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Chemical Composition, Antioxidant, Antibacterial, Antibiofilm, and Cytotoxic Activities of Robusta Coffee Extract (*Coffea canephora*)

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ABSTRACT

Coffee extracts are a rich source of potential compounds with numerous biological activities. This study aimed to investigate the potential of chemical compounds derived from robusta coffee extract (*Coffea canephora*) and its dominant compound, caffeine, to scavenge free radicals, inhibit bacterial growth and biofilm formation, and cytotoxic properties in human breast adenocarcinoma cell line. Chemical constituents of coffee extract were analysed quantitively for total phenolic, flavonoid, and alkaloid contents, along with Liquid Chromatography Quadrupole-Mass Spectrometry (LC/MS–MS) analysis. Antioxidant activity was measured by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis; 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) methods. Antibacterial pair with antibiofilm properties against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* were also determined, as well as cytotoxic activity on the MCF7 cell line model. The LC/MS–MS analysis of coffee extracts revealed high levels of caffeine; thus, the caffeine standard is used in all subsequent assays. Notably, robusta coffee extract showed remarkable antioxidant activity and selective inhibition of the growth against gram-positive and negative bacteria, along with the best inhibition of *P. aeruginosa* biofilm. However, compared with the caffeine standard, robusta coffee extract had lower cytotoxic activity and different bacterial targets in antibacterial and antibiofilm properties. Our results indicate that Robusta coffee extract is potentially a functional food due to its high alkaloid, phenolic, and flavonoid content and antioxidant activity, besides being used for natural compounds against bacterial infections.

1. Introduction

Coffee is one of the most widely consumed beverages in the world, with over two billion cups being enjoyed every day. The two common coffee species, *C. canephora* (robusta) and *Coffea arabica* are the most preferred and common coffee types globally. A review of the literature confirms the beneficial effects of coffee on human health (Poole *et al.* 2017). Coffee consumption has been shown to correlate with lower incidence of cardiovascular causes, type 2 diabetes, neurodegenerative diseases, and cancer (i.e., endometrial cancer, melanoma skin cancer, oral cancer, prostate cancer, and liver cancer). The reasons for consuming coffee include improving concentration and cognitive abilities

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and reducing sleepiness and fatigue (Gokcen and Sanlier 2019). Generally, the coffee extract is reported as having numerous biological properties, including antibacterial, antioxidant, and cytotoxic activities. Coffee arabica extract from Ethiopia showed antioxidant and antibacterial activity against some bacterial tested, such as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, and *Salmonella typhimurium* (Tasew *et al.* 2020). In addition, coffee arabica and robusta from Greece exhibited cytotoxic activity on myoblast and endothelial cell lines (Priftis *et al.* 2018). The beneficial effects of coffee extract concerning non-infectious/cell disorders are attributed to coffee's antioxidant properties.

These numerous biological activities of coffee are determined by the presence of the most widely known and scientifically analysed ingredient of coffee, the alkaloid caffeine (Borota *et al.* 2014). Caffeine is an alkaloid, a secondary plant metabolite that stimulates the centers of the nervous system (Fredholm *et al.* 2017). Robusta green beans generally have between 1.2 and 2.4% of alkaloid caffeine. Arabica green beans contain, on average, 0.9 to 1.5% dry weight of caffeine (Olechno *et al.* 2021). Epidemiologic and worldwide studies exhibited that caffeine could reduce the risk of Parkinson's disease, depression, liver fibrosis, cirrhosis, and several types of cancer (e.g., breast and prostate cancer, melanoma skin cancer, and hepatocellular carcinoma) (Bae *et al.* 2014). Following a drug repurposing approach, caffeine was proposed to treat SARS-Cov2 infections and was detected with therapeutic effects on hypertension (Siminea *et al.* 2022; Wu *et al.* 2022). Caffeine has also been reported to have antioxidant and antibacterial activities against some bacterial including *S. aureus, Bacillus subtilis, E. coli*, and *Klebsiella pneumoniae* (Nonthakaew *et al.* 2015; Rathi *et al.* 2022; Yashin *et al.* 2013). However, the caffeine content also depends on the origin, and type of coffee, the place (soil type) of cultivation, environmental conditions, the processing of the beans (cleaning and roasting process), and the time and conditions of storage. The method of its measurement also reported could affect the caffeine content (Olechno *et al.* 2021).

Robusta coffee (*Coffea canephora*) is one of the species of the coffee genus that has long been used in the manufacture of instant and mixed coffee. Lampung province is one of Indonesia's largest robusta coffee centers, with production reaching 118.000 tons in 2021 (BPS 2021). The abundance of robusta coffee in this province can encourage the potential for using and drinking it. This study investigated the chemical composition and determined robusta coffee extract's total phenolic, flavonoid, and alkaloid contents. Since caffeine was identified as the main compound in this coffee extract, we further utilized caffeine standard in all subsequent assays, including to evaluate its activity as antioxidant, antibacterial, antibiofilm, and cytotoxic properties.

2. Materials and Methods

2.1. Sample and Extraction

The Robusta coffee beans used in this study are collected from Mangarai Village, West Lampung, Indonesia (GPS location: 5°06'33.1"S 104°23'21.8" E). The Coffee beans were roasted using drum roasters where the seeds contact a hot surface of which the temperature reaches 180±10°C. Then, the roasted coffee was well-ground in the coffee grinder. The extraction of Coffee beans was carried out maceration method using 99% ethanol and the extraction process using a rotary vacuum evaporator at 60°C. Caffeine standard (anhydrous) was purchased from CSPC Innovation Pharmaceutical CO., Ltd (USA).

2.2. Measurement of Total Phenolic and Flavonoid

Total polyphenol content (TPC) and total flavonoid content (TFC) were determined according to the Folin-Ciocalteu colorimetric method and aluminum chloride colorimetric methods with slight modification (Prastya *et al.* 2019). Briefly, 500 µL of sample solution (1,000 µg/ml) was mixed with 250 µL of the Folin-Ciocalteu reagent and 3.5 ml distilled water before being homogenized and incubated at room temperature for 8 min. Further, 750 µL of 20% sodium bicarbonate solution was added and incubated for 2 hours at room temperature. The absorbance was measured at 765 nm using an ELISA reader Thermo Scientific Varioskan Flash (Thermo Fischer). The TPC was obtained from the calibration curve of gallic acid and expressed as mg gallic acid equivalent (GAE)/mg extract. As for TFC, a 500 µL sample (1,000 µg/ml) was mixed with 2.45 ml of distilled water and 150 µL of 5% $NaNO_2$ and homogenized and incubated at room temperature for 2 minutes. Subsequently, the mixtures were

added with 150 µL of 10% aluminum chloride and homogenized again before being incubated for 8 minutes at room temperature. Soon after that, 2 ml of 1M NaOH was added, and the absorbance of the reaction mixtures was determined at 510 nm. The concentration of total flavonoid content in the test samples was expressed as mg quercetin equivalent (QE)/mg sample.

2.3. Total Alkaloid Analysis

Total alkaloid was performed using a method as described elsewhere with slight modifications (Ajanal *et al.* 2012). Briefly, the coffee extract was prepared by dissolving in ethanol at several concentrations: 3, 9, and 15 µg/ml. As for caffeine standard (Sigma) was diluted in ethanol and set to various concentrations for the standard curve (1, 3, 6, 9, 12, and 15 µg/ml). At the same time, Bromocresol Green (BG) solution was made by heating 7 mg BG with 300 µL of 2N NaOH and 500 µL distilled water, and the solution was diluted to 100 ml of distilled water. Total alkaloid measurement was conducted by mixing 2 ml of extract or standard with 5 ml of BCG and 5 ml of phosphate buffer (pH 4.7) and shaking until wholly diluted. Subsequently, the mixture was extracted with 1, 2, 3, and 4 ml of chloroform by vigorous shaking. The BCG-caffeine complex layer was then collected and measured at 470 nm in Thermo Scientific Varioskan Flash (Thermo Fischer). The standard absorbance was further regressed to obtain a linear regression equation and used to calculate the total alkaloids of the sample. Total alkaloids are represented as mg caffeine equivalents per gram extract.

2.4. Liquid Chromatography Quadrupole-Mass Spectrometry (LC-MS/MS) Analysis

The extract was analyzed using Xevo G2-XS QTof (Quadrupole Time-of-Flight) mass spectrometry instrument (Waters, USA) via an electron spray interface (ESI). Chromatographic separation conditions were conducted using an LC system in the form of Ultra Performance Liquid Chromatography (UPLC)/QTof MS analytical system (Waters). Separation was examined by stepwise gradients from 95% A and 5 B to 5% A and 95% B for 16 minutes (A contained 0.1% formic acid + distilled water; B contained acetonitrile + 0.1% formic acid). Mass spectrometry of each peak was analyzed using the type of electrospray ionization (ES) Xevo G2-S QTof (Waters) with Quadrupole Time-of-Flight mass spectrometry in positive ion mode. The specific mass and fragment ions were identified using the UNIFI software library.

2.5. Antioxidant Activity

The antioxidant activity of the extract was carried out using both radicals of DPPH and ABTS following the method (Prastya *et al.* 2020). In short, 100 µL of sample in different concentrations was mixed with 100 µL of 125 µM DPPH solution and incubated for 30 minutes before observed. As for the ABTS assay, 7 mM ABTS solution was oxidized by 2.45 mM potassium persulfate (1:1) with an incubation period of 12-14 hours in the dark condition at room temperature to produce ABTS radicals before reacting with the extract. Subsequently, 20 µL of the extract was mixed with 180 µL ABTS radical and incubated for 30 minutes. The absorbance of these assays was determined using Thermo Scientific Varioskan Flash (Thermo Fischer) at 515 nm and 734 nm for DPPH and ABTS activity, respectively. The inhibition values were calculated using the formula as follows:

$$\text{DPPH/ABTS inhibiton value (\%)} = \frac{A_1 - A_2}{A_1} \times 100\%$$

A_1 represents the absorbance of DPPH/ABTS blank (without samples), and A_2 = the absorbance of samples. The results are reflected as the inhibitory concentration of 50% (IC_{50}). Ascorbic acid and quercetin were used for the positive control.

2.6. Antibacterial Activity by Disc Diffusion Method

The antibacterial activity of extracts was conducted using the disc diffusion method following Priyanto (2022) and using a Nutrient Agar medium. We used 4 bacterial strains, including *Escherichia coli* (FNCC-0195), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (FNCC-0047), and *Bacillus subtilis* (FNCC-0059), which purchased from the culture collection of Food and Nutrition Study Program, Gadjah Mada University, Indonesia. Shortly, a suspension of bacterial inoculum was adjusted to McFarland standard 0.5 in 0.85% NaCl and introduced to Nutrient Agar (NA) (Himedia) plate medium with sterile cotton buds until evenly distributed and allowed to dry. Subsequently, sterile filter paper discs approximately 6 mm in diameter were impregnated with extracts of 2,000 µg/ml concentration and placed on the surface of the inoculated agar plate. After incubation for 24 h at 37°C, the antibacterial

activity was evaluated by measuring the diameter of inhibition zones for microbial growth surrounding the discs. Based on CLSI, MHA media is the recommended media for testing. However, based on the results of Arora and Kaur (2007), there is no significant difference between MHA and NA media during antimicrobial testing, so that antimicrobial testing can use MHA or NA media. What is essential, on the medium, the test microbes can grow well. All test bacteria can grow well on the NA medium in this study.

2.7. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC value was carried out using a standard micro-dilution assay with slight modifications (Priyanto *et al.* 2022). Briefly, two-fold dilutions of extract (volume of 100 µL) were supplemented to the 96-well sterile microtiter plate, which was previously filled with 100 µL of Mueller Hinton Broth (MHB) medium. Further, the bacterial cultures were set up in 0.85% NaCl and adjusted to McFarland standard 0.5, equivalent to 1 × 10^8 CFU/ml. This particular bacterial suspension was applied to each 96 well with 100 µL, which was the standard dilution to achieve the cell number of 5 × 10^7 CFU/ml and a total treatment volume of 200 µL. This plate was further incubated at 37°C for 24 hours. MIC values were determined to be the lowest extract concentration that could suppress bacterial growth based on a clear medium around colonies of bacteria. MBC value was measured from each well after inoculation on NA medium followed by incubation at 37°C for 24 h. MBC value is the lowest concentration of the extract in which a total (100%) of bacteria tested were killed. Tetracycline (Sigma-Aldrich) was utilized as the positive control.

2.8. Antibiofilm Activity

The extract's potency to prevent bacterial biofilm formation was examined on a 96-well sterile microplate following the method (Priyanto *et al.* 2022). Briefly, the bacterial suspensions (100 µL) were mixed into the 96 wells containing extract at various concentrations (¼ MIC, ½ MIC, MIC, and 2 MIC), and the mixture of Brain Heart Infusion (BHI) medium followed with the incubation for 24 hours at 37°C. Further, the bacteria cells were removed and washed twice with 0.85% NaCl. The wells were further stained with 0.1% crystal violet (200 µL) and followed by incubation for 30 minutes at 37°C. The plate was then rewashed with 0.85% NaCl, and the stained biofilms were solubilized with 99% DMSO (200 µL). The absorbance which reflected the biofilm formation was measured at 595 nm, and the percentage of biofilm formation was then calculated. The untreated bacterial suspension was applied as a negative control.

2.9. Eradication of Cells Biofilm

The capacity of the extract to eliminate the bacterial biofilm was carried out using an MTT assay following the method with some modifications (Priyanto *et al.* 2022). Briefly, 200 µL bacterial suspension in 0.85% NaCl was set to the 0.5 McFarland standard, equivalent to 1 × 10^8 CFU ml^{-1} was grown into a sterile 96-well plate followed by incubation for 24 hours at 37°C for 5 days. For 5 days incubation period, the medium was periodically changed daily with fresh 200 µL BHI liquid medium (enriched with 0.25% glucose). Sample in several concentrations of ½ MIC, MIC, 2 MIC, and 4 MIC diluted with liquid media was added to each well, followed by incubation for 24 hours at 37°C. Subsequently, the medium was removed and supplemented with 10 µL of 5 mg/ml MTT solution (Roche) and incubation for 3 hours at 37°C. The insoluble formazan crystals were diluted with 200 µL of 99% DMSO and absorbance was measured at 595 nm using ELISA Thermo Scientific Varioskan Flash (Thermo Fischer).

2.10. Cytotoxic Properties

In this study, we utilized cancer cell lines, namely MCF7 (human breast adenocarcinoma) (ATCC HTB-22), which was obtained from the Laboratory of Biochemical and Natural Product Developments, Research Centre for Pharmaceutical Ingredients and Traditional Medicine, KST BJ. Habibie, Sarpong (BRIN). MCF7 cells were cultured in Dulbecco's modified eagle medium (DMEM; Sigma) with 5% fetal bovine serum (FBS; Sigma) to maintain the growth conditions. MCF7 cell lines were further treated with 1% penicillin-streptomycin (Sigma) in a humidified atmosphere with 5% CO_2 at 37°C entire the assay. Cell viability was analysed by an MTT colorimetric assay (3- [4, 5-dimethylthiazol-2-2,5 diphenyl tetrazolium bromide assay) (Roche) following the method as described elsewhere with some modifications (Herlina *et al.* 2019). In short, the cells were seeded in 96-well plates at 1 × 10^4 and incubated at 37°C for

24 h until the cells reached more than 90% confluence. Subsequently, the medium was removed, and the cells were treated with fresh medium containing 100 µg/ml of sample diluted on DMSO and incubated for 48 h at 5% CO_2; 37°C. Control cells were supplemented with 0.05% DMSO (v/v) utilized as control treatment. Soon after that, the supernatants were removed, and MTT (0.5 mg/ml) was added to each well following incubation for 3 h. The remaining formazan crystals were further dissolved in 99% DMSO. The absorbance of each well was measured on Thermo Scientific Varioskan Flash (Thermo Fischer) at the wavelength of 570 nm, and percent inhibition was further determined.

2.11. Statistical Analysis

All data are presented as the mean ± standard deviation of at least triplicate repetitions. Further analysis was performed with one-way ANOVA followed by multiple Duncan test ranges. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Total Phenolic, Flavonoid, Alkaloid, and LC-MS/MS Profile of the Coffee Extract

In the preliminary analysis, we demonstrated that coffee extract has a total phenolic, flavonoid, and alkaloid content of 143.84±1.74 mg GAE/g extract, 127.33±3.79 mg QE/g extract, and 356.90±41.51 mg Caffeine equivalent/g extract (Table 1). In addition, LC-MS/MS analysis showed that coffee extract was dominated by at least 5 putatively identified compounds, including trigonelline, 4-O-caffeoylquinic acid, caffeine, erythrocentaurin, and glucopyranosylatractyligenin (Table 2). Interestingly, caffeine is the most dominant compound in this extract. Therefore we utilized the caffeine standard in all subsequent assays to determine whether the main components inside the coffee extract were responsible for the promising activity (Figure 1).

3.2. Antioxidant Activities

To understand the antioxidant properties of coffee extract and caffeine standard, we performed the DPPH scavenging and ABTS radical assays. This activity was performed as an IC_{50} value, indicating that a low value related to the high activity. The results showed that coffee extract has slightly stronger antioxidant activity than caffeine standard with an IC_{50} value of 26.24±1.76 and 43.11±1.93 for DPPH and ABTS radicals, respectively. Even though those activities are still lower than ascorbic acid and quercetin standards as positive controls (Table 1).

3.3. Effect of Coffee Extract and Caffeine on Antibacterial Activity

Based on disc diffusion assay, both coffee extract and caffeine standard showed a broad-spectrum antibacterial activity which was able to inhibit the growth of Gram-positive (*B. subtilis* and *S. aureus*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*). The Clear zone around the paper disc indicated the active constituents from coffee extract or caffeine standard could inhibit the growth of bacteria (Figure 2). Furthermore, MIC and MBC analysis results indicated that caffeine standard has a stronger inhibitory value

Table 1. Total phenol, flavonoid, alkaloid and antioxidant activities of Robusta coffee extract and caffeine

Code	Total phenolic (mg GAE/g extract)	Total flavonoid (mg QE/g extract)	Total alkaloid (mg Caffeine equivalent/g extract)	Antioxidant activities	
				DPPH IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)
Extract	143.84±1.74	127.33±3.79	356.90±41.51	26.24±1.76	43.11±1.93
Caffeine	-	-	-	73.61±2.73	94.52±1.54
Ascorbic acid	-	-	-	5.13±0.73	18.21±1.55
Quercetin	-	-	-	2.91±0.92	7.92±0.96

"- " not determined

Table 2. Putative identified compounds of coffee extract by LC-MS/MS analysis

Compounds identified	Formula	Retention time	Observed m/z	Relative abundance (%)
Trigonelline	$C_7H_7NO_2$	0.51	138.0551	4.93
4-O-caffeoylquinic acid	$C_{16}H_{18}O_9$	2.48	355.1035	3.31
Caffeine	$C_8H_{10}N_4O_2$	2.57	195.0877	72.72
Erythrocentaurin	$C_{10}H_8O_3$	3.01	177.0550	2.33
Glucopyranosylatractyligenin	$C_{25}H_{38}O_9$	3.34	505.2390	1.68
Others	-	-	-	15.00

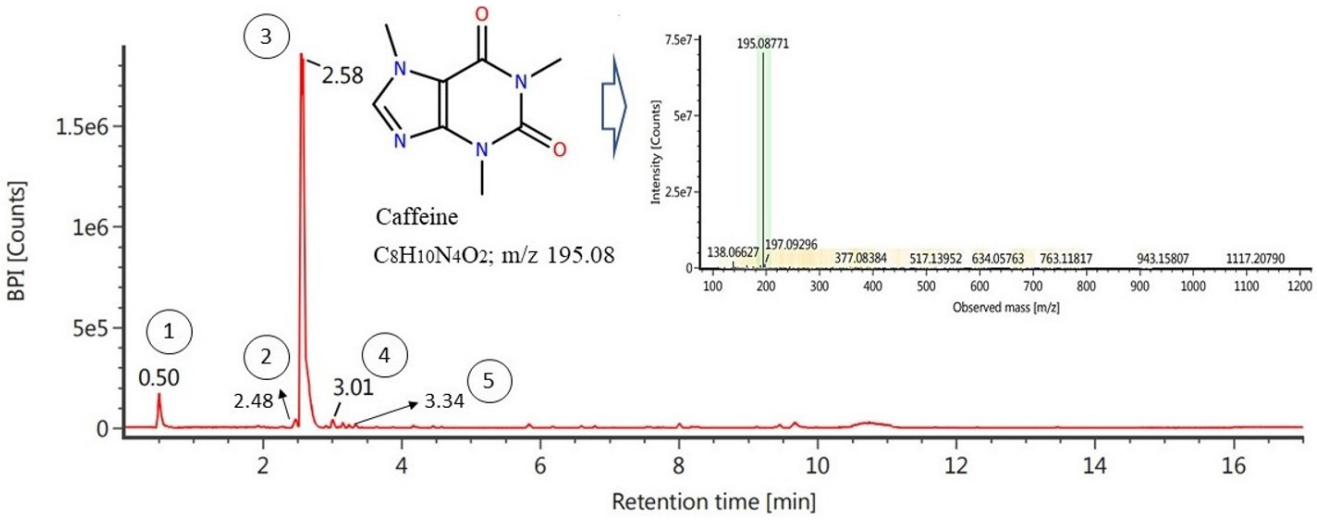

Figure 1. LC-MS/MS profile of coffee extract

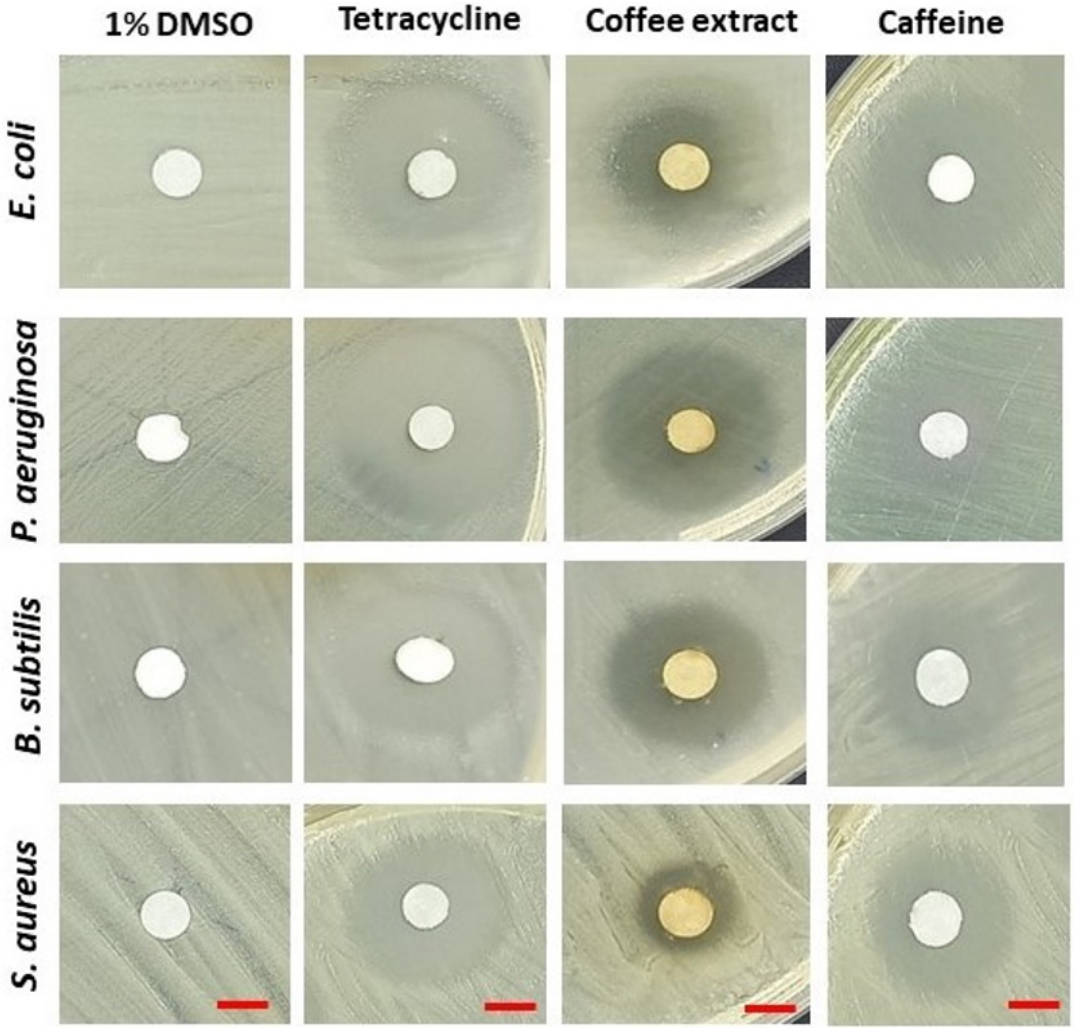

Figure 2. Representation of antibacterial activity via disc diffusion method. Coffee extract and caffeine applied on each concentration of 2,000 µg/ml. 1%DMSO and tetracycline (200 µg/ml) used for negative and positive control, respectively. Bars represent 6 mm

than coffee extract against all bacteria tested. Those results are depicted by the lower MIC value of the caffeine standard with a range of 62.5–125.0 µg/ml against all bacteria tested. For detailed MIC and MBC values of investigated coffee extract and caffeine standard, refer to Table 3.

3.4. Coffee Extract and Caffeine Showed Antibiofilm Properties

The results of antibiofilm activity against bacteria tested are presented in Figure 3. Samples resulting in inhibition of biofilm formation above 50% were considered to have good activity, whereas those with inhibition between 0 and 50% indicated poor activity.

Table 3. MIC and MBC values of Robusta coffee extract and caffeine

Sample	Bacterial tested			
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*
	MIC/MBC values (µg/ml)			
Extract	375/750	187/>375	375/750	375/>750
Caffeine	62.5/125	125/250	125/250	125/125
Tetracycline	7.81/15.62	7.81/15.62	7.81/15.62	7.81/15.62

As for coffee extract, the concentration of 2x MIC value could inhibit the biofilm formation and eradicate cells biofilm at best activity against *P. aeruginosa* biofilm with the inhibition values of 34% and 28%, respectively (Figure 3A and C). Interestingly, caffeine standard (2x MIC value) showed different activity which could inhibit the biofilm formation against both *E. coli* and *S. aureus* with the inhibition value and eradicate cells biofilm activity with the range of 29-34% and 36-39%, respectively (Figure 3B and D). Nevertheless, coffee extract and caffeine standard had lower antibiofilm activity (<50% inhibition).

3.5. Cytotoxic Activity of Coffee Extract and Caffeine

In this study, we applied DMSO 1% as a solvent, proving that this did not affect the MCF7 cell line (Figure 4A and B). On the concentration applied from samples and positive control of 100 µg/ml, cisplatin as positive control had the highest inhibition value (the strongest activity) of 96%. Caffeine standard and coffee extract had 74% and 52% inhibition values, respectively

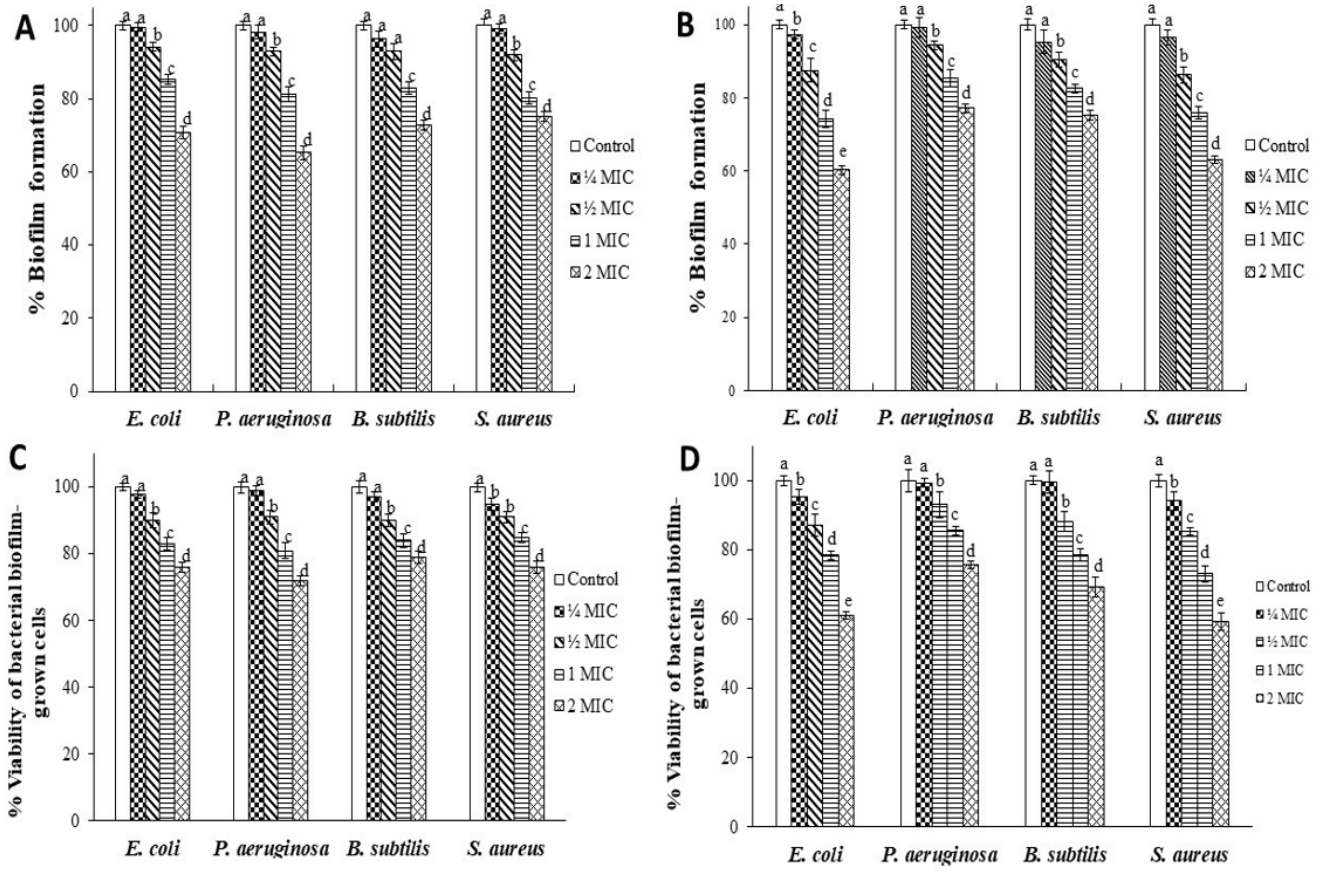

Figure 3. Antibiofilm activity of (A) coffee extract, (B) caffeine and eradication cells biofilm properties of (C) coffee extract, (D) caffeine

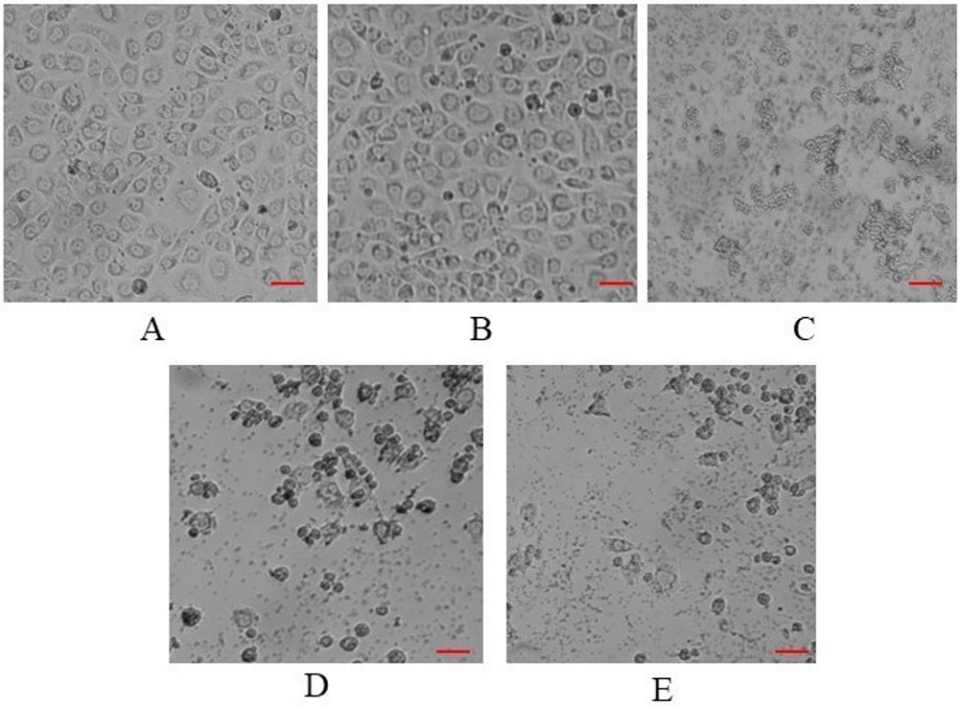

Figure 4. MCF-7 cell line on (A) DMEM medium, treatment with (B) 1% DMSO, (C) Cisplatin (positive control), (D) Coffee extract, (E) Caffeine. C-D were applied on concentration of 100 µg/ml. 1%DMSO and cisplatin used for negative and positive control, respectively. Bars represent 30 µm

(Figure 4C-E). This indicates that the caffeine standard showed stronger cytotoxic activity than coffee extract on the MCF7 cell line model.

4. Discussion

Based on the chemical composition analysis results, it is known that this coffee extract contains high total phenols, flavonoids, and alkaloid contents. The total phenolic content of robusta coffee reached 143.8 mg GAE/g extract (Table 1). This is higher than Panusa *et al.* (2013) and Mussato *et al.* (2011), who exhibited the total phenolic content of spent coffee from 16.0 to 35.5 mg GAE/g extract. Interestingly, the total alkaloid content of this coffee extract is also high, reaching 356.90 mg of caffeine equivalent/gram extract. This result is confirmed by the LC-MS/MS analysis, which showed that caffeine alkaloid is the most dominant compound with an abundance of up to 72%. Caffeine is generally found as a white crystalline anhydrous powder. It is a water-soluble and heat-stable molecule having a melting point of around 235°C. The caffeine molecule contains an imidazole ring and a pyrimidinedione, which contribute to its xanthine core (Choi and Koh 2017). Because caffeine was the predominant compound, we used caffeine standard to compare in all subsequent assays while determining the bioactivity of robusta coffee extract, including antioxidant, antibacterial, antibiofilm, and cytotoxic properties.

Antioxidants are substances that present at mild concentrations, compared to an oxidizable substrate could significantly inhibit the oxidation of those substrates. Antioxidant molecules have played a significant role in removing excess free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Gebeyehu and Bikila 2015). Previous studies demonstrated that consuming vegetables, coffee, tea, and fruits is essential for reducing the risk of diseases caused by free radicals. Thus, coffee as one of them also has provided much attention and several studies have proven that the biologically antioxidant active components of the coffee extract, such as caffeine (alkaloids), chlorogenic acid, feruloylquinic acid, caffeoylquinic acid, and p-coumaroylqunic acid (Duarte *et al.* 2010; Truong *et al.* 2007). Although caffeine is well-known for producing bioactive symptoms that increase alertness and include fatigue reduction, it also has antioxidant activity, as demonstrated by its affinity to scavenge superoxide radicals and hydroxyl radicals and to reduce the activity of ROO− using the ORAC

assay (Liang and Kitts 2014). In our study, we used 2 types of radicals, DPPH and ABTS which measure the ability of samples to stabilize DPPH or ABTS radicals via hydrogen or proton donors, respectively (Belaqziz *et al.* 2017). The results indicated that robusta coffee extract had stronger antioxidant activity on DPPH and ABTS radicals than the caffeine standard (Table 1). Of note, these results are in line with several previous studies that state that caffeine has low antioxidant activity against DPPH and ABTS radicals (Brezova *et al.* 2009; Rivelli *et al.* 2007). Therefore, the strong antioxidant activity of robusta coffee extract might be due to the presence of other compounds, such as phenols or flavonoids (Table 2). Notably, the antioxidant capacity of robusta coffee extract reported in this study is stronger than other coffee extracts such as a green coffee extract from Ethiopia, arabica coffee from Guatemala, and robusta coffee from Vietnam (Ludwig *et al.* 2012; Tasew *et al.* 2020). Therefore, we conducted further analysis to determine the potential bioactivity of this robusta coffee extract, including antibacterial and antibiofilm activities.

The antibacterial test showed that coffee extract and caffeine standard had activity in inhibiting bacterial growth in a different capacity. The coffee extract had stronger activity against *P. aeruginosa* and *B. subtilis*, whereas caffeine had stronger against *E. coli* and *S. aureus* (Figure 2). As for coffee extract, these results are in line with Valenzuela *et al.* (2019) and Pedras *et al.* (2019) described the noteworthy antibacterial activity of the coffee extract against gram-positive and gram-negative bacteria. Nonetheless, caffeine is also consistent with results showing its activity against pathogenic bacteria, including *E. coli, S. aureus*, and *Klebsiella pneumoniae* (Cogo *et al.* 2008; Mohammed and Al-Bayati 2009). The difference between the two results might be due to differences in the mechanism of caffeine and compounds in coffee extracts containing phenol groups. Caffeine is reported to inhibit bacteria by several mechanisms, such as increasing the generation time, changing cell morphology, and affecting DNA synthesis (Sledz *et al.* 2015). On the other hand, the coffee extract also contains phenolic compounds, which are reported to be able to deactivate bacterial cellular enzymes, which depend on the rate of penetration of substances into cells caused by changes in membrane permeability (Akhlaghi *et al.* 2019).

Furthermore, the results of the antibiofilm assay showed that coffee extract could only inhibit the formation of *P. aeruginosa* biofilms. In contrast, according to the results of the antibacterial test, caffeine consistently inhibited *E. coli* and *S. aureus* biofilm formation (Figure 3). Biofilms consist of complex microbial communities that allow them to survive in extreme conditions such as desiccation and nutrient depletion. Biofilm-associated infections such as urethritis, vaginitis, implant-associated infections, and cystic fibrosis are generally chronic treatment is challenging because cells are embedded in a matrix of extracellular polymeric substance (EPS), making them less susceptible to antimicrobials agents, and host immunity (Wu *et al.* 2015). Therefore, compounds that could inhibit biofilm formation have the potential to be developed further. The results obtained from our study show the consistency of antibacterial and antibiofilm activity of caffeine in line with several previous studies. Previous reports have shown that caffeine inhibits the growth of *Enterobacter aerogenes, E. coli, P. aeruginosa*, and *S. aureus* (Al-Janabi 2011; Yssel *et al.* 2017). *E. coli* biofilm formation could also inhibit by caffeine treatment by regulating curli assembly and thus may be used as an alternative therapeutic strategy for treating *E. coli* biofilms-related infections (Rathi *et al.* 2022).

In the following step, coffee extract and caffeine were administered to MCF7 cell lines to test their potential cytotoxic effects using the MTT assay. Interestingly, caffeine was considered more cytotoxic than coffee extract at the same 100 µg/ml concentration, but it had a lower inhibition value than the positive control (Figure 4). However, our study is in line with previous reports, which showed caffeine could inhibit the growth of the human breast cancer lines (MCF7) by inducing apoptosis in a dose dependent-manner (Rosendahl *et al.* 2015). In addition, caffeine derivative compounds also exhibited cytotoxic activity on the MCF7 cell line with an IC_{50} value of 100 µM (Jasiewicz *et al.* 2018). In general, caffeine has been reported to affect some mechanistic cell cycle functions, such as inducing programmed cell death or apoptosis, along with perturbing key regulatory proteins, namely the tumor suppressor protein, p53, which is related to the DNA damage regulation (Bode and Dong 2007).

In conclusion, the results obtained in this study indicate that robusta coffee extracts are rich in alkaloid, phenolic, and flavonoid compounds, along with possessing high *in vitro* antioxidant activity. A selective concentration of coffee extract could inhibit the best activity against some bacterial growth, including *B. subtilis* and *P. aeruginosa*, and inhibition of the *P. aeruginosa* biofilm formation. However, compared with the caffeine standard, the coffee extract had lower activity in cytotoxic against the MCF7 cell line and different capacities in showing antibacterial and antibiofilm, which caffeine exhibited higher activity against *E. coli* and *S. aureus*. To the best of our knowledge, this is the first report on the bioactivity of robusta coffee extract for its antibacterial and antibiofilm properties that may be of great use for developing therapies against common infectious bacterial isolates.

Conflict of Interest

None declare.

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