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ANTIMICROBIAL METABOLITE OF ZINGIBERACEAE ESSENTIAL OILS USING RESAZURIN A RAPID COLORIMETRIC DETECTION

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Abstract: *An antimicrobial assay using broth microdilution is the method accepted and approved by Clinical and Laboratory Standards Institute (CLSI). This technique was in vitro and minimized a volume of the medium that was performed the susceptibility of antimicrobial activity using resazurin rapid colorimetric detection. The technique had performance testing antimicrobial activities of essential oil from Zingiberaceae; Curcuma longa L, C. zedoaria (Christm.) Roscoe, C. aromatica Salisb, C. comosa Roxb., and Zingiber cassumunar Roxb. An approval this technique was confirmed using agar disc diffusion assay. All the essential extracted had bioactives against all testing pathogenic gram positive and gram negative bacteria and infectious yeast Candida albicans especially C. aromatica and C. comosa had inhibited Pseudomonas aeruginosa more than Ampicillin, antibacterial drug. Hence, it is expected that, from these results, C. aromatica and C. comosa rhizomes from different habitats should be used more rationally and effectively as natural pharmaceutical, culinary and cosmetic additives in the future.*

Keywords: *resazurin, antimicrobial, colorimetric, Zingiberaceae*

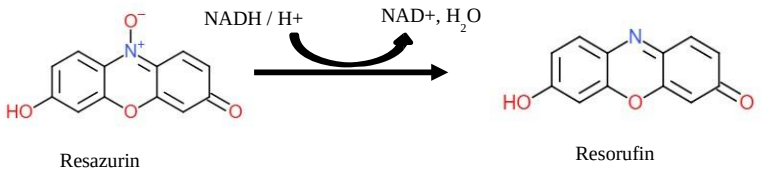
INTRODUCTION

Zingiberaceae, the well-known flower plant family had classification containing about 52 genera and more than 1,200 species [29]. These aromatic herbs grow in moist areas of the tropics and subtropics, including some regions that are seasonably dry. Most of the herbaceous perennial plant of the family Zingiberaceae, probably native to particularly in Southeast Asia. Its aromatic, pungent rhizome (underground stem) used as a spice, flavoring, food, and traditional medicine.

Curcuma longa L have demonstrated various health-related biological activities and several essential oil have turmeric rhizomes shown numerous beneficial effects for health maintenance and treatment of diseases [19, 23, 28]. Several Curcuma species are used medicinally in Bangladesh, Malaysia, India, Nepal, and Thailand [28] for treating pneumonia, bronchial complaints, leucorrhea, diarrhea, dysentery, infectious wounds or abscesses, and insect bites [19, 23, 28]. *Curcuma zedoaria* (Christm.) Roscoe, has been used in gastric treatment as traditionally in folk medicine in Thailand as piperine is the principal alkaloid in black peppers (*Piper nigrum* L.), which is a commonly included used anti-diarrheal formulation [20]. This plant had bioactive metabolite efforts to screen traditional medicinal plants exhibiting pharmacological potential and to characterize the compounds involved the anti-inflammatory effects [15], against gastric cancer [7], and antifungal activities [33]. *Zingiber cassumunar* Roxb. or current scientific name as *Z. montanum* (J.Koenig) Link ex A. Dietr. and is a synonym *Z. purpureum* Roscoe known locally as “Plai” in Thailand. It is a perennial herb, consisting of

underground rhizomes [13, 17, 26]. The rhizome extract metabolite had inhibition the gastric lesions, anti-inflammatory trend of muscle and ankle pain, and protecting normal hematopoietic cells from radiation-induced damage [4, 6, 18]. *Z. cassumunar* essential oils obtained has been *in vitro* analyzed antibacterial, antifungal and antiviral activity such as *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Cyptococcus neoformans*, and a potential of antiviral avian influenza virus (H5N1) [13, 27]. *Curcuma aromatica* Salisb rhizomes has excellent antimicrobial activities against *E. coli*, *P. aeruginosa*, *S. aureus*, *C. albicans* and *S. cerevisiae*. Essential oils more outstanding anti-inflammatory activities than ibuprofen, by remarkable suppressing the expression of inflammatory cytokines (TNF-α and COX-2). Consequently, the phytochemicals and bioactivities are significantly different from each other due to their different growth environment, and thus *C. aromatica* rhizomes and essential oils from different habitats should be discriminating utilized as pharmaceutical, food, culinary and cosmetic additives and in the future [8]. *C. aromatica* oil is a volatile oil extracted act as an anti-inflammation, anti-virus, and antioxidation agent that had the anti-carcinogenic properties of *C. aromatica* oil have been identified however, there have been in vitro mouse model evaluated the anti-tumour activity [11, 24, 25, 32]. *Curcuma comosa* Roxb. (Zingiberaceae) is an indigenous medicinal plant of Thailand. It is contributing agent to the total observed antioxidant activity [1]. *C. comosa* extracted metabolite prevents bone loss induced by estrogen deficiency. Therefore, *C. comosa* would be a potential alternative treatment for prevention of postmenopausal osteoporosis [12]. Rhizomes have been used for treatments of hemorrhoids, postpartum uterine bleeding, perimenopausal bleeding, uterine inflammation and promoting lactation [2, 3, 12]. From previous phytochemical studies, diarylheptanoids showed nematocidal activity [21] and estrogenic activity of phloracetophenone glucoside exhibited choleretic activity [3,2]. The *C. comosa* extract also increased plasma high density lipoprotein (HDL)-cholesterol and decreased plasma low density lipoprotein (LDL)-cholesterol. These results suggest that the *C. comosa* extract exerts a hypolipidaemic action by acceleration of lipid mobilization from extrahepatic tissue to the liver which subsequently increases excretion of cholesterol via the bile for excretion [21].

Antimicrobial screening practice had a variety technique for studying the microbial physiology, microbial growth, microbial metabolism, microbial drug susceptibility, and cultured cells and also had been established. Most common techniques are important to be able to reproductivity estimates, precision and can be easily observed. There must be performed viability of the population as the percentage of cells living or cells inhibition. The microbial growth or its inhibition can be measured by culturing techniques, e.g. viable counts, direct technique microscopic counts, cells turbidity measurement, bioluminescence and or fluorimetry. The most common technique using the reduction of tetrazolium salts from colorless or weakly colored. The dyes are dissolved in aqueous solutions to brightly colored derivatives known as formazans. This has been the basis of their use as vital colors in redox histochemistry and in biochemical applications for more than fifty years [16]. Resazurin (7-Hydroxy-3Hphenoxazin-3-one 10-oxide or IUPAC name, 7-hydroxy-10-oxidophenoxazin-10-ium-3-one,) show in Fig. 2 also known in other names Alamar Blue, Vybrant, and UptiBlue is a blue dye, itself weakly fluorescent [5] until it is irreversibly reduced to the pink colored and highly red fluorescent resorufin as shown in Fig. 1. It is used as an oxidation-reduction indicator in cell viability assays for both aerobic and anaerobic respiration of bacterial growth and survival [10]. Usually, it is available commercially as the sodium salt that easily to dissolve in water.



Resazurin Resorufin

Fig 1: Reszurin dissolved oxidizing agent with polar un chart molecule and dark blue colors, then reduction be uptake an electron oxidized form to pink color, Resorufin [5].

MATERIALS AND METHODS

A. Plant materials and preparation of extracts

Five selected Zingiberaceae are freshly picked one-year-old rhizomes of *C. longa* L obtain from Sakon Nakhon province, Earthen part of Thailand (Fig. 2 A, B), *C. zedoaria* (Christm.) Roscoe obtains from Nakhon Phanom Earthen part of Thailand (Fig. 2 C,D), *Z. cassumunar* Roxb. obtain from Kanchanaburi, Western part of Thailand (Fig. 2 E, F), *C. aromatica* Salisb obtain from Khon Kaen, Earthen part of Thailand (Fig. 2 G, H), and *C. comosa* Roxb. obtain from Chaiyaphum, Earthen part of Thailand (Fig. 2 I, J). There were collected during winter, December 2017 to March 2018. Rhizomes of Zingiberaceae plants were cleaned, cut into small pieces and put in a 5L flask. Sterile water was added at a ratio of 1:2 (w/v) and the plant materials were extracted for essential oils by a water distillation method in a modified Clevenger type apparatus for 6-8 h. the oils after they were extracted by mixing them with anhydrous sodium sulphate. The water was removed from essential oil by mixing them with a little of anhydrous sodium sulfate, and then stored at 4 °C for further use.

B. Bacterial strains and growth conditions

Antimicrobial activity of selected compounds was tested against the foodborne pathogens. The bacteria culture was grown in Mueller–Hinton agar (MHA) and broth (MHB) medium (pH 7.3). Vribrio were using Mueller–Hinton agar (MHA) medium and broth (MHB) plus 1.5% NaCl (pH 7.3). Candida was using Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) as a medium. Most of the testing cultures came from American Type Culture Collection (ATCC) that recommended for drug susceptibility testing isolates. The bacteria were using *Bacillus subtilis* (ATCC6633) stand for gram-positive, rod-shaped bacteria, *Enterococcus faeca*lis (ATCC2921) stand for gram-positive, diplococci and single cocci, *Staphylococcus aur*eus (ATCC25923); gram-positive, round-shaped and grape-like clusters, *Pseudomonas aeruginos*a (ATCC741), *Klebsiella pneumoniae* (TISTR1843), *Escherichia coli* (ATCC25922), *Salmonella typh*i (clinical isolate) *Vibrio parahaemolyticus* (5HP,

Centex Shrimp Mahidol), V. parahaemolyticus (NX89, Centex Shrimp Mahidol) gram-negative, rod-shaped, and infected yeast *Candida albicans* (ATCC90020). Plates performed incubation overnight at 37 °C for 18-24 hr in ambient. Chosen the fresh single 4-5 colonies for the MIC procedure.

C. Disk Diffusion Method

The essential oil extract of Zingiberaceae plants was tested against the above Gram-positive, Gram-negative bacteria and infectious yeast by the disk agar diffusion method. The method for antibacterial disk diffusion susceptibility following a manual of antimicrobial susceptibility testing in a manual of antimicrobial susceptibility testing guideline [22, 16] and antifungal *C. albicans* disk diffusion susceptibility testing following a manual method for antifungal disk diffusion susceptibility testing of yeasts in NCCLS guideline [22]. These bacteria were grown on Mueller–Hinton agar (MHA) medium (pH 7.3) except *Vibrio* spp. were supplement with 1.5% w/v NaCl and yeast using Sabouraud dextrose agar (SDA). Agar media were poured into the plates to uniform depth of 5 mm and allowed to solidify. The microbial suspensions were prepared by spectrophotometer using culture broth adding the sufficient sterile medium to adjust the transmittance to that produced by a 0.5 McFarland standard match to an optical density (OD) 0.1 at 625 nm wavelength. This procedure will bacterial yields stock suspension $1x10^8$ cfu/ml and yeast stock suspension of $1x10^6$ to $5x10^6$ cfu/ml. The microbial suspension was streaked over the surface of media using a sterile cotton swab to ensure the confluent growth of the organism. The disks used were Whatman® No. 1 papers, 6 mm in diameter were then aseptically applied to the surface of the agar plates at well-spaced intervals. The plates were incubated at 37 °C for 24 h and observed growth inhibition zones, including the diameter of the disks, were measured. Control disks impregnated with 10 µl of the solvent DMSO [30, 31].

D. Determination of the minimum inhibitory concentration (MIC)

The bacterial cultures were grown in broth MHB using the horizontal orbital incubator shaker (Forma®435) at 150 rpm and 37 °C for several hours till logarithmic phase (O.D.625 ~0.1) was reached. After growth, the cells suspensions were prepared by spectrophotometer using culture broth adding a sufficient sterile medium to adjust the transmittance that related turbidity standard equivalent to a 0.5 McFarland standard match to an optical density (OD) 0.1 at 625 nm wavelength [12]. This result in a suspension containing approximately 1-2 × 10^8 colony-forming units (CFU)/mL [22, 30, 31]. Optimally within 15 minutes of preparation, dilute the adjusted inoculum suspension in MHB so, after inoculation, each well contains approximately 5 × 10^5 CFU/mL

(range 2-8 × 10^5 CFU/mL). The MIC was determined by using flat-bottom, polystyrene, non-tissue-culture-treated 96-well microliter plates. For each well, 100µl of MHB were added. Then the experimental essential oil extract, with an initial volume of 100µl, were added to their corresponding wells, followed by a series of serial dilutions with 100µl each time to achieve the desirable concentrations from highest to the lowest level. Then 100µl of microorganism inoculum dilution were added to each well. The micro dilution suspension was diluted the inoculum volume of 0.5 McFarland suspension match to 1:20 yield as 1-1.5 × 10^8 CFU/mL which indicated a final concentration of approximately 15 × 10^5 CFU/mL following on Clinical and Laboratory Standards Institute [22, 30, 31].

E. Colorimetric viability assay

Resazurin sodium salt (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) was purchased from Sigma-Aldrich (cat. R0717). Resazurin was prepared by dissolves in sterile distilled water with 50 µM concentration then added to 30 µl in each 96-well plate from the total volume 200 µl. At the end of 20-30 min incubation, color development were observation was reduction dark blue to pink color.

RESULTS AND DISCUSSIONS

A. Plant extraction and yields

The results of essential oils extraction from five Zingiberaceae herbal plants (F.g.1), *C. longa* L, *C. Zedoaria* (Christm.) Roscoe, *Z. cassumunar* Roxb., *C. aromatica* Salisb, and *C. comosa* Roxb., were shown in Table 1. Fresh rhizome were obtained 0.3-0.4% yield (w/w) by a water distillation method in a modified Clevenger type apparatus for 6-8 h. The essential oils from *C. longa* and *C. zedoaria* gave pale-yellow and fresh fragrant, *Z. cassumunar* Roxb. was colourless and soft spicy, *C. aromatica* Salisb was brown color and sharp foul-smelling, and *C. comosa* Roxb. was yellow colour and mild fragrant.

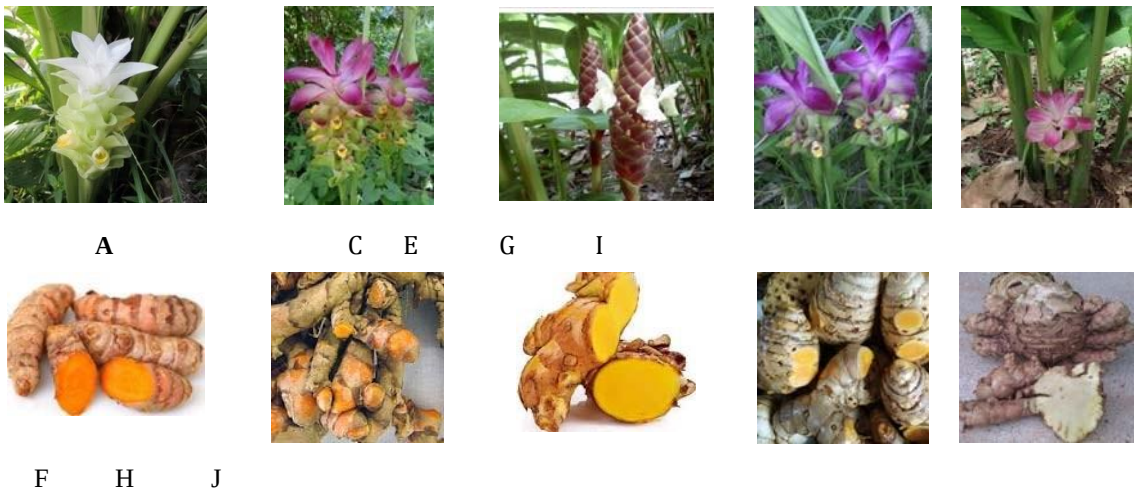


Fig 2: Five Zingiberaceae herbal plants, C. longa L show flower (A) and rhizome (B), C. zedoaria (Christm.) Roscoe show flower (C) and rhizome (D), Z. cassumunar Roxb. show flower (E) and rhizome (F), C. aromatica Salisb show flower (G) and rhizome (H), and C. comosa Roxb. show flower (I) and rhizome (J).

Table 1: The essential oil extract from herbal plant, Zingiberaceae

Plant species	Plant part	essential oils	Percent yield (%)
Curcuma longa L	rhizome	pale yellow	0.358 ± 0.052
Curcuma zedoaria (Christm.) Roscoe	rhizome	pale yellow	0.332 ± 0.033
Zingiber cassumunar Roxb.	rhizome	colorless	0.410 ± 0.061

Curcuma aromatica Salisb	rhizome	brown	0.472 ± 0.087	
Curcuma comosa Roxb.	rhizome	yellow	0.381 ± 0.054	

B. Agar Disk Diffusion Sensitivity Testing

Development of disk diffusion assay for determining the microbial susceptibility was modified from NCCLS guideline in 2015. A filter-paper disk, impregnated with the essential oil extracted compound to be tested, is placed on the surface of the agar. The bioactive compound diffuses from the filter paper into the agar. The concentration of the compound will be the highest next to the disk and will decrease as a distance from the disk increases. If the compound is effective against microbes at a certain concentration, no microbial colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is where the zone of inhibition was measured in Table 2. Fives essential oil of *C. longa, C. zedoaria, C. aromatica, C.comosa, and Z. cassumunar* with concentration 15-30 mg were inhibited all gram positive bacteria, *B. subtilis, E. faecalis, and S. aureus.* The gram negative bacteria had effective found in *C. aromatica* especialy *P. aeruginosa. V. parahaemolyticus* (5HP) and *V. parahaemolyticus* (NX89) were shown clear zone of in hibition in all testing. *C. albicans* was the effective clear zone 10-18.00 mm of essential from five selecetd Zingiberaceae (Table 1).

Table 2: Agar disk diffusion sensitivity testing of essential oils, Zingiberaceae

	Inhibition zone of essential oil extract from rhizome (mm)				
Zingiberaceae Anti-microorganism	*C. longa* (15 mg)	*C. zedoaria* (30 mg)	*C. aromatica* (30 mg)	*C.comosa* (15 mg)	*Z. cassumunar* (30 mg)
B. subtilis	12.5 ± 0.5	12.8 ± 0.5	17.6 ± 1.5	14.3 ± 1.0	9.8 ± 0.5
E. faecalis	9.5 ± 0.5	8.3 ± 0.5	10.8 ± 0.5	9.8 ± 1.0	6.2 ± 0.5
S. aureus	10.0 ± 0.5	9.8 ± 1.0	15.3 ± 1.5	15.0 ± 1.0	13.3 ± 1.0
P. aeruginosa	NI	NI	8.0 ± 1.0	6.0 ± 0.5	NI
E. coli	6.0 ± 0.5	6.0 ± 0.5	8.25 ± 1.0	6.75 ± 1.0	8.0 ± 1.0
S. typhi	6.0 ± 0.5	6.0 ± 0.5	6.0 ± 1.0	NI	8.0 ± 1.0
V. parahaemolyticus XN89	6.0 ± 0.5	6.0 ± 0.5	7.5 ± 1.0	11.5 ± 1.0	9.5 ± 1.0
V. paraparahaemolyticus 5HP	10.0 ± 1.5	8.8 ± 1.0	12.8 ± 1.0	10.5 ± 1.0	8.8 ± 1.5
C. albicans	10.3 ± 1.5	11.3 ± 1.5	11.0 ± 1.0	11.3 ± 0.5	18.0 ± 2.5

NI = non inhibition

C. The minimum inhibitory concentration (MIC)

In our experiment using whole part of fresh rhizome extraction in order to primary screening the antibacterial and infectious yeast activity. The result of essential oil extract screening for antimicrobial of the minimum inhibitory concentration (MIC) where show in Table 3. The result indicated that essential oil of *C. aromatica* Salisb and *C. comosa* Roxb. had effective against all testing gram positive bacteria, gram negative bacteria, and infectious yeast *C. albicans*. Most of the essential oils had strong effective gram positive bacteria and infectious yeast *C. albicans*.

D. The colorimetric viability assay for minimum inhibitory concentration (MIC)

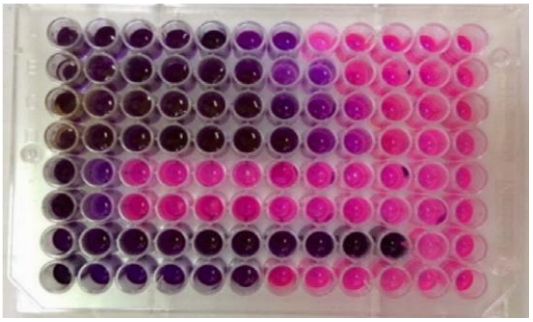
The MICs obtained by the rapid colorimetric assay using resazurin dectection. The broth microdilution methods had performed crude essential oils from plant extract to interpretive categories of anti-bacterial and infectious yeast susceptible inhibition, intermediate, and resistant strains show in Table 3 and Fig. 3. Resazurin has developed the colorimetric viability assay for minimum inhibitory concentration (MIC) by changing from dark blue color to reduced form pink color (Fig. 3) in an example of gram positive bacteria *E. faecalis* and gram negative bacteria *V. paraparahaemolyticus* 5HP which had not interference from 1.5% salt supplement. The detection was performed by dissolves resazurin hydrochloride in sterile distilled water with 50 µM concentration then added to 30 µl in each 96-well plate and incubation for 20-30 min. The detection of infectious yeast, *C. albicans* was quite difficult detection, it had long incubation more than 1-2 hr and the color was changing from violet color to reduced form pale pink color

Table 3: Comparative MIC values of five essential oils against bacteria and infectious yeast by microdilution broth asay.

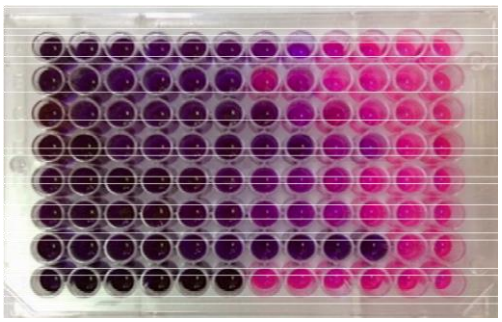
Minimum inhibitory concentration (MIC) of essential oil (µg)

Plant species Anti-microorganism	*C. longa*	*C. zedoaria*	*C. aromatica*	*C.comosa*	*Z. cassumunar*	Ampicillin
B. subtilis	195	195	24	98	6,250	98
E. faecalis	781	391	391	391	25,000	98
S. aureus	1,563	1,563	195	391	781	24
P. aeruginosa	NI	NI	12,500	50,000	NI	NI
E. coli	25,000	25,000	3,125	3,125	3,125	195
S. typhi	50,000	50,000	50,000	50,000	12,500	98
V. parahaemolyticus XN89	50,000	12,500	50,000	6,250	50,000	391
V. paraparahaemolyticus 5HP	781	1,563	391	781	781	98
C. albicans	195	50,000	12,500	6,250	12,500	NI

NI = non inhibition



E. faecalis



V. paraparahaemolyticus 5HP

Fig 3: Example of colorimetric viability assay for minimum inhibitory concentration (MIC) in 96-well plate comparison using Resazurin detection anti E. faecalis and V. paraparahaemolyticus 5HP. Resazurin has a

dark blue color for bacterial inhibition and then reduced to pink color that means the non-inhibition concentration of testing well.

CONCLUSION

Zingiberaceae plants, *C. longa* L, *C. zedoaria* (Christm.) Roscoe, *Z. cassumunar* Roxb., *C. aromatica* Salisb, and *C. comosa*

Roxb. had been using in to assess traditional Thai claims about the therapeutic potential of medicinal plants and to select plants for future phytochemical research. Nonpolar volantile essential oil extraction from fresh rhizome had a potential extracted bioactive metabolite gave 0.3-0.4 % (w/w) yield. Their properties had potential antibiotic susceptibility tests from broth agar disc diffusion assay and broth microdilution methods that provide the MIC used to define anti Gram-positive spore forming *B. subtilis*, anti the bacterium inhabiting the gastrointestinal tracts of humans *E. faecalis*, and anti *S. aureus*, which a common cause of skin infections including abscesses, respiratory infections such as sinusitis, and food poisoning [9]. This bacterium is Gram-negative that considerable medical importance, a multidrug resistant pathogen recognized for its ubiquity, and its intrinsically advanced antibiotic resistance mechanisms such as *P. aeruginosa*, *E. coli*, *S. typhi*, and *V. paraparahaemolyticus* were significant inhibition by the essential oils of *C. aromatica* Salisb, and *C. comosa* Roxb. whereas antibacterial drug Ampicillin was not effective in *P. aeruginosa*. All of essential oils had also effective in *C. albicans* compare to antibacterial drug Ampicillin was not effective. The colorimetric assay using Reszurin had been enhancing the viability visual for clear-cut MIC broth microdilution assay in 96-well plate. The concentration of them was 50 mM with minimal volume 30 µl per total 200 µl volume. According to reduction reaction to have uptake an electron from living microorganism changing dark blue color grained electron from living bacteria changed to reducing from Resorufin had pink color (Fig. 1). From the result of the colorimetric assay, Resazurin had advantage for bacterial detection whereas anti yeast detection could be long incubation period and increasing the concentration of resazurin. However, more studies are needed to fully these of potential herbal plant to recommend extraction and purification its wide use for bioactive metabolite for adapted drug susceptibility testing methods or screening anti-microbial assay in future.

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