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ANTIMICROBIAL ACTIVITY METHANOL EXTRACT OF *IN VITRO* PLANTLETS OF *Curcuma xanthorrhiza* Roxb. AND *Zingiber aromaticum* Vahl.

Melati Yulia Kusumastuti[1], Chan Lai Keng[2], Gunawan Indrayanto[3]

[1] FMIPA, Universitas Muslim Nusantara, Jl Garu II no. 93, Medan, 20147,Indonesia
[2] School of Biological Science,Universiti Sains Malaysia, Jalan Sungai Dua, Penang, 11800, Malaysia
[3] Faculty of Pharmacy, Airlangga University, Kampus B Jl. Dharmawangsa Dalam, Surabaya, 60286, Indonesia
E-mail: melati.biotech07@gmail.com

Abstract

Curcuma xanthorrhiza and *Zingiber aromaticum* are members of the Zingiberaceae family. These plants are being used in traditional medicine and found to be effective in the treatment of several diseases. Tissue Culture Technique can be used as an alternative for the production of secondary metabolite. In this study, the antimicrobial test of methanol extract of rhizome and *in vitro* plantlets of *C. xanthorrhiza* and *Z. aromaticum* was carried out by disc-diffusion assay using Gram-negative,-positive and fungal organism. Methanol extract from rhizome and *in vitro* plantlets of *Z. aromaticum* showed antimicrobial activity againts *Escherichia coli* and *Pseudomonas aeruginosa*. Minimum Inhibitory Concentration (MIC) of methanol extract from mother plant and *in vitro* plant to inhibit the growth of *E. coli* was 625 µg/mL and MIC for *P. aeruginosa* was 1250 µg/mL and 625 µg/mL. Through GC/MS scanning, derivates curcumin, xanthorrhizol and 2-methoxy-4-vinylphenol were detected in methanol extract of *in vitro* plantlets of *C. xanthorrhiza* while 2-methoxy-4-vinylphenol, α-Humulene and limone dioxide were foud in *in vitro* plantlets of *Z. aromaticum.*

Keywords*: in vitro*, plantlets*, C. xanthorrhiza, Z. Aromaticum*, disc-diffusion, MIC, GC/MS.

A. INTRODUCTION

Curcuma xanthorrhiza Roxb. and *Zingiber aromaticum* Vahl. are members of the Zingiberaceae family. They are found to be effective in the treatment of several diseases. Recent study indicated that *C. xanthorrhiza* and *Z. aromaticum* have antimicrobial activities. Xanthorrhizol is active compound from *C. xanthorrhiza*. It possess antibacterial activity against *Streptococcus* species that causes dental caries [1]. *Z. aromaticum* was also reported that their extract had potential activity in inhibiting the growth of *Helicobacter pylori* [2]. *Z. aromaticum* had also been proven to inhibit human microsomal cytochrome P450 3A4 (CYP 3A4) and CYP2D6 which oxidized and metabolized the clinical used drug [3].

Most plants do not produce secondary metabolites such as curcumin, xanthorrhizol, and essential oil in large amount. *In vitro* Culture Techniques can be used as alternatives for the production of secondary metabolite. Micropropagation is an *in vitro*

culture technique that is commonly used as the true-to-type propagation of selected genotypes. This technique can be achieved via various ways, such as shoot proliferation (shoot culture), node culture, de novo formation of adventitious shoots through shoots organogenesis or somatic embryogenesis, depending on the plant species and culture conditions [4]. Microprogation has many advantages which inculde rapid clonal multiplication of plantlets, especially the endangered species and economic crops recovery of disease-free clones and conservation of germsplasm, genetic improvement of crops and production of pharmaceutical or other natural product (secondary metabolites) in medicinal plant [5].

Based on present study above, the aim of this study is to compare the antimicrobial activity of the shoot culture of *Curcuma xanthorrhiza* and *Zingiber aromaticum* with antimicrobial activity of rhizome from both species.

B. MATERIAL AND METHOD

1. Extraction Method

Dried rhizome of field grown plants of *C. xanthorrhizza* and *Z. aromaticum* and dried *in vitro* shoots of *C. xanthorrhiza* and *Z. aromaticum* derived from solid medium were ground into powder form. One gram dried powder of each sample was extracted with 10 mL methanol using sonicator for 10 minutes. This procedure was repeated three times. The extracts were concentrated using the rotary evaporator (Eyela) at 40^0C.

2. Disc-diffusion Assay

Six microorganism, *Staphylococcus aureus* (Gram positive), *Bacillus substilis* (Gram positive), *Escherichia coli* (Gram -), *Pseudomonas aeruginosa* (Gram -), *Aspergillus niger* (fungi) and *Candida albican* (fungi) were used for assay.

The plant extracts were dissolved with methanol for the preparation of 100 mg/mL extract. Single colony of bacteria and fungi were spread on their suitable medium (NA, SDA and PDA medium). Sterile filter paper disc (6 mm in diameter) were impregnated with 25 µL extract (2500 µg/disc) and placed on inoculated agar. Methanol as the solvent for extract was used as negative control. Kanamycin (30 µg/mL), Penicillin (30 µg/mL), Vancomycin (30 µg/mL), Miconazole(30 µg/mL) and Amphotericin B (30 µg/mL) were used as positive control to determine sensitivity of each tested microbial species. Three replicates were used for each tested microbial species. All the inoculated plates were incubated at 32^0C for 24 hours for bacteria and 48 hours for fungus. Antimicrobial activity was determinate by measuring the inhibition zone for each type of microorganism.

3. Minimum Inhibitor Concentration (MIC)

The extract that showed antimicrobial activity in disk diffusion method was tested for minimum concentration of the extract for microbial inhibition. The MIC test protocol was conducted based on Sumathy et al. (2011). Bacteria, *E. coli* and *P. aeruginosa,* were cultured into nutrient broth and incubated overnight to get the turbidity of microbe equal to 0.5 McFarland standards (10^6 CFU/ml). Two-fold serial dilution of active crude extract (10-5000 µg/mL) was prepared in Nutrient Broth (NB) medium. Stock of bacteria (0.5 mL) was cultured into each concentration of crude extract and incubated at 32 - 34^{0}C. After 24 hours, the mixed solution of extract and microorganism were spread on NA medium and incubated at 32 - 34^{0}C for another 24 hours. The lowest concentration of extract that inhibited the growth of bacteria on the medium after incubation period was regarded as minimum inhibitory concentration.

4. Detection of active compound

Dried *in vitro* plantlets of *C. xanthorrhiza* and *Z. aromaticum* were grinded into powder form and the dried powder (0.2 g) of each species was extracted with 2 mL methanol using vortex for a few minutes. This procedure was repeated three times. The extracts were air dried in the fume cupboard and the concentrated extract was diluted with 1 mL methanol and filtered with 0.2 µm before injecting in the GC/MS machine (Agilent Technologies 6890 Series). The column used was HP-5 (5% phenyl methyl xyloxane) column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) and 5973 MSD detector. Helium was used as the carrier gas, at a flow rate 1mL/min. The column temperature was set on 150 ^{o}C for 2 min and then raised to 300^{0}C at 4^{0}C min^{-1}. The samples (1 µL) were injected with splitless technique at 280^{0}C. The chromatograms and the mass spectra of the samples were matched with the library data of GC/MS system for authenticity.

C. RESULT AND DISCUSSION

In this study, the methanol extract from the rhizomes and *in vitro* shoot of *Z. aromaticum* showed antimicrobial activity against *Pseudomonosa aeruginosa* and *Escherichia coli* (gram negative bacteria) (Table 1). It showed that *Z. aromaticum* contains bioactive compounds that possess antimicrobial activities. Studies have been indicated that several derivates of zerumbone were found to inhibit the growth of Gram-positive bacteria [6]. Flavonol glycoside [kaempferol-3-*O*-(2,3-di-*O*-acetyl-α-L-rhamnopyranoside) and kaempferol-3-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranoside)] isolated from the extract of *Z. aromaticum* inhibited the growth of human microsomal cytochrome CYP3A4 and CYP2D6 [7]. Methanol extract of Zingiberaceae plants from Taiwan showed antimicrobial activity against all food microorganisms (*Escherichia coli*, *Staphylococcus aureus*,

Salmonella enterica and *Vibrio parahaemolyticus*) [8]. The ethanolic extracts of plants belonging to Zingiberaceae family were also tested for antifungal activity. *Alpinia galangal*, *Curcuma zedoaria* and *Zingiber purpureum* have showed antifungal activity against wide variety fungal pathogen [9].

MIC test showed that extract from *in vitro* shoot of *Z. aromaticum* has the same activity as the mother plant extract with inhibition of *E. coli* growth at 625 µg/mL. Otherwise, *in vitro* shoot extract of *Z. aromaticum* has better inhibition (MIC 625 µg/ml) against bacteria *P. aeruginosa* than the methanol extract of mother plant (MIC 1250 µg/ml) (Table 2). This result showed that tissue culture technique could be use as an alternative to produce secondary metabolites from plants. Secondary metabolites of *Berberis buxifolia* (isoquinoline alkaloid berberine) produced via tissue culture techniques showed antimicrobial activity as that of the mother plant [10]. Methanol extract from *in vitro* and *in vivo* leaves of *Stevia rebaudiana* showed similar antimicrobial activity [11].

Table 1. Zone inhibition (mm) of *c xanthorrhiza* and *z. Aromaticum* crude extract

Samples	*Escheria coli*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*	*Candida albicans*	*Aspergillus niger*
Mother plant of *C. xanthorrhiza*	--	--	--	--	--	--
Mother plant of *Z. aromaticum*	10 ± 0.4	11 ± 0.3	--	--	--	--
In vitro plant of *C. xanthorrhiza*	--	--	--	--	--	--
In vitro plant of *Z. aromaticum*	11 ± 0.5	11 ± 0.3	--	--	--	--
Kanamycin (+ control)	15 ± 0.2	18 ± 0.4	ND	ND	ND	ND
Penicillin (+ control)	ND	ND	18 ± 0.8	ND	ND	ND
Vancomycin (+ control)	ND	ND	ND	15 ± 0.3	ND	ND
Miconazole (+ control)	ND	ND	ND	ND	14 ± 0.3	ND
Amphotericin B (+ control)	ND	ND	ND	ND	ND	19 ± 0.3

-- : no zone inhibition, ND: not determined

Table 2. Mic (minimum inhibitor concentration) of *z. Aromaticum* metahnol crude extract againts *e. Coli* and *p. Aeruginosa*

Bacteria	Methanol extract of *Z. aromaticum*	MIC (µg/ml)
E. coli	Mother plant	625
E. coli	*in vitro* plant	625
P. aeruginosa	Mother plant	1250
P. aeruginosa	*In vitro* plant	625

Through GC/MS scanning some compounds were detected in the methanol extracts of *in vitro* plantlets of *C. xanthorrhiza* and *Z. aromaticum* as shown in the total ion chromatogram (Fig. 1 and Fig. 2). Derivatives of curcumin compunds such as ar-curcumin and dihydrocurcumin were detected on *in vitro* plantlets of *C. xanthorriza* in the rhizomes of *C. xanthorriza* with other curcuminoids compounds (demethoxycurcumin and bis-demetoxycurcumin) (Table 3)**.** Curcumin is one of curcumionoid compound found in abundance in *Curcuma* species. It was reported that curcumin was found in *Curcuma longa* which was responsible for its anti-bacteria, anti HIV, antioxidant, anti-inflammatory and anti-tumor activities [12]. Xanthorrhizol was also detected in *in vitro* plantlets of *C. xanthorrhiza* (Table 3). Xanthorrhizol is one the volatile oil compound in rhizome of *C. xanthorrhiza*. This compound is only found in this species, but not in other *Curcuma* species [13]. This paper [14] reported that 1 mg ethanolic extract of *C. xanthorrhiza* contained 0.1238 mg of xanthorrhizol through standardization method using GC/MS. 2-methoxy-4-vinylphenol, a derivatives of 4-vinylphenol was found in *in vitro* plantlets extract of *C. xanthorrhiza* and *Z. aromaticum* (Table 3). They are widely used in industrial application such as flavouring compound for perfume, food and beverage [15]. This compound or their derivatives also reported found on *Oryza sativa* [16] and *Eupatorium betonicaeforme* [17]. It showed that *in vitro* plantlet of *C. xanthorrhiza* could produce the same secondary metabolites as their mother plant.

1,3-propanediol was one of the compounds that were detected in the *in vitro* plantlets extract of *Z. aromaticum* (Table 3). Derivatives of this compound also found in Soybean nodules as serinol (2-amino-1,3-propanediol) [18]. 1,3-propanediol is a bifunctional organic compound which can be used to produce polyesters, polyethers and polyurethanes [19]. α-Humulene and limonene dioxide was also detected in the *in vitro* plantlets extract of *Z. aromaticum* (Table 3). Based on [20] 27% of α-humulene was detected in the volatile oil of *Z. nimmonii* rhizome. Limonene (1.14%) was also detected in

the essential oil from leaves of *Zingiber zerumbet* [21]. This paper [22] reported that limonene and α-humulene possess antimicrobial activity. Hence both of these compounds found in the *in vitro* plantlet extract of *Z. aromaticum* might be responsible for the antimicrobial activity.

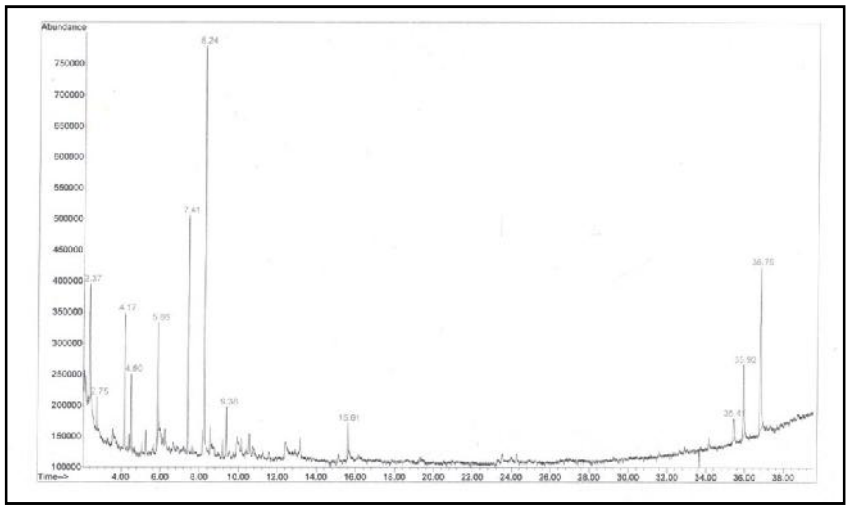

Figure 1. Total ion chromatogram of extract from *in vitro* shoot of *C. xanthorrhiza*

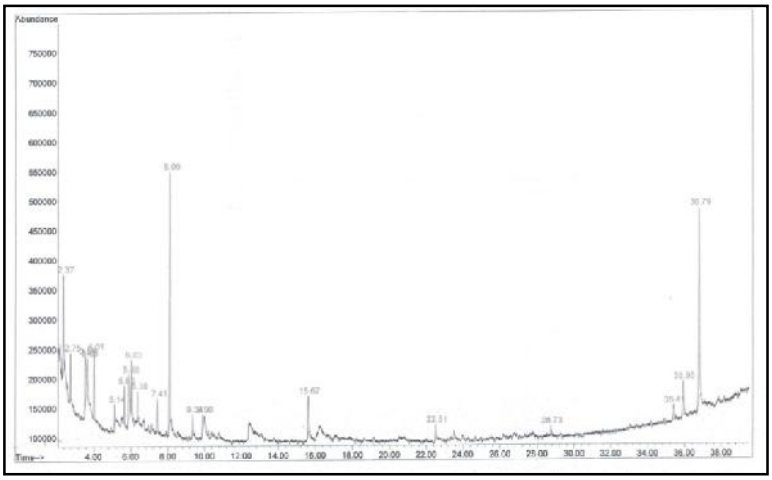

Figure 2. Total ion chromatogram of extract from *in vitro* shoot of *Z. aromaticum*.

Table 3. Active compounds detected from methanol extract of *in vitro* plantlets of *C. xanthorrhiza* and *Z. aromaticum* with GC/MS.

Sample	Compound Name	Retention Time (minutes)	Database Library Match (%)
C. xanthorrhiza	1. (+)-a-ar-curcumene, 2. dihydrocurcumene, 3. Xanthorrizol, 4. 2-methoxy-4-vinylphenol	4.172 4.500 8.234 2.749	99 95 95 91
Z. aromaticum	1. 2-methoxy-4-vinylphenol, 2. 1,3-propanediol, 3. α-humulene, 4. limonene dioxide, 5. Zerumbone	2.753 3.563 4.013 5.646 8.093	99 95 99 93 54

D. CONCLUSION

Mother plant and *in vitro* plant of *Z. aromaticum* have same bioactivity againts *E. Coli* and *P. aeruginosa*. The phytochemical of *in vitro* plant of *C. xanthorrhiza* and *Z. aromaticum* were same with their mother plant.

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