extract was gently blot dry on a paper towel. It was then submerged to the bottom of the 3% hydrogen peroxide solution in the test tube. The disc was released and the time it took for the disc to float to the surface of the hydrogen peroxide solution was recorded as the catalase activity in mm (length of the H_2O_2 solution in the tube = distance traveled by the disc) per second. The procedure was repeated for all the test tubes with the different aqueous extracts. Sterile distilled water served as the negative control. The catalase, if present, will react with the hydrogen peroxide solution breaking it down into water and oxygen. The oxygen will accumulate on the surface of the disc, making it buoyant and float to the surface of the hydrogen peroxide solution.

Non-Enzymatic Antioxidant Assay

DPPH (2,2-Diphenyl-1-picrylhydrazyl) scavenging activities of the extracts were determined using the method developed by Brand-Williams *et al*. (15) with slight modifications. The stock solution of DPPH (30mg/300mL) was prepared using 95% ethanol as solvent, and the initial absorbance was read in a UV spectrophotometer at 517nm. To start the reaction, 3.9 mL of 0.01% DPPH solution was added to 0.1 ml of the extracts. It was then shaken for 15 minutes and left to stand at room temperature in the dark for 60 minutes to give enough time for the reaction of the cellular antioxidants with DPPH. Afterwards, the absorbance was read at 517nm using 95% ethanol as blank. Ascorbic acid was used as the positive control at decreasing concentrations (0.05%, 0.04%, 0.03%, 0.02%, and 0.01%) with 10% (5g/50mL) stock solution while sterile distilled water served as the negative control. Three replicates per concentration (100%, 80%, 60%, 40%, and 20%) of the aqueous extract (leachate, decoction, tea, homogenized) were employed. Calculation of the scavenging activity was carried out as the percent inhibition of DPPH radical scavenging activity using a standard formula as follows. The data were shown as the IC50 calculated from the point of 50% inhibition against the concentration plot.

$$\% \text{ DPPH Scavenged} = (A_{cont}-A_{test})/A_{cont} \times 100$$

A_{cont} = absorbance of the negative control (sterile distilled water) and

A_{test} = absorbance in the presence of the sample of the extracts

Experimental Design and Statistical Analysis

The experiments were all carried out after a randomized design (CRD). The statistical significances among the

averages of the replicates were assessed using two-way analysis of variance (ANOVA) for antibacterial and antioxidant assays, and one-way analysis of variance (ANOVA) for anticoagulation assay, followed by Tukey's HSD-test. All the values were shown as mean SD, and the values with $p<0.05$ were regarded highly significant. A statistician was consulted to ensure the precise analysis of the data. SPSS version 23.0 was utilized.

Results and Discussion

Antibacterial Activities of *C. ternatea* Aqueous Extracts

The antibacterial assay of the crude aqueous extracts of *C. ternatea* flowers exhibited antibacterial activity against *S. enterica* and *S. aureus* (Fig.1). To further compare the antibacterial activities of the extracts, the means of their zones of inhibition were obtained and statistically analyzed (Table 1). The analysis revealed that the antibacterial activities of the extracts were not noticeably distinct from each other and were significantly lower than the commercial antibiotic Ciprofloxacin. These results can be attributed to the fact that the preparations made from the flowers were micro-extraction methods. A study conducted by Indrianingsih *et al*., (16) indicated that dichloromethane and ethyl acetate soluble fractions of C. ternatea flower has higher minimum inhibitory and bactericidal concentrations than its aqueous extract. Moreover, the crude extracts possibly contain other compounds that could have interfered with the active antibacterial compounds.

Another factor that might have contributed to the differences in the results is the fact that the treatments were delivered through the agar well using a 1 mL syringe, while the positive control was delivered through a disk placed on the surface of the hardened

Table 1: Comparison of the antibacterial activities (ZOI±SD) of the *C. ternatea* flower aqueous extracts.

Treatment	Average Zone of Inhibition (mm) ±SD	
	***S. enterica* (G-)**	***S. aureus* (G+)**
Ciprofloxacin (+C)	24.50 ±2.78[a]	22.83 ±3.33[a]
Leachate	6.67 ±3.79[b]	3.50 ±3.50[b]
Decoction	3.34 ±3.06[b]	5.00 ±4.44[b]
Tea	9.84 ±6.71[b]	1.16 ±2.02[b]
Homogenate	3.67 ±6.35[b]	2.83 ±4.91[b]
Sterile Distilled Water (-C)	-	-

+C = Positive Control, -C = Negative Control, means followed by the same letters are not significantly different at 5% level

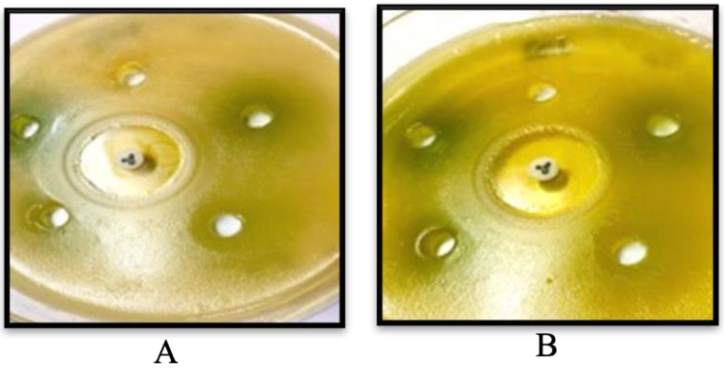

Figure 1. Representative plates showing growth inhibition of aqueous extract from tea on (A) Salmonella enterica and (B) Staphylococcus aureus.

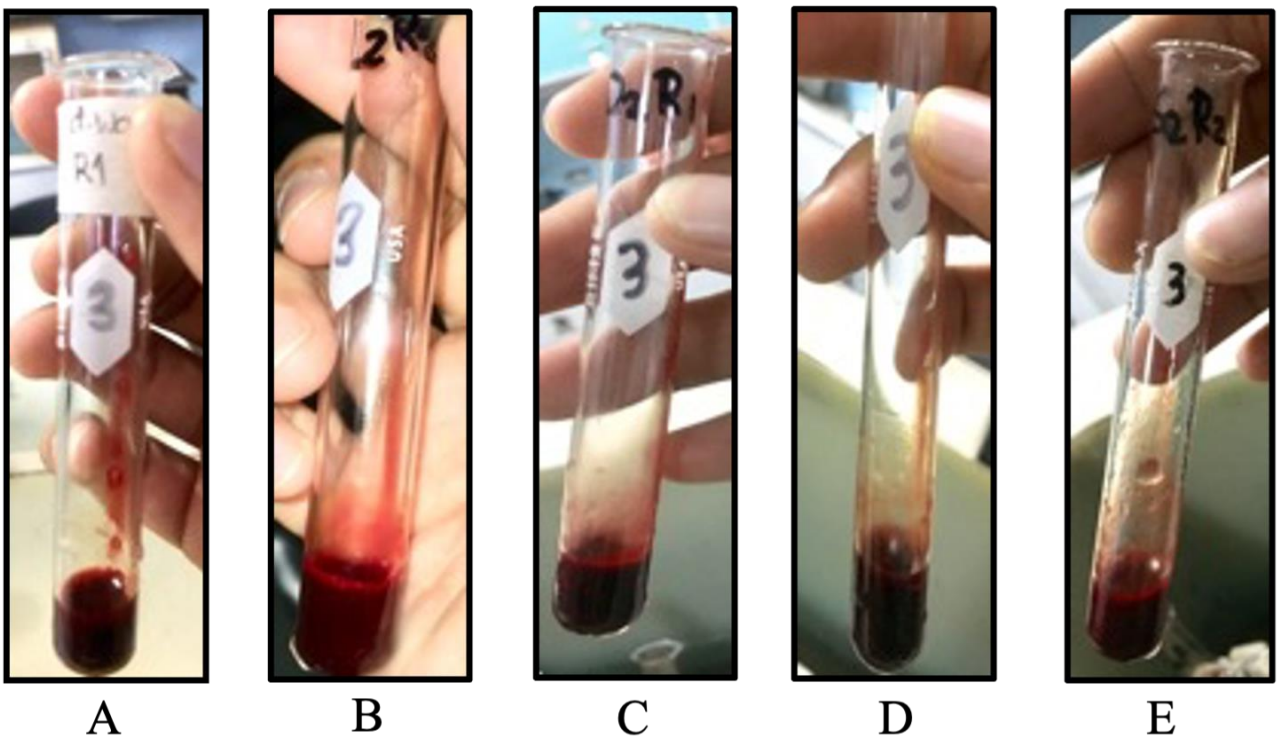

Figure 2. Human blood type O+ exposed to (A) sterile distilled water, (B) leachate, (C) decoction, (D) tea and (E) homogenate preparations of C. ternatea flowers.

Table 2: Coagulation times (mean ±SD) of human blood type O^+ exposed to different aqueous extracts of *C. ternatea* flowers.

Treatments	Coagulation Time (min)
CPDA-1 (+C)	No Coagulation
Sterile distilled water (-C)	5.06 ± 0.36^{ns}
Leachate	5.69 ± 0.97^{ns}
Decoction	5.01 ± 0.64^{ns}
Tea	5.55 ± 0.88^{ns}
Homogenate	5.44 ± 0.37^{ns}

-C = Negative Control, +C = Positive Control, ns = not significantly different at $\alpha = 0.05$

agar. The extracts were absorbed by the agar, but since the inoculation was done by swabbing the bacterial culture on the surface of the agar, the extracts did not show large zones of inhibition. The extracts might have shown larger zones of inhibition if they were also delivered through the paper disc, or if the bacterial culture was incorporated in the agar.

The antibacterial activities of the aqueous extracts of the *C. ternatea* can be due to the presence of flavonoids as previously reported by Manjula *et al.* (17) when they screened the methanolic extracts of *C. ternatea* obtained through Soxhlet-extraction. The potential broad spectrum of antibacterial activity of the *C. ternatea* aqueous extracts agrees with the reported results of Dzoyem *et al.* (18) when they screened acetone leaf extracts of nine Fabaceae tree species wherein all the leaf extracts showed inhibitory activity

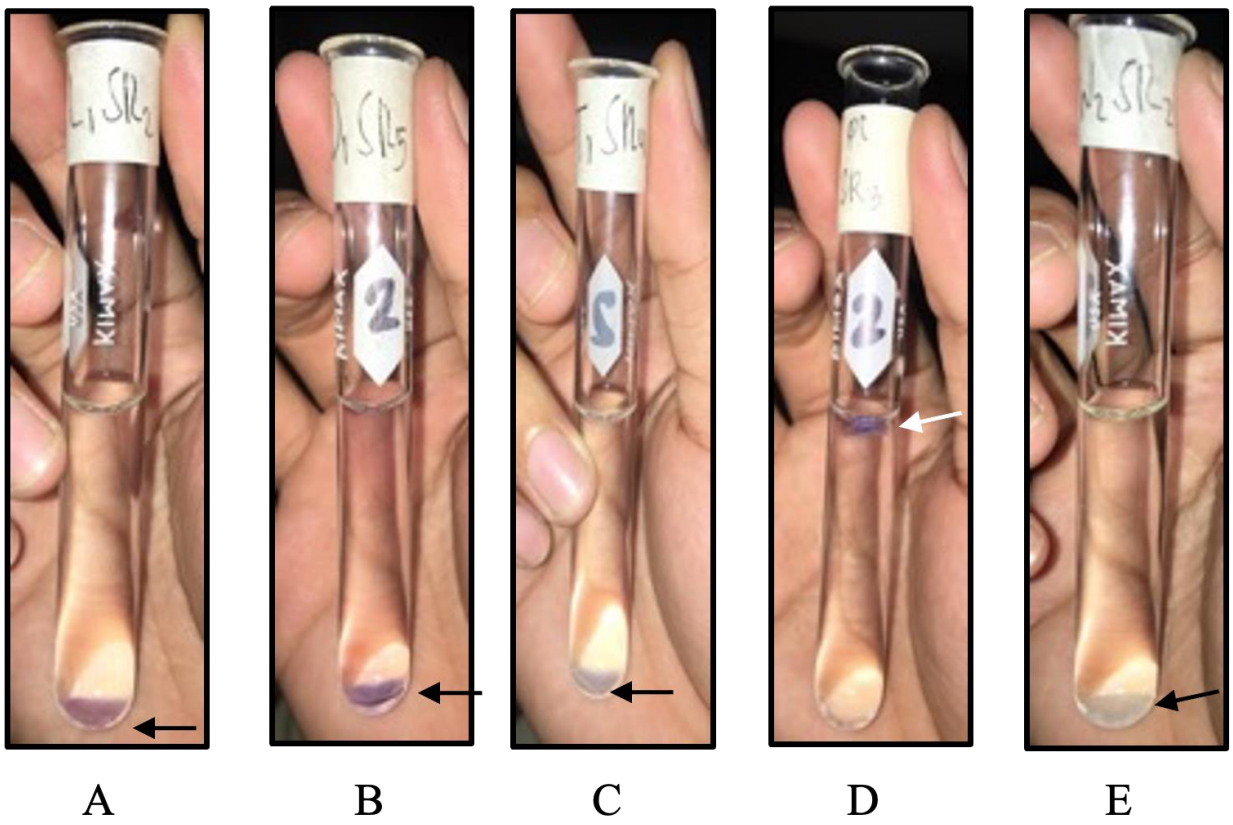

Figure 3. *C. ternatea* flower aqueous extracts (A) leachate, (B) decoction, (C) tea, (D) homogenate tested for their catalase activities through floating disc assay on 5 mL 3% hydrogen peroxide, having (E) sterile distilled water as the negative control. Floating disc (white arrow) means positive catalase activity, while the discs that stayed at the bottom of the tube (black arrow) indicates absence of catalase activity.

against all tested bacterial strains (*S. aureus, E. faecalis, B. cereus, E. coli, P. aeruginosa, S. typhimurium*).

It was an advantage to use the wells in this setup because the extracts were obtained from different preparations that did not involve the use of a rotary evaporator to obtain powder for dissolution. Using paper discs to deliver the aqueous extracts means that we need an accurate concentration of the extracts that will be absorbed by the disc. Also, the only available broad-spectrum antibiotic during the conduct of the experiment was the Ciprofloxacin in the disc form. The assay proceeded with the use of the Ciprofloxacin discs as a positive control because the procurement of a commercial antibiotic drug requires a medical prescription. The results, however, showed that even though there was an inconsistency in the delivery of the treatments, the extracts did not fail to show their potential antibacterial activity against the examined microorganisms. Hence, the researcher still believes that the results are something worth informing the public about. Furthermore, this study is focused only on the "screening" of aqueous extracts for potential bioactivities as reported by previous studies that involve the use of other solvents aside from sterile distilled water. The researcher mainly wants to know if there is or there is not any antibacterial activity, in this case, of the aqueous extract obtained from the household preparations. Since the positive control used in experimentation was delivered through a paper disc on the surface of the agar, it is recommended to use the disc diffusion method to avoid the differences in the delivery of the treatments.

Anticoagulant Activities of *C. ternatea* Aqueous Extracts

Figure 2 presents the results of the anticoagulant

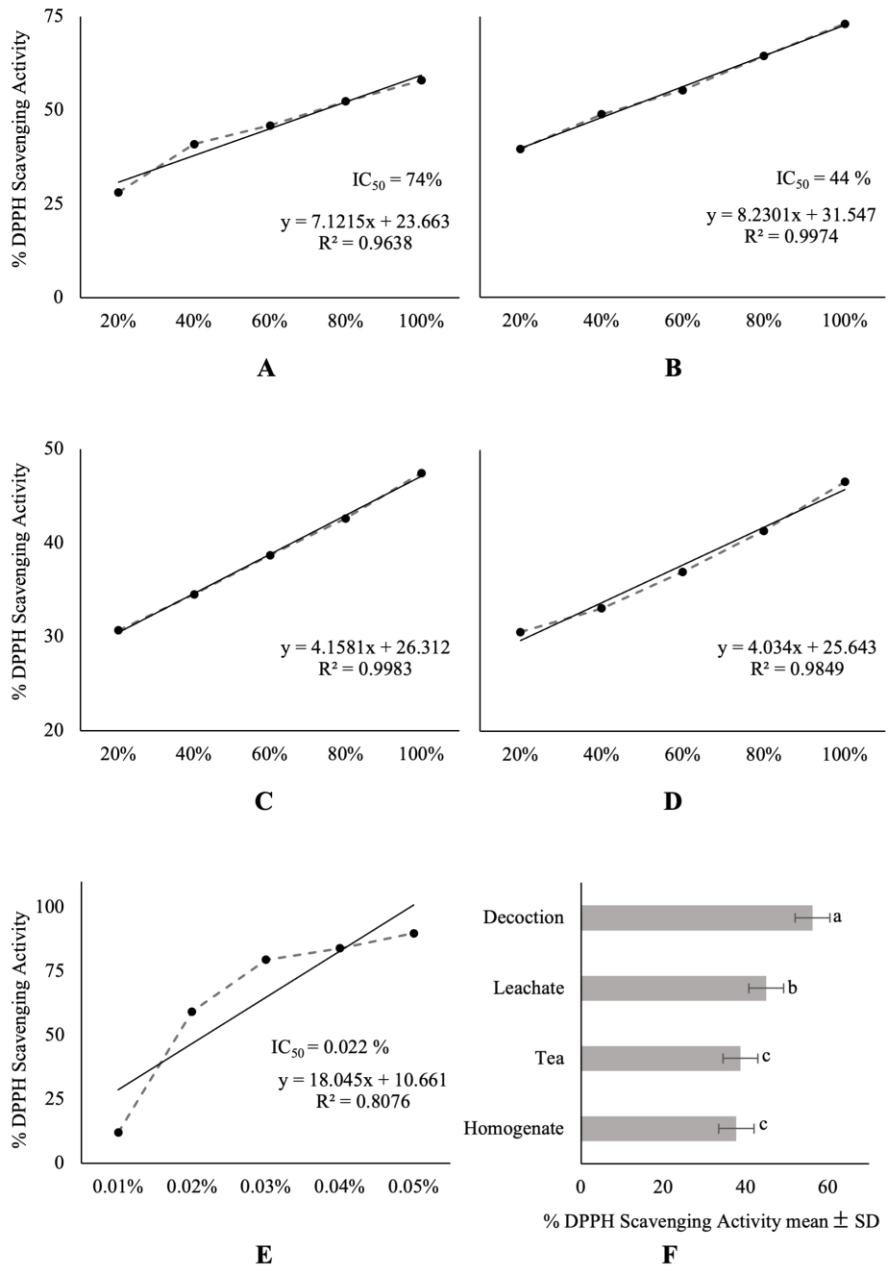

Figure 4. DPPH scavenging activity of the varying concentrations of (A) leachate, (B) decoction, (C) tea, (D) homogenate and (E) ascorbic acid, and the (F) comparison of their harmonic means. Treatments with the same letters are not significantly different at α = 0.05.

screening of *C. ternatea* aqueous extracts on type O^+ blood samples. No significant differences were observed between the average coagulation times of blood exposed to different crude aqueous extracts of the *C.* ternatea flower. On the other hand, no coagulation was observed in the blood exposed to CPDA-1, a known blood preservative, even in varying concentrations (100%, 50%, 25%). These results indicate that *C. ternatea* flower aqueous extracts have no anticoagulant properties (Table 2). Exposing the blood to different extracts made the blood coagulate faster, though. According to Sutton (19), the normal coagulation time of the whole blood is around 8 – 9 minutes, almost the same as the average coagulation time of blood samples without any treatments at 7.56 ±0.25 during the trial experiments. However, this

potential pro-coagulation activity cannot be attributed to the compounds in the extracts because the blood exposed to sterile distilled water also coagulated faster. The non-occurrence of the anticoagulation activity of the aqueous extracts in this experiment correlates with the results of the phytochemical analysis of *C. ternatea* methanolic extracts by Manjula *et al.* (17) that detected the presence of tannins in the flowers. Tannins have an astringent property which may lead to faster healing of wounds and inflamed mucous membranes (20).

A similar wound-healing effect, comparable to cotrimoxazole ointment, was observed by Al-snafi (5) when he tested *C. ternatea* seed and root extracts in rats via topical administration. He found out through HPLC analysis that *C. ternatea* leaf acetone extract and ethyl acetate fraction are enriched with taraxerol 5.32% w/w and 4.55% w/w, respectively. He attributed this compound to the wound healing capacity of *C. ternatea*.

Enzymatic Antioxidant Activity of *C. ternatea* Aqueous Extracts

Floating disc assay revealed that catalase is only present in the homogenized *C. ternatea* flowers (Fig. 3). Catalase is responsible for reducing hydrogen peroxide into water and oxygen. It detoxifies free radicals before they react with lipids, proteins, and nucleic acids.

The presence of enzymatic antioxidants in the homogenate compared with the leachate might be due to the use of a blender, which agrees with the reported results of McMillan *et al.* (21) in terms of cell breakage. Since one of the factors of extraction is the particle size of plant tissues, the use of a blender in preparing the homogenate agrees with the basic principle of extraction– to grind the plant material (wet in this case) so that the surface area for extraction will be increased, and consequently raising the rate of extraction as well (22). On the other hand, the introduction of heat during the extraction process in decoction and tea might have affected the presence of catalase in the final extracts. Since catalase is an enzyme, it can become denatured or inactivated when exposed to high temperatures (23).

In 2001, Jiang and Huang (24) observed a decrease in antioxidant enzyme activities in sods of tall fescue (*Festuca arundinacea* L.) and Kentucky bluegrass (*Poa pratensis* L.) exposed to drought and heat stress. The catalase activity in both species declined continuously during the entire experimental period in all stress treatments.

Non-Enzymatic Antioxidant Activities of *C. ternatea* Aqueous Extracts

Results of the DPPH scavenging assay revealed that all the *C. ternatea* aqueous extracts exhibited increasing antioxidant activity with increasing concentrations. The same was true with the ascorbic acid. As shown in Figure 4, *C. ternatea* flower decoction has the highest antioxidant activity, scavenging 50% of the DPPH solution at only 44% concentration, while leachate preparations did the same at 74% concentration. IC_{50} values were not computed from tea and homogenate preparations since both were not able to scavenge more than 50% of the DPPH solution even at 100 % concentrations. The antioxidant activity can only be attributed to the aqueous extracts because the negative control, containing the solvent used for the aqueous extracts, did not show any antioxidant activity to the DPPH solution. However, the antioxidant activities of *C. ternatea* flower aqueous extracts were lower compared to that of the ascorbic acid (IC_{50}=0.022%) that served as the positive control.

Since the antioxidant activities of *C. ternatea* flower aqueous extracts were not properly compared based on their IC_{50} values, the harmonized means of all the concentrations of each extract were statistically analyzed. Analysis of variance (ANOVA) followed by post-hoc comparison of means through Tukey's HSD confirmed the previous analysis that *C. ternatea* flower decoction has the highest non-enzymatic antioxidant activity at 56.24%, followed by the flower's leachate extracts at 45.03%, and the tea and homogenate as the least at 38.79% and 37.74%, respectively. A more detailed investigation was conducted by Indrianingsih *et al*. (16). Their results of the DPPH radical scavenging activities of different extracts of C. ternatea revealed that their $IC5_0$ is at >800 g/ml. This is significantly lower than the extracts of *Theobroma cacao* pod husk and *Annona muricata* leaves.

The non-enzymatic antioxidant activity of the aqueous extracts of the *C. ternatea* flower can be due to high amounts of anthocyanins, as reported in the evaluation by Vankar and Srivastava (25). It can be noted also that decoction, which used a blender to produce powder

from oven-dried flowers and boiled to reduce the volume of solvent during extraction, obtained the highest antioxidant activity, followed by leachate, which involved soaking chopped flowers in the water for 24 hours.

The two extracts that showed significant antioxidant activity were obtained from different preparations. Following the established decoction method, the flowers were exposed to heat prior (oven-drying) and during (boiling) the extraction process. However, heat cannot be attributed as the main factor for this difference because phenolic compounds are relatively stable at high temperatures (60ºC, 80ºC, and 100ºC) for up to 4 hours (26). Furthermore, the aqueous extract obtained from tea was also exposed to heat during the infusion process.

The increase in surface area and cell breakage cannot be attributed to the results for the leachate because the homogenate was also subjected to mechanical damage to increase the surface area for extraction and maximum breakage of cells by using a blender.

Nonetheless, the results agree with the findings of Rabeta and Nabil (27). This was correlated to the high amount of phenolic compounds found in the aqueous extracts of *C. ternatea* flowers. The hydrogen atoms in phenolic compounds can capture free radicals and convert them into more stable molecules thereby terminating the oxidation process (15). Furthermore, the pigments present in *C. ternatea* flowers are highly stable in aqueous solutions, making them more favorable for coloring food and beverages (28).

Conclusion

The aqueous extracts showed antibacterial inhibition against *S. enterica* and *S. aureus* but were not as potent as the control. The aqueous extracts showed no significant anticoagulant activity when applied to human blood type O^+. Enzymatic antioxidant activity was observed only in homogenized preparation. On the other hand, non-enzymatic antioxidant activity was observed in all the preparations. Decoction showed significant non-enzymatic antioxidant activity at IC50= 74% concentration and leachate showed significant non-enzymatic antioxidant activity at IC50= 44% concentration. However, the harmonic means of tea and homogenized preparations were not significantly different at 5% (THSD). Ascorbic acid, the positive control, used in this assay obtained IC50 = 0.022%. This study showed potential antibacterial, pro-coagulant, and antioxidant activities of the aqueous extracts of *C. ternatea* flowers obtained through micro-extraction procedures. It is highly recommended to slightly upscale the extraction process to obtain aqueous extracts that might exhibit potent biological activities for further investigations such as anti-inflammatory, anti-angiogenic, and phytotoxicity assays, as well as testing the extracts to other bacteria such as *Escherichia coli* and other fungal organisms. It is also recommended to subject the aqueous extracts to phytochemical screening to confirm the presence of compounds that can be attributed to the bioactivities of the flowers. Lastly, it is recommended to screen the white petal variety of the *C. ternatea* for the same potential and to expose the aqueous extracts to other human blood types as well.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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