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Evaluation of In-Vitro Antimicrobial Effects of Various Solvents Extracts of Curcuma Zedoaria (Kunyit Putih) Leaves on Selected Gram-Negative and Gram-Positive Bacterial Strains

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A B S T R A C T

Members of the family Zingiberaceae have generally attracted interest since the ancient times of mankind, and their uses have developed successively. *Curcuma zedoaria* is a distinct example that has a vast array of antimicrobial properties. Leaves of *C. zedoaria* were extracted using 90% each of methanol and ethanol and 30% isopropanol. They were subjected to antimicrobial susceptibility testing using the agar-disc diffusion technique at concentrations of 10 mg/mL, 25mg/mL, and 50mg/mL against both of Gram-positive bacteria including *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 33019), *Staphylococcus aureus* (ATCC 29737), *Propionibacterium acne* (ATCC 6919), *Streptococcus mutans* (ATCC 27351), and *Listeria monocytogenes*, and gram-negative bacteria including *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 13773), and *Acinetobacter anitratus* (A9). Their minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were also determined. All the extracts exhibited antimicrobial effects at all concentrations on *S. mutans*, with inhibition zones ranging from 7.5 to 9.83 mm. There was no activity on all the extracts at a concentration of 10 mg/mL. However, all the extracts exhibited activity against *B. cereus* and *B. subtilis* at 25 mg/mL and 50mg/mL, with inhibition zones ranging from 7.67 to 9.67 mm. Isopropanol, methanol, and ethanol extracts showed activity only at 50mg/mL against *A. anitratus* with inhibition zones of 7.5, 7.67 and 7.83 mm, respectively. However, these extracts exhibited no effect on *E. coli*, *K. pneumonia*, *S. aureus*, *L. monocytogenes*, or *P. aeruginosa* at all concentrations. Except for *A. anitranus*, the MIC and MBC values of ethanol and isopropanol extracts were the same for all the test organisms. The effect was the same in the case of methanol extract, with MIC values of 1.25 mg/mL against *S. mutants*, *B. cereus*, and *B. subtilis*, and MBC value of 2.5mg/mL. Accordingly, methanol extract had the lowest MIC value of 1.25 mg/mL against *B. cereus* while ethanol and Isopropanol extracts had the lowest MIC values of 0.625 mg/mL against *S. mutans*. Meanwhile, all these extracts exhibited the lowest of MIC of 1.25 mg/mL against all organisms except for *A. anitranus*, with values of 5.0mg/mL. It can be indicated that there is a possibility of exploiting the leaf extract of this plant as an antagonist to the pathogenic effects of these types of bacteria.

تقييم التأثيرات المضادة للميكروبات في المختبر لمستخلصات المذيبات المختلفة لأوراق الكركم الأبيض (**Curcuma Zedoaria**) على سلالات بكتيرية سالبة وموجبة الجرام المختارة

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الملخص

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النشاط المختبري
مستخلصات المذيبات
الكركم الابيض
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أعضاء عائلة الزنجبيلية (**ZINGIBERACEAE**) قد جذبت اهتمامًا عامًا منذ العصور القديمة للبشرية، وتطورت استخداماتها بشكل متتابع. الكركم الزنجبيل هو مثال مميز يتمتع بمجموعة واسعة من الخصائص المضادة للميكروبات. تم استخراج أوراق **C. ZEDOARIA** باستخدام 90% من الميثانول والإيثانول و30% من الأيزوبروبانول. تم إخضاعها لاختبار الحساسية المضادة للميكروبات باستخدام تقنية انتشار القرص في الأجار بتركيزات 10 ملغ/مل، 25 ملغ/مل، و50 ملغ/مل ضد كل من البكتيريا موجبة الجرام بما في ذلك **BACILLUS SUBTILIS (ATCC 6633)**، **BACILLUS CEREUS (ATCC 33019)**، **STAPHYLOCOCCUS AUREUS (ATCC 29737)**، **PROPIONIBACTERIUM ACNE (ATCC 6919)**، **STREPTOCOCCUS MUTANS (ATCC 27351)**، و**LISTERIA MONOCYTOGENES**، والبكتيريا سالبة الجرام بما في ذلك **ESCHERICHIA COLI (ATCC 25922)**، **KLEBSIELLA PNEUMONIA (ATCC 13773)**، و**ACINETOBACTER ANITRATUS (A9)**. تم تحديد تركيزاتها الأدنى المثبطة (**MICs**) وتركيزاتها الأدنى القاتلة للبكتيريا (**MBCs**) أيضًا. أظهرت جميع المستخلصات تأثيرات مضادة للميكروبات عند جميع التركيزات على **S. MUTANS**، مع مناطق تثبيط تتراوح من 7.5 إلى 9.83 مم. لم يكن هناك أي نشاط لجميع المستخلصات بتركيز 10 ملغ/مل. ومع ذلك، أظهرت جميع المستخلصات نشاطًا ضد **B. CEREUS** و **B. SUBTILIS** عند تركيز 25 ملغ/مل و50 ملغ/مل، مع مناطق تثبيط تتراوح بين 7.67 إلى 9.67 مم. أظهرت مستخلصات الأيزوبروبانول والميثانول والإيثانول نشاطًا فقط عند تركيز 50 ملغ/مل ضد **A. ANITRATUS** مع مناطق تثبيط بلغت 7.5 و7.67 و7.83 مم على التوالي. ومع ذلك، لم تُظهر هذه المستخلصات أي تأثير على **E. COLI** أو **K. PNEUMONIA** أو **S. AUREUS** أو **L. MONOCYTOGENES** أو **P. AERUGINOSA** عند جميع التركيزات. باستثناء **A. ANITRANUS**، كانت قيم **MIC** و**MBC** لمستخلصات الإيثانول والإيزوبروبانول هي نفسها لجميع الكائنات الحية المختبرة. كان التأثير هو نفسه في حالة مستخلص الميثانول، حيث كانت قيم **MIC** تبلغ 1.25 ملغ/مل ضد **S. MUTANTS** و**B. CEREUS** و**B. SUBTILIS**، وقيمة **MBC** تبلغ 2.5 ملغ/مل. وبالتالي، كان لمستخلص الميثانول أقل قيمة **MIC** تبلغ 1.25 ملغ/مل ضد **B. CEREUS** بينما كان لمستخلصات الإيثانول والإيزوبروبانول أقل قيم **MIC** تبلغ 0.625 ملغ/مل ضد **S. MUTANS**. وفي الوقت نفسه، أظهرت جميع هذه المستخلصات أقل قيمة لتركيز المثبط الأدنى (**MIC**) وهي 1.25 ملغ/مل ضد جميع الكائنات الحية باستثناء **A. ANITRANUS**، التي كانت قيمتها 5.0 ملغ/مل. يمكن الإشارة إلى أنه هناك إمكانية لاستغلال مستخلص أوراق هذه النبتة كمضاد للتأثيرات الممرضة لهذه الأنواع من البكتيريا.

Introduction

The emergence of plants and their products as important sources of natural products has been as old as man himself [1], and their antimicrobial potencies against certain microbes have also been studied by various studies [2-5]. Such antimicrobial properties are linked to the presence of secondary metabolites such as phenolic compounds and tannins in plants and their products [6]. It has been documented by the WHO that during the previous decades, about 80% of the world population depended largely on traditional medicine that employed plant extracts as panaceas for a variety of their illnesses [7]. Antibiotic resistance by pathogenic microbes has always been a global concern, with its increasing incidences as a threat to the health of humans and animals. Once antibiotic resistance occurs, the potential for treatment of disease due to the antibiotic- resistant organism is strongly reduced [8].

The use of plants in traditional medicine started when resistance to antibiotics persistently increased. Thus, plants are equally explored persistently as alternatives to the failed antibiotics. The plant *Curcuma zedoaria* has been widely used as a spice and coloring agent that possesses medicinal properties [9]. It is a member species of the rhizomatous Zingiberaceae family, commonly known as ginger [10]. Various parts of this plant have been used in the treatment of various illnesses such as diarrhea, stomach diseases, coryza, skin disorders, blood stagnation, rheumatism, and liver protection as well as facilitating menstruation [11,12]. Activities such as antibacterial, antifungal and anti-inflammatory activities have been reported for *C. zedoaria*, *C. longa*, *C. amada*, and *C. aromatic* species [13-15]. This study therefore aimed to determine the potential of the ethanol, methanol, and isopropanol extracts of *C. zedoaria* leaves as antibacterial agents with the objective of investigating their activity against *Streptococcus mutans*, *Bacillus cereus*, *Escherichia coli*, *Bacillus subtilis*, *Acinetobacter anitratus*, *Klebsiellapneumoniae*, *Staphylococcusaureus*, *Listeria monocytogenes* and *Propionibacterium acnes*. This was conducted with a view to finding alternative to the conventional antibiotics being used, as they face resistance by both Gram positive and negative bacterial strains.

Materials and methods

Phytochemical Screening

Phytochemical screening of C. zedoaria leaf extract was conducted using solvent extraction with methanol, ethanol and isopropanol as the solvents to detect the presence of three main phytochemicals namely; flavonoid, antioxidant and phenolic compounds. They were extracted by weighing 0.1 g of the kunyit putih (C. zedoaria) powdered leaves and dissolved in 10 mL of various concentrations (0, 10, 30, 50, 70, 90 and 100%) of each solvent and shook at 90 rpm for an overnight. The solutions were filtered at room temperature using Whatman's filter papers (0.2 µm) and subjected to secondary metabolites analysis to find out the plant content (leaves) of both total phenol content (TPC) total flavonoid content (TFC) and the percentage of antioxidants.

Impressive results and accurate conclusions were drawn from those extended experiments. The preference in general was for both methanol and ethanol (90 %), in addition to isopropanol (30 %). They are the ones that were used (as crude extracts) here, as they have given the best results for the contents of the aforementioned materials.

Sample collection and preparation of plant extracts

The leaves of C. zedoaria were obtained from a farm in

Temerloh, Pahang, Malaysia. The procedure was carried out according to [16], with some modifications. The leaves were washed three times thoroughly with clean water to remove dust, cut into pieces, placed in perforated paper bags, and air-dried at room temperature for 24 hours in an oven gradually at varying temperature of 40oC for three days. With the aid of a laboratory grinder, the dried leaves were ground to powder, and 15 g of the powder was soaked in 250 mL of the solvents; ethanol (90%), methanol (90%) and isopropanol (30%) (Chosen based on the results of the phytochemical screening) in a 500 mL volumetric flask plugged and covered with aluminium foil and shaken using ultrasonic path sonicator (Branson, model 8510E-MTH, Danbury, USA). The mixture was left to stand for 1 hour in a shaking water bath maintained at 40 °C and filtered. The filtrate was then evaporated under reduced pressure at 40 oC to obtain the extracts of each solvent using a rotary evaporator. The extracts obtained were weighed to determine the total extractable compounds and then stored in desiccators away from light. Moreover, 100 mg of the extracts of the solvents were dissolved in 1 mL of dimethylsulfoxide (DMSO) (Fisher Scientific, Leicestershire, United Kingdom) to obtain a stock solution with a concentration of 100 mg/mL. Further dilutions were made with the DMSO to obtain solutions with concentrations of 50, 25, and 10mg/mL (50,000, 25,000, and 10,000 µg/mL). The DMSO served as a negative control.

Test Organisms

The bacterial strains used in the study were members of both Gram-positive and gram-negative bacteria (GeneBank, NCBI). The gram-positive ones include Bacillus subtilis (ATCC 6633), Bacillus cereus (ATCC 33019), Staphylococcus aureus (ATCC 29737), Propionibacterium acne (ATCC 6919), Streptococcus mutans (ATCC 27351), and Listeria monocytogenes. The gram-negative ones include Escherichia coli (ATCC 25922), Klebsiella pneumoniae (ATCC 13773) and Acinetobacter anitratus (A9).

Inoculum Standardization

All the test organisms were subcultured in Mueller Hinton broth (MHB, Difco, Sparks, Maryland, UnitedStates) and incubated at 30 °C for 24 hours prior to the susceptibility testing. The resultant suspension was standardized using fresh MHB, as described by [17].

Antimicrobial Susceptibility Testing

The crude extracts of C. zedoaria were tested for antimicrobial activity against the test organisms based on the Clinical and Laboratory Standard Institute (CLSI) guidelines [18]. A swab of overnight culture was aseptically spread onto Mueller -Hinton agar (MHA) for all the bacterial tested strains using a sterile cotton swab. A Sterile paper disc of 6 mm diameter was placed on the agar, and about 10 µl of each prepared concentration of 10mg/mL (10,000 µg/mL), 25mg/mL (25,000 µg/mL), and 50 mg/mL (50,000 µg/mL) of leaf crude extract was pipetted onto the disc. Chlorhexidine with a concentration of 0.1 mg/mL (100µg/mL) was used as positive control. The negative control was 10 µl of DMSO. The plates were incubated at 30 °C for 24 hours. Zones of inhibition as clear zones around the disc were observed, and their diameter was measured in mm. All bioassays were conducted in triplicate, and the results were recorded.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

This was conducted according to [19] and in accordance with the CLSI (2012). This was carried out in a96-wellU-shaped microtitre plate using the two-fold standard broth micro-dilution method with an inoculum density of approximately 105– 107CFU/mL. The stock solution (10mg/mL) of the crude extract of C. zedoaria leaves was mixed in a two-fold dilution in the medium containing the standardized inoculum. Column 12 of the microtiter plate contained the highest concentration of extract (5mg/mL) while column 3 contained the lowest concentration of extract (0.0097mg/mL). Column 1 served as negative control (only medium, no inoculum, and no antimicrobial agent). Meanwhile, column 2 served as positive control for all samples (only medium and inoculum or an antimicrobial agent-free well). Microtiter plates were incubated at 30 °C for 24 hours. The MIC was defined as the lowest concentration of an antimicrobial agent that resulted in the completed inhibition of visible growth [19].

The statistical analysis used in this study was not specified in the information provided. It would be important to refer to the original research paper or source to determine the specific statistical methods employed. Statistical analysis in research studies can vary and may include descriptive statistics, inferential statistics, regression analysis, t-tests, ANOVA, chi-square tests, or other appropriate statistical techniques depending on the study design and research question.

Results and discussion

Antimicrobial activity of the methanol, ethanol, and isopropanol extracts C. zedoaria leaves:

The results of the antimicrobial activity of methanol, ethanol, and isopropanol extracts of the leaves of C. zedoaria against nine bacterial isolates are shown in Table 3.1. It can be seen that the activity increases with increasing concentration. The mechanism of action of these extracts can be either bacteriostatic or bactericidal depending on the magnitude of their concentration and contents of the antimicrobial active principles. The activities against S. mutans of ethanol and isopropanol extracts were similar at 50mg/mL concentrations, with mean zone of inhibition of 9.83±0.75mm. However, at 10mg/mL isopropanol had the highest activity (7.83±0.75mm) followed by methanol extract with 7.67±0.82mm and finally ethanol extract with 7.50±0.55mm although the differences in the inhibition zones are not remarkably enough to show differences (Figure 1). At a 10mg/mL concentration, all the extracts showed no activity against B. cereus while methanol extract exhibited the highest activity against the organism. In this regard, the methanol extract showed the highest activity at both 25mg/mL and 50mg/mL followed by the ethanol extract and finally the isopropanol extract (Figure 2). Based on the above activity of the extracts of C. zeodaria leaves against S. mutans and B. cereus, it can be clearly understood that the activity largely depends on the concentration and on the organism tested [16].

There was no activity at all against E. coli in all the extracts and at all the concentrations. This work does not agree with the study of [16] and it is perhaps a different species. This indicates that E. coli was found to be resistant to C. zeodaria leaf extract. However, crude extracts of C. zeodaria may have an inhibitory effect against the E. coli, and there was also no activity recorded against B. subtilis by the ethanol, methanol and isopropanol extracts of C. zedoaria at 10mg/mL concentration. This may be the concentration is too small to

show activity or as said earlier, the extracts are too crude to exhibit inhibition. The highest activity against B. subtilis was observed in the ethanol extract with 9.67±0.52mm at 50mg/mL concentration followed by methanol extract (9.17±0.75mm) and finally isopropanol extract with 9.00±0.89 inhibition zone. The zones of inhibition herein, are not much different to indicate significance. However at 25 mg/mL concentration, there was equal activity observed by ethanol and isopropanol extracts against the B. subtilis isolate with 7.83±0.75mm mean zones of inhibition respectively. The methanol extract exhibited 7.67±0.52mm against the B. subtilis.

However, A. anitranus was found to be resistant to the methanol, ethanol and isopropanol extracts of leaves of C. zedoaria at 10mg/mL and 25mg/mL concentrations but 7.67±0.82, 7.83±0.75 and 7.50±0.55mm were observed respectively against the organism at 50 mg/mL concentration. This shows that the ethanol extract was the most active followed by the methanol extract and finally the isopropanol extract. There was no activity observed against the isolates of K. pneumoniae, S. aureus, L. monocytogenes and P. aeruginosa at all concentrations and by all extracts.

The antibacterial effect of C. zedoaria against the tested organisms is dependent on the organism, the solvent extract and the concentration. The higher the concentration used, the higher the antimicrobial activity. This is evident from the results as 10mg/mL was not active against some organisms that were sensitive at 25 mg/mL and 50 mg/mL and against others. The antimicrobial activity of C. zedoaria against bacterial strains such as S. aureus, B. cereus was reported by [20] where similar results were obtained as in this study. Similarly, their study showed no effect on E. coli and P. aeruginosa as observed in this study. In addition, various curcuma species such as C. longa, C. amada, C. aromatic and C. malabarica were found to have anti-inflammatory, antifungal and antibacterial activities [14-16] and they have been utilized due to the presence of starch in them which is invaluably utilized as food for the recuperating patients as well as diet for infants [21].

It is noticeable that the failure of the effect of the lower concentration (10%) in the case of both Bacillus cereus and Bacillus subtilis indicates an inability to rein in the growth of these bacteria at this level. While the higher concentration (25%) can show a reasonable effect, which can be relatively magnified by moving up to the higher concentration (50%), in a clear indication that the active substance has no effect (not working in such a case), the need to increase the concentration of the extract appears to be able to influence as a clear connotation of the inability of the leaf extracts with the mentioned solvents to push them to limit their activity. Other than that, the effect of the extract is less severe in the case of Acinetobacter anitratus, so that it does not show any

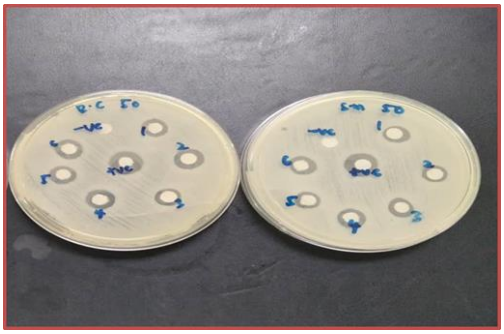

Fig.1: Inhibition zones of C. zedoaria leaves Isopropanol crude extracts against Streptococcus mutans (these plates indicate the 50 mg/mL concentrations of the extracts).

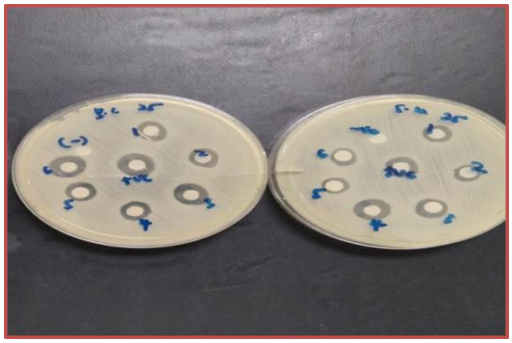

Fig.2: Inhibition zones of C. zedoaria leaves Methanol crude extracts against Streptococcus mutans (these plates indicate the 25 mg/mL concentrations of the extracts).

Table 1: Antimicrobial activity of the methanol, ethanol, and isopropanol extracts of C. zedoaria leaves.

Bacteria	Concentration	Methanol 90%	Ethanol 90%	Isopropanol 30%
Streptococcus mutans	10mg/mL	7.67±0.82	7.5±0.55	7.83±0.75
	25mg/mL	8.83±0.75	8.67±0.82	8.83±0.75
	50mg/mL	9.67±0.82	9.83±0.75	9.83±0.98
	10mg/mL	-	-	-
Bacillus cereus	25mg/mL	8.83±0.75	8.5±0.55	8.33±0.52
	50mg/mL	9.00±0.63	8.83±0.75	8.67±0.52
	10mg/mL	-	-	-
Escherichia coli	25mg/mL	-	-	-
	50mg/mL	-	-	-
	10mg/mL	-	-	-
Bacillus subtilis	25mg/mL	7.67±0.52	7.83±0.75	7.83±0.75
	50mg/mL	9.17±0.75	9.67±0.52	9.00±0.89
	10mg/mL	-	-	-
Acinetobacter anitratus	25mg/mL	-	-	-
	50mg/mL	7.67±0.82	7.83±0.75	7.50±0.55
Klebsiella pneumoniae	10mg/mL-50mg/mL	-	-	-
Staphylococcus aureus	10mg/mL-50mg/mL	-	-	-
Listeria monocytogenes	10mg/mL-50mg/mL	-	-	-
Pseudomonasaeruginosa	10mg/mL-50mg/mL	-	-	-

effect except with the highest concentration (50%), as if the bacteria exhibit higher resistance, thus we need to increase the concentration, till its end, to show a little effect. Variously, it can be inferred that in all cases where the ability of the extract popped up (wherever the inhibition occurred) {as in the case of Streptococcus mutans}, it reflected the existence of some substances that would be resistant to these bacteria. It is noticeable that this effect increases as the concentration of the extract used increases.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Solvent Extracts of C. zedoaria leaves:

All MICs values for methanol extracts against S. mutans, B. cereus and B. subtilis were the same by 1.25, while the values for ethanol and isopropanol were identical for S. mutans, B. cereus, B. subtilis with the following values, 0.625, 2.5 and 1.25 respectively. The MBC values were also similar for all the solvents used (methanol, ethanol and isopropanol) against the aforementioned bacteria with the following values 1.25, 2.5 and 2.5. Nevertheless, the MIC and MBC values were high against the Acinobacter bacteria, with values of 5.0 or >5, in an indication of the weak effect of different leaf extracts on these bacteria (Table 2).

Table 2: Minimum Inhibition Concentration (MIC; mg/mL) and Minimum Bacterial Concentration (MBC; mg/mL) of different solvents leaf extracts

Crude Samples	Solvent used	Methanol		Ethanol		Isopropanol	
	Bacteria\ Concentrations (mg/mL)	MIC	MBC	MIC	MBC	MIC	MBC
Bacteria	*Streptococcus Mutants*	1.25	1.25	0.625	1.25	0.625	1.25
	Bacillus Cereus	1.25	2.5	2.5	2.5	2.5	2.5
	Bacillus Subtilis	1.25	2.5	1.25	2.5	1.25	2.5
	Acinobacteranitratus	5.00	>5	5.0	>5	>5	>5

Conclusion

The leaf extracts of C. zedoaria were found to contain phenolic compounds, flavonoids and antioxidants at various concentrations. This are believed to have exhibited antimicrobial properties against the organisms tested; S. mutans, B. cereus, B. subtilis and A. anitranus. exhibited antimicrobial properties against S. mutans, B. cereus, B. subtilis, and A. anitranus. There was no activity recorded against S. aureus, E. coli, K. pneumoniae, L. monocytogenes and P. aeruginosa. This may not indicate total inactivity, as an increase in concentration and the use of industrially refined extracts may indicate efficacy.

However, there may or may not be potential side effects or toxicity associated with these extracts as relative toxicity test was not within the scope of this study. In addition, there was no animal study neither was there any clinical trial conducted to determine their effectiveness in combating microbial infections. Hence, it is recommended that relative toxicity be conducted to determine whether the extracts are toxic for human health.

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