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Preliminary phytochemical analysis, antibacterial and anti-biofilm activities of *Curcuma zedoaria* (Christm.) Roscoe extracts

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ABSTRACT

Aims: Plant extracts are a rich source of natural compounds that have some degree of antimicrobial efficacy and have less side effects compared to antibiotics. The aim of this research was to screen the phytochemical compounds and investigate the potency of *Curcuma zedoaria* (Christm.) Roscoe rhizome (CZR) extracts to inhibit the growth and biofilm formation of some pathogenic bacteria.
Methodology and results: Antimicrobial and antibiofilm effects of CZR extracts in different solvents were examined by agar well diffusion and the broth microdilution method after phytochemical screening. The 95% ethanolic extract of CZR exhibited broad-spectrum antibacterial properties against Gram-negative and Gram-positive bacteria with inhibition zones of 7.25 ± 0.58-12.00 ± 0.26 mm and MIC values ranging from 50-200 mg/mL. The extract also showed rapid bacteriostatic and bactericidal activities towards *Enterococcus faecalis* DMST 4736 and *Staphylococcus aureus* ATCC 25923 by time-kill assays. Moreover, the 95% ethanolic extracts of CZR also acted as a potent anti-biofilm agent against *E. faecalis* DMST 4736, *S. aureus* ATCC 25923, *S. epidermidis*, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853 and *Proteus mirabilis* DMST 8212 (54.62 ± 0.30-71.25 ± 0.20% inhibition of biofilm formation). The bioactive potency of compounds of the crude 95% ethanolic extract (tannins, flavonoids, cardiac glycosides, steroids, terpenoids and alkaloids) play important roles in the observed antibacterial and anti-biofilm activities.
Conclusion, significance and impact of study: *Curcuma zedoaria* (Christm.) Roscoe extract had broad-spectrum antibacterial activity. The ethanolic CZR extract revealed bacteriostatic and bactericidal capacities, depending on time of exposure and concentration of the extracts. Thus, the present results indicate that *C. zedoaria* (Christm.) Roscoe rhizomes are a potential natural alternative antibacterial agent for preventing bacterial diseases.

Keywords: Phytochemical screening, antibacterial activity, anti-biofilm activity, *Curcuma zedoaria*, time-kill curves

INTRODUCTION

Generally, the treatment of infectious diseases in humans and animals primarily uses antibiotics, but they often have side effects on beneficial microorganisms (normal flora), or the pathogenic bacteria develop resistance if used improperly. Nowadays, human populations around the world have turned to herbal medicines for primary health care. The antimicrobial properties of plants have been investigated in numerous studies around the world. Many studies have examined these as alternative therapies due to their biological activities like antimicrobial, anti-inflammatory, and antioxidant properties (Dosoky and Setzer, 2018; Owusu *et al*., 2021). These properties are due to secondary metabolites of the plants, such as phenolics, flavonoids, tannins, steroids, essential oils, etc. (Altemimi *et al*., 2017). However, the selection of plant extracts to play a role in treatment or therapy still needs to be studied in terms of safety and no/minimal side effects.

Curcuma zedoaria (Christm.) Roscoe is commonly known as zedoary and belongs to the Zingiberaceae family. It is one of the *Curcuma* genera which is widely used as a medicine and an ingredient in cooking. *C. zedoaria* (Christm.) Roscoe has a great variety of components such as essential oils, therapeutic compounds, and other constituents with a broad spectrum of biological properties, including anti-cancer (Rita *et al*., 2019), anti-inflammatory (Rahaman *et al*., 2021), antipyretics (Azam *et al*., 2014), antioxidant (Rahman *et al*., 2014) and antimicrobial (Islam *et al*., 2017) activities. However, the antibiofilm formation of *C. zedoaria* (Christm) Roscoe rhizomes extracts had not yet been evaluated.

Biofilm formation is one of the resistance strategies of

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many pathogenic strains. Biofilms enable surface-attached microorganisms to persist even under adverse conditions such as natural host defenses and antimicrobial agents (Jamal *et al*., 2018). Several strains of human pathogenic bacteria form biofilms that make them difficult to treat, namely *Bacillus*, *Escherichia*, *Staphylococcus*, *Pseudomonas*, *Salmonella*, *Listeria*, etc. (Chakraborty *et al*., 2018; Somarathna *et al*., 2020). Bacterial biofilm formation is one of the mechanisms to escape from antibiotics. Plants are a source of natural substances that can inhibit or hinder the growth or biofilm formation of pathogenic bacteria. There have been few or no reports of the anti-biofilm activity and time killing assay of CZR until now. Therefore, this study aimed to investigate the phytochemical compounds and assess the antimicrobial and antibiofilm activities of different solvent extracts of *C. zedoaria* (Christm.) Roscoe rhizomes on various pathogenic strains in terms of destroying bacterial biofilms, which is an important strategy for blocking the pathogenesis on host cells. This is beneficial information for development of food or pharmaceutical products in the future.

MATERIALS AND METHODS

Plant material

Fresh rhizomes of *C. zedoaria* (Christm.) Roscoe were purchased from a Thaprachan herb shop with raw material from Khao Kho District, Phetchabun Province, Thailand. The rhizomes were washed and cut into small pieces, dried at a temperature not exceeding 40 °C and pulverized into a coarse powder. The coarse powders were macerated with 95% ethanol (ratio=1:4), 75% acetone (ratio=1:4) for 5 days and boiled in distilled water (ratio=1:5) at 60 °C for 30 min. The mixtures were filtered through Whatman No. 1 filter paper and centrifuged at 4000× *g* for 10 min. Then, each filtrate was subsequently concentrated under vacuum in a rotary evaporator and kept at –20 °C under a dark condition until further analysis. The % yield (w/v) of all extracts was calculated after evaporation.

Preliminary phytochemical analysis

Curcuma zedoaria (Christm.) Roscoe rhizome extracts were subjected to qualitative phytochemical screening for the presence and/or absence of different phytoconstituents like saponins, flavonoids, tannins, steroids, alkaloids, terpenoids, anthraquinones and cardiac glycosides (Harborne, 1998).

Bacterial strains and culture conditions

The microorganisms used in this study were 9 pathogenic strains, including 5 Gram-negative (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* DMST 8212 and *Salmonella typhimurium* ATCC 13311) and 4 Gram-positive (*Bacillus cereus* DMST 5040, *Enterococcus faecalis* DMST 4736, *Staphylococus aureus* ATCC 25923 and *Staphylococcus epidermidis*) bacteria. These bacterial strains were kindly provided by laboratory of the Department of Biotechnology, King Mongkut's University of Technology North Bangkok, Thailand. All strains were maintained on brain heart infusion (BHI, Difco) agar at 37 °C.

Evaluation of antimicrobial activity

Agar well diffusion method

The antimicrobial activity of plant extracts was determined using the agar-well diffusion assay (Teanpaisan *et al*., 2017). Briefly, the overnight cultures of tested bacterial strains were adjusted to a concentration of approximately 1×10^7 CFU/mL (OD_{600}=0.2). The BHI agar plates were overlaid with 5 mL of BHI soft agar (0.75%) seeded with 100 µL of the indicator strains. After solidifying BHI agar, the different concentrations of each crude extract (100 µL) were dropped into punched wells before being incubated at 37 °C. All plates were observed, and the inhibition diameter was measured in millimetres at 1, 3 and 5 days.

Broth micro-dilution method

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by a modified broth microdilution assay (Tinrat, 2015). Briefly, the stock solution of each extract was diluted in the BHI broth in two-fold serial dilutions to obtain concentrations from 1,600 to 3.125 mg/mL at a total volume of 200 µL per well in 96-well microtiter plates. Next, 5 µL of each of the tested bacteria at a final concentration of 1×10^7 CFU/mL were added to each well and incubated at 37 °C. The lowest concentration of the extracts that showed no visible growth after 24 h of incubation was considered the MIC. The MBC values were determined by sub-culturing visually clear broth dilution from the MIC well to HBI agar (Difco) and incubating at 37 °C for 24 h. The concentration showing the complete absence of bacterial growth was considered the MBC.

Time-kill curve assay

In vitro bactericidal ability of crude extracts with significant antimicrobial activity was determined using time-kill curves (Tinrat, 2015). Crude extracts of *C. zedoaria* (Christm.) Roscoe rhizomes (1/2× MIC, 1× MIC and 2× MIC) and tested bacterial strains (early log phase; 3×10^7 CFU/mL) were mixed and incubated at 37 °C. Surviving bacteria were observed at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 h by the drop plate technique. Curves were plotted as the viable cells ($\log_{10}$ of the numbers of CFU/mL) versus time. Bactericidal activity is defined as a 3 $\log_{10}$ decrease in the CFU/mL or a 99.9% kill over a specified time (May *et al*., 2000).

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Anti-biofilm assay (Anti-attach activity)

The effects of *C. zedoaria* (Christm.) Roscoe rhizomes extracts on biofilm formation were evaluated as described by Noumi *et al*. (2017) with some modifications. Approximately 200 µL of BHI broth containing pathogenic strains (early log phase; 10^7 CFU/mL; OD_{600}=0.2) were transferred to flat-bottomed 96-well microplates. 200 µL of each crude extract (2× MIC value) were added to the microplate and incubated at 37 °C for 24 h to culture the biofilms. Broth medium without inoculation with microorganisms was used as a negative control. The broth culture medium was drained, and each well was washed with 200 µL of phosphate-buffered saline (pH 7.6) to remove all traces of medium and bacterial cells. The biofilm formed was stained for 15 min at room temperature with 0.1% crystal violet solution after well drying for 5-10 min. The excess staining solution was washed with sterile distilled water. The 96-well microplate was dried for 30 min, and then the optical density at 570 nm was recorded using a microplate reader. The percentage inhibition of biofilm was calculated as follows:

Percentage inhibition = [1 – (A_{570} of the test/A_{570} of non-treated control)] × 100

Statistical analysis

Experimental results are expressed as mean ± standard deviation (SD) in at least three replicates. In addition, differences between samples and control were analyzed using one-way analysis of variance (ANOVA). The significant difference between the means was tested using Tukey's multiple comparisons or paired *t*-test at α=0.05. Graphpad prism 9.0 software was used to analyze the data.

RESULTS AND DISCUSSION

% Yield of various extracts of *C. zedoaria* (Christm.) Roscoe rhizomes

The percentage yield of various extracts from the plant mainly depended on the type of solvent used in the extraction procedure. The % yield of the three extracts (distilled water, 95% ethanol and 75% acetone) of *C. zedoaria* (Christm.) Roscoe rhizomes (CZR) are summarized in Table 1. The % yield of CZR extracts was highest in the crude aqueous extract (4.78% w/v), followed by 75% acetone (2.81% w/v) and 95% ethanol (2.48% w/v) extracts on a dry weight basis. Differences in the percentage yield of these crude extracts may be due to differences in environmental conditions. This study found that the aqueous extract of this rhizome plant had a higher yield than other solvent extracts. These results revealed that the available active ingredients from the CZR extracts were highly soluble in polar solvents. Use of water as a solvent is popular in herbal extraction among traditional healers and practitioners (Junsongduang *et al*., 2020).

Preliminary phytochemical constituents of the *C. zedoaria* (Christm.) Roscoe rhizomes extract

Phytochemical screening from plant samples is a very important parameter because it provides extensive information for the discovery of bioactive agents or finding new commercial compounds. The solvents used during the extraction process have an influence on the amount of extracted secondary metabolites. Various phytochemical constituents were tested for in the different solvent extracts of CZR with standard procedures. The results are summarized in Table 1 and show the presence of different secondary metabolites. According to the observed results, saponins, tannins, flavonoids, cardiac glycosides, steroids, terpenoids and alkaloids were found in both crude 95% ethanolic and 75% acetonic extracts of CZR. In the case of the aqueous extract, the results indicated the presence of saponins, flavonoids, cardiac glycosides, steroids, terpenoids and alkaloids. Anthraquinones were absent in all crude rhizome extracts of *C. zedoari*a (Christm.) Roscoe. Previous reports showed that phytochemical analysis of 96% ethanol extract of CZR revealed the presence of flavonoid, saponin, steroid, triterpernoid, quinone and volatile oil (Desmiaty *et al*., 2018). As with previous research, *Curcuma zedoaria* has major secondary metabolites in the form of terpenoids (Azam *et al*., 2014). The presence of tannins, saponins, alkaloids, terpinoids and steroids in *C. zedoaria* (Christm.) Roscoe has been reported by Azam *et al*. (2014). The presence of alkaloids in plant samples are responsible for antitumor, antimicrobial, and anti-inflammatory properties. The flavonoids and tannins in plant samples possess antibacterial, antifungal, antioxidant, antimicrobial, and anthelmintic activities (Brodowska, 2017). Saponins are responsible for the antimicrobial, anti-inflammatory, and cytotoxic activity of compounds in medicinal plants (Sparg *et al*., 2004). Steroids derived from plants are known to have antibacterial, antifungal, and insecticidal properties (Ke, 2018). The selection of solvents for the extraction process is critical in maximizing the extract yield and bioactivity of the plant extracts (Waszkowiak *et al*., 2015). Solvents can be classified according to their polarity such as polar, semi-polar and non-polar. These results suggest that *C. zedoaria* (Christm.) Roscoe rhizomes have soluble metabolites in very polar (distilled water and ethanol) and non-polar (acetone) solvents.

Evaluation of antimicrobial activity by the agar well diffusion method

Preliminary antimicrobial tests were performed by the well diffusion method. The results showed variability between each solvent extract of *C. zedoaria* (Christm.) Roscoe rhizomes ranging from 7.25 ± 0.58 to 12.00 ± 0.26 mm at a concentration of 400 mg/mL (Table 2). At the concentrations of 25, 100 and 200 mg/mL, all crude extracts of CZR had no antibacterial effect (no inhibition zone) against the tested pathogenic strains. The 95% ethanolic extract displayed potent antibacterial activity

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Table 1: Phytochemical screening results of *Curcuma zedoaria* (Christm.) Roscoe extracts in different solvents.

Phytochemical compounds	Distilled water	95% Ethanol	75% Acetone
Saponins			
Froth formation test	+++	+++	+++
Tannins			
Ferric chloride test	-	+	+
Flavonoids			
Shinoda test	+	++	+
Lead acetate test	++	+++	++
Anthraquinones			
Borntrager's test	-	-	-
Steroids			
Libermann test	++	+++	++
Terpenoids			
Salkowski test	+	+	+++
Cardiac glycosides			
Keller-Killiani test	+	+	+
Alkaloids			
28% NH_4OH	++	+++	+
Dragendoff's reagent	+	+++	+++

+: Low intensity reaction; ++: Medium intensity reaction; +++: Strong intensity reaction; -: No reaction;
+: Presence of secondary metabolite; -: Absence of secondary metabolite.

Table 2: Antimicrobial activity of crude extracts of *Curcuma zedoaria* (Christm.) Roscoe rhizomes by the agar well diffusion method.

Pathogenic strains		Zone of Inhibition in mm ± SD (∅=6 mm)			Ampicillin (10 µg)
		Distilled water 400 mg/mL	95% Ethanol 400 mg/mL	75% Acetone 400 mg/mL	
Gram positive strains	*B. cereus* ATCC 11778	–	–	–	20.67 ± 0.58
	E. faecalis DMST 4736	–	–	–	30.00 ± 0.00
	S. aureus ATCC 25923	–	9.33 ± 0.43^{b}	–	21.67 ± 0.58
	S. epidermidis	–	7.67 ± 0.58^{c}	7.67 ± 0.58^{c}	23.33 ± 0.58
Gram negative strains	*E. coli* ATCC 25922	–	–	–	23.00 ± 0.00
	K. pneumoniae	–	–	–	20.67 ± 0.58
	P. aeruginosa ATCC 27853	10.00 ± 0.58^{b}	7.25 ± 0.58^{c}	–	23.00 ± 0.00
	P. mirabilis DMST 8212	–	12.00 ± 0.26^{a}	–	24.17 ± 1.04
	S. typhimurium ATCC 13311	–	–	–	27.00 ± 0.00

^{abc}: Values with different superscripts differed significantly (p<0.05) when comparing among columns;
–: No zone of inhibition.

against five pathogenic strains including *E. faecalis* DMST 4736, *S. aureus* ATCC 25923, *S. epidermidis*, *P. aeruginosa* ATCC 27853 and *P. mirabilis* DMST 8212, while aqueous and 75% acetonic extracts showed appreciable antimicrobial activities against *P. aeruginosa* ATCC 27853 and *S. epidermidis* with inhibition zones (IZ) of 10.00 ± 0.58 and 7.67 ± 0.58 mm at a 400 mg/mL concentration, respectively. *Bacillus cereus* ATCC 11778, *E. coli* ATCC 25922, *K. pneumoniae* and *S. typhimurium* ATCC 13311 were the microorganisms that were not inhibited by any of crude extracts of CZR. Among the three *C. zedoaria* (Christm.) Roscoe rhizomes extracts, the antimicrobial activity of the 95% ethanolic extracts were significantly stronger than the aqueous and 75% acetonic extracts (p<0.05). The 95% ethanolic extract showed significant antimicrobial activity against some of the tested Gram-negative and Gram-positive bacteria while the aqueous and 75% acetonic extracts exhibited little antimicrobial activity against some Gram-negative (*S. epidermidis*) and Gram-positive bacteria (*P. aeruginosa* ATCC 27853), respectively (p<0.05). The Gram-positive bacteria, *E. faecalis* DMST 4736, *S. aureus* ATCC 25923 and *S. epidermidis* were shown to be susceptible to the 95% ethanolic CZR extract. *E. faecalis* DMST 4736 was found to be the most sensitive strain to 95% ethanolic CZR extract with IZ of 11.33 ± 0.52 mm. While *B. cereus* ATCC 11778 was the most resistant strain to all crude extracts due to its endospore formation which permits survival under sub-optimal conditions or environmental stress. However, the well diffusion assay is considered a

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qualitative technique and is mainly used for the screening of most antimicrobial active extracts when the inhibition zone diameter is ≥10 mm (Usman *et al*., 2009). Based on the well diffusion assay, it is important to recognize that the size of inhibition zones of different extracts could be due to the compound's polarity, since a more diffusible but less active extract could give a bigger diameter of inhibition than a non-diffusible but more active extract (Savaroglu *et al*., 2011). As can be seen from the results of this study, if the extracts of CZR produced an inhibition zone against pathogenic bacteria less than 10 mm, it did not necessarily mean that these extracts had no antibacterial effect. Other methods should also be considered. The broth microdilution assay was also carried out to assess the antimicrobial effects of compounds in this study. This method studied the antimicrobial activity in the broth medium but may have affected the distribution of extracts and the clarity of the results. These research results (IZs of <10 mm) are in agreement with the study of Wilson *et al*. (2005) which found that the antibacterial activity of an ethanol extract of CZR against *Proteus mirabilis* and *Klebsiella pneumoniae* produced an inhibition zone of 8.00-9.00 mm (3.75 mg/well), but this extract showed MIC values at low concentrations (0.08-0.94 mg/mL). Islam *et al.* (2017) reported antibacterial activity from an ethanol extract of *C. zedoaria* Rosc against 8 pathogenic bacteria namely *Bacillus cereus*, *Staphylococcus aureus*, *Sarcina lutea*, *Bacillus magaterium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella boydi* with inhibition zones of 10.00 ± 0.50, 9.00 ± 0.40, 7.00 ± 0.70, 10.00 ± 0.50, 12.00 ± 0.50, 15.00 ± 0.50, 12.00 ± 1.90 and 10.00 ± 0.90 mm, respectively, at a concentration of 400 µL. But, Chachad *et al*. (2015) reported antimicrobial activity of an ethanolic *C. zedoaria* Rosc extract against *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus albus* and *Streptococcus pyrogenes* by agar disc diffusion with 9, 16, 7 and 10 mm inhibition zones, respectively.

Evaluation of antimicrobial activity by broth microdilution assay

The MIC and MBC values of *C. zedoaria* (Christm.) Roscoe rhizome extracts were evaluated by the broth micro-dilution assay and are shown in Table 3. The results showed that the crude extracts in different solvents showed strong antibacterial activity against the pathogenic strains. The MIC values for all crude extracts ranged between 50-400 mg/mL. The aqueous and 95% ethanolic extracts showed MIC values of 50-200 mg/mL, while the 75% acetonic extract revealed MIC values of 50-800 mg/mL. *Staphylococcus epidermidis*, *S. aureus* ATCC 25923 and *E. faecalis* DMST 4736 with MIC values of 50 mg/mL were the most significantly susceptible of the Gram-positive bacteria to aqueous, 95% ethanolic and 75% acetonic extracts of CZR in this study (p<0.05). Of the Gram-negative bacteria, *E. coli* ATCC 25922 was the most significantly susceptible to the 95% ethanolic extract with a MIC value of 50 mg/mL (Table 3) (p<0.05). The minimum bactericidal concentrations (MBC) are listed in Table 3. The results showed that the MBC values for all crude extracts ranged from 100 to >1,600 mg/mL. Among the three crude extracts, the bactericidal concentrations were significantly higher than the minimum inhibitory concentration (p<0.05). Based on the MIC and MBC results of aqueous and 95% ethanolic extracts, *S. aureus* ATCC 25923, *S. epidermidis*, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *P. mirabilis* DMST 8212 were the most susceptible strains with MIC/MBC values of approximately 50-100/400-800 mg/mL, respectively. In the case of the 75% acetonic extract, the most susceptible strains were *E. faecalis* DMST 4736, *S. aureus* ATCC 25923 and *S. epidermidis* with MIC/MBC values of approximately 50-100/800 mg/mL. This observation supports the findings of Islam *et al*. (2017) who reported that an ethanol extract of *C. zedoaria* rhizomes possessed antimicrobial activity against *Escherichia coli* and *Bacillus cereus*, with MIC values of 64 and 128 µg/mL, respectively. Wilson *et al*. (2005) reported that acetonic and ethanolic rhizome extracts were highly active against two Gram-positive bacteria, *Bacillus subtilis*, *Micrococcus luteus* and two Gram-negative bacteria, *Proteus mirabilis* and *Klebsiella pneumoniae*. The MIC values of these extracts were 0.01-0.15 mg/mL. In this study, the MIC/MBC values of ampicillin as the positive control against bacterial strains were 0.039-0.313/0.078-1.25 µg/mL (data not showed). However, lower MIC values showed better inhibitory activity.

Based on both the antimicrobial activity results, Gram-negative bacteria had a higher resistance to the plant extracts than Gram-positive bacteria. This may be attributed to the distinct feature of the cell membrane morphology of Gram-negative bacteria which differs from that of Gram-positive bacteria. Gram-negative bacteria have an outer membrane composed of hydrophilic lipopolysaccharides that are highly resistant to the penetration of antibacterial agents (Sperandeo *et al*., 2017). Among the plant extracts tested, the 95% ethanolic extract of CZR had a broad-spectrum antibacterial activity by the agar well diffusion method while the 95% ethanolic and 75% acetonic extracts had broad spectrum activity against all the tested bacteria by the micro-dilution diffusion assay.

Time-kill assay of *C. zedoaria* (Christm.) Roscoe rhizomes extracts against pathogenic bacteria

Although there are many reports of antimicrobial effects of CZR rhizome extracts on pathogenic strains, research concerning their anti-biofilm activity and time-kill assays are sparse. Therefore, this study investigated the inhibition of biofilm formation and time-kill curves of these extracts against nine bacterial strains. Based on agar well diffusion and MIC/MBC values, *E. faecalis* DMST 4736, *S. aureus* ATCC 25923, *S. epidermidis*, *P. aeruginosa* ATCC 27853 and *P. mirabilis* DMST 8212 were selected in time-kill studies with the 95% ethanolic extract of CZR. A range of concentrations of 95% ethanolic extract from

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Table 3: MIC and MBC of *Curcuma zedoaria* (Christm.) Roscoe rhizomes extracts against pathogenic strains by the broth micro-dilution assay.

Pathogenic strains	Distilled water		95% Ethanol		75% Acetone	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
Gram positive strains						
B. cereus DMST 5040	200 ± 0.00^{c}	800 ± 0.00^{C}	200 ± 0.00^{c}	800 ± 0.00^{C}	800 ± 0.00^{d}	>1600 ± 0.00
E. faecalis DMST 4736	200 ± 0.00^{c}	800 ± 0.00^{C}	200 ± 0.00^{c}	800 ± 0.00^{C}	50 ± 0.00^{a}	800 ± 0.00^{C}
S. aureus ATCC 25923	100 ± 0.00^{b}	800 ± 0.00^{C}	50 ± 0.00^{a}	400 ± 0.00^{B}	100 ± 0.00^{b}	800 ± 0.00^{C}
S. epidermidis	50 ± 0.00^{a}	100 ± 0.00^{A}	100 ± 0.00^{b}	400 ± 0.00^{B}	100 ± 0.00^{b}	800 ± 0.00^{C}
Gram negative strains						
E. coli ATCC 25922	100 ± 0.00^{b}	800 ± 0.00^{C}	50 ± 0.00^{a}	800 ± 0.00^{C}	100 ± 0.00^{b}	>1,600 ± 0.00
K. pneumoniae	50 ± 0.00^{a}	800 ± 0.00^{C}	100 ± 0.00^{b}	800 ± 0.00^{C}	200 ± 0.00^{c}	>1,600 ± 0.00
P. aeruginosa ATCC 27853	100 ± 0.00^{b}	800 ± 0.00^{C}	100 ± 0.00^{b}	800 ± 0.00^{C}	100 ± 0.00^{b}	>1,600 ± 0.00
P. mirabilis DMST 8212	100 ± 0.00^{b}	400 ± 0.00^{B}	100 ± 0.00^{b}	400 ± 0.00^{B}	100 ± 0.00^{b}	>1,600 ± 0.00
S. typhimurium ATCC 13311	200 ± 0.00^{c}	800 ± 0.00^{C}	200 ± 0.00^{c}	800 ± 0.00^{C}	800 ± 0.00^{d}	>1,600 ± 0.00

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration;
^{abcd}: Values in the MIC determination with different superscripts differed significantly ($p \leq 0.05$);
^{ABC}: Values in the MBC determination with different superscripts differed significantly ($p \leq 0.05$).

1/2× MIC to 2× MIC (25 to 400 mg/mL) was tested by the time-kill assay. The results are shown in Figure 1. The time-kill kinetics profile of CZR extracts against *E. faecalis* DMST 4736, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *S. epidermidis* and *P. mirabilis* DMST 8212 showed reduction in the number of viable cells over the first 6, 6, 9, 12 and 15 h at 1× and 2× MIC of crude extract. The control (without crude extracts; 3.0-3.34 $\log_{10}$ CFU/mL at an initial count) displayed exponential growth at 6-18 h depending on the tested pathogenic strains (Figure 1). The complete killing of *E. faecalis* DMST 4736 treated with 1/2×, 1× and 2× MIC of the 95% ethanolic extracts occurred within 9, 6 and 6 h, respectively. This result was similar to the action of hexane gac fruit aril extract (1.56 mg/mL) against *E. faecalis* DMST 4736 which displayed a remarkable bacteriocidal effect after 9 h of incubation (Tinrat and Sila-Asna, 2016). The 95% ethanolic extracts at the concentrations of 1/2×, 1× and 2× MIC, exhibited rapid bacteriostatic activity against *E. faecalis* DMST 4736 at 3 h with a significant decrease in viable counts of 3-6 $\log_{10}$ CFU/mL when compared with the initial count (p<0.05). *Staphylococcus aureus* ATCC 25923 was killed after 12, 6 and 6 h at 1/2×, 1× and 2× MIC of the 95% ethanolic extracts, respectively. At 1× and 2× MIC, the results revealed a rapid decrease in viable counts of 3-6 $\log_{10}$ CFU/mL at the first 3 h of incubation (p<0.05). At 1/2×, 1× and 2× MIC, *P. aeruginosa* ATCC 27853 was killed after 15, 12 and 9 h, respectively. The viable cells rapidly decreased to 4-5 $\log_{10}$ CFU/mL after 3 h at 1× and 2× MIC of the crude extracts compared with the initial count, whereas there was a reduction of only 2 $\log_{10}$ CFU/mL at 1/2× MIC. Previous studies have also reported that aqueous *Asparagus racemosus* root extract (1,600 mg/mL) showed microbicidal activity against *P. aeruginasa* ATCC 27853 at 6 h intervals after incubation (Tinrat and Sila-Asna, 2017). For *S. epidermidis*, it exhibited a decrease in viable counts of 3-5 $\log_{10}$ CFU/mL after 3 h of incubation. At 3 h the viable counts exhibited a significant reduction of 1 $\log_{10}$ CFU/mL from the initial count at all studied concentrations and a slight decrease to 2-3 $\log_{10}$ CFU/mL after 6 h of testing (p<0.05). There are few reports on time-kill kinetic studies of *C. zedoaria* (Christm.) Roscoe rhizome extracts, and several studies of the natural product extracts have been reported (Tinrat, 2015; Teanpaisan *et al.*, 2017). However, the kill-curve generated from this study showed microbial reduction at various time intervals, which would be highly beneficial in commercial production if the extract was developed as a natural preservative or even products to inhibit pathogenic bacteria.

Biofilm inhibition assay (Anti-attach activity)

The ability of *C. zedoaria* (Christm.) Roscoe rhizome extracts to inhibit biofilm formation was measured using a microplate-based assay. The activities of plant extracts (2× MIC) with good anti-attachment activity on bacterial biofilm formation in 24 h are presented in Table 4. The values between 0 and 100% indicate biofilm inhibition, while enhancement of biofilm formation was reflected with values below 0%. Above 50% inhibition means good activity, while values of 0 and 49% mean poor activity (Famuyide *et al.*, 2019). The results showed that the 95% ethanolic extract of CZR had percentage inhibition values above 50% in all tested pathogenic strains, except *B. cereus* ATCC 11778 and *S. typhimurium* ATCC 13311 (Table 4). Aqueous and 75% acetonic extracts had significantly poor anti-biofilm activity against *B. cereus* ATCC 11778 (21.94 ± 0.50-36.18 ± 0.10% of biofilm inhibition), *K. pneumoniae* (33.17 ± 0.40-44.55 ± 0.40% biofilm inhibition) and *S. typhimurium* ATCC 13311 (38.24 ± 0.50-46.41 ± 0.30% biofilm inhibition). None of the *C.*

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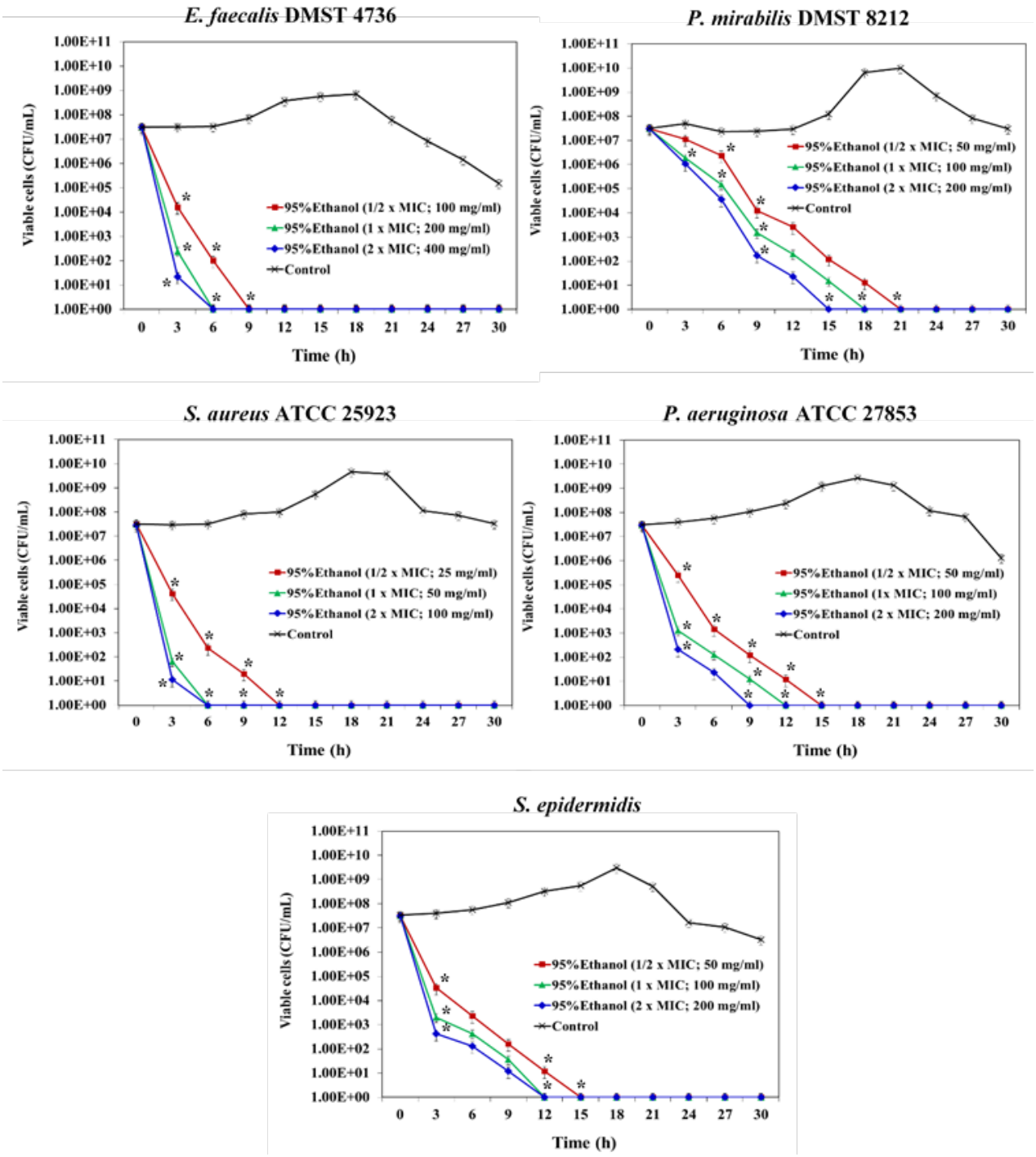

Figure 1: Time-killing curves of different concentrations of *Curcuma zedoaria (Christm.)* Roscoe extracts. CFU = Colony Forming Units; * = Indicate statistically significant difference between groups (p<0.05).

zedoaria (Christm.) Roscoe rhizome extracts could completely inhibit biofilm formation.

Escherichia coli biofilm formation was significantly inhibited by the aqueous extract of CZR (31.95 ± 0.20% biofilm inhibition) (p<0.05). Based on Gram-positive bacterial strains, all the three crude extracts of CZR significantly revealed the highest biofilm inhibition of *S. aureus* ATCC 25923 at 62.95 ± 0.40-71.25 ± 0.20% while showing the lowest biofilm inhibition of *B. cereus* ATCC 11778 (21.94 ± 0.50-39.70 ± 0.40%) (p<0.05). *Proteus*

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Table 4: Anti-biofilm activity of crude extracts of *Curcuma zedoaria* (Christm.) Roscoe against pathogenic strains.

Pathogenic strains		% Biofilm inhibition		
		Distilled water	95% Ethanol	75% Acetone
Gram positive strains	*B. cereus* ATCC 11778	21.94 ± 0.50^{cI}	39.70 ± 0.40^{aI}	36.18 ± 0.10^{bG}
	E. faecalis DMST 4736	56.98 ± 0.20^{bE}	63.31 ± 0.40^{aE}	52.70 ± 0.20^{cC}
	S. aureus ATCC 25923	62.95 ± 0.40^{cB}	71.25 ± 0.20^{aA}	64.68 ± 0.30^{bA}
	S. epidermidis	58.29 ± 0.10^{bD}	67.02 ± 0.10^{aD}	53.12 ± 0.40^{cC}
Gram negative strains	*E. coli* ATCC 25922	31.95 ± 0.20^{cH}	56.98 ± 0.20^{aF}	53.08 ± 0.30^{bC}
	K. pneumoniae	33.17 ± 0.40^{cG}	54.62 ± 0.30^{aG}	44.55 ± 0.40^{bF}
	P. aeruginosa ATCC 27853	61.03 ± 0.20^{bC}	66.04 ± 0.10^{aC}	50.39 ± 0.10^{cD}
	P. mirabilis DMST 8212	65.02 ± 0.50^{bA}	69.27 ± 0.20^{aB}	55.83 ± 0.20^{cB}
	S. typhimurium ATCC 13311	38.24 ± 0.50^{cF}	48.69 ± 0.50^{aH}	46.41 ± 0.30^{bE}

^{abc}: Means with different superscripts in the same row show significant differences ($p \leq 0.05$);
^{ABCDEFGHI}: Means with different superscripts in the same column show significant differences ($p \leq 0.05$).

mirabilis DMST 8212 (55.83 ± 0.20-69.27 ± 0.20%) and *K. pneumoniae* (33.17 ± 0.40-44.55 ± 0.40%) were the Gram-negative bacterial strains that had the highest and lowest percentage of biofilm inhibition, respectively. The 95% ethanolic extract of CZR caused a strong inhibition of biofilm formation in all pathogenic strains tested (>54% biofilm inhibition), except *B. cereus* ATCC 11778 and *S. typhimurium* ATCC 13311(<50% of biofilm inhibition). The % anti-attach activity of CZR extracts varied on tested bacterial strains in this study. The results represented good anti-adherent properties of the CZR extracts (aqueous, 95% ethanol and 75% acetone extracts) on three Gram-positive bacteria and two Gram-negative bacteria. These results are consistent with study of Rabe and van Staden (1997) who reported that plant extracts had more anti-adhesion activity against Gram-positive bacteria than Gram-negative bacteria.

Biofilm formation represents a prominent safety issue in the food industry because bacterial biofilm formation is associated with pathogenic bacteria. Anti-biofilm activity consists of two distinct parts: antibiofilm formation (anti-attach property) and biofilm removal. This study evaluated the ability of CZR extracts to prevent biofilm formation of bacteria species at 24 h. *Enterococcus faecalis* DMST 4736 and *P. mirabilis* DMST 8212 are common biofilm formers usually implicated in urinary tract infections. All rhizome extracts of *C. zedoaria* (Christm.) Roscoe demonstrated good biofilm inhibition against both bacterial strains (>50% anti-biofilm activity). *Staphylococcus aureus* is frequently related to oral problems (Botelho, 2000). The 95% ethanolic and 75% acetonic extracts of CZR extracts had excellent anti-biofilm activity against *S. aureus* ATCC 25923 at 24 h. Moreover, the three rhizome extracts of CZR had good anti-attachment property against *P. aeruginosa* ATCC 27853. This strain can produce biofilms that cause the highest number of acute and chronic infections in several systems, especially excretory and respiratory systems (Ahmed *et al.*, 2018). The presence of an extracellular polymetric matrix provides a strong adhesion by microorganisms on surfaces, resulting in lower antibiotic penetration. Plant extracts may produce substances that interfere with cell-to-cell communication strategies called quorum sensing, which is a bacterial biofilm formation mechanism (Merghni *et al*., 2018).

CONCLUSION

The present study concludes that *C. zedoaria* (Christm.) Roscoe rhizome extracts show a good source of biologically active compounds (tannins, flavonoids, cardiac glycosides, steroids, terpenoids and alkaloids). Furthermore, the 95% ethanolic extract of CZR showed broad spectrum antimicrobial and antibiofilm activities, especially against *S. aureus* ATCC 25923, an important opportunistic human pathogen. Of interest, the 95% ethanolic extract of CZR not only showed strong and rapid bacteriostatic and bactericidal activities against *E. faecalis* DMST 4736 and *S. aureus* ATCC 25923 by time kill study, but it also exhibited strong biofilm inhibitory activity against *E. faecalis* DMST 4736, *P. mirabilis* DMST 8212 and *P. aeruginosa* ATCC 27853. These are pathogenic strains that are the most frequently found in urinary tract and oral infections. Thus, *C. zedoaria* (Christm.) Roscoe extracts can be used as natural antibacterial agents for the therapy of infectious diseases caused by pathogenic bacteria or development of new pharmaceutical products.

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