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Comparative Antibacterial Effectiveness of Orange Peel Extracts from Five Varieties Against Strain of Multi Drug Resistance *Escherichia coli*

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ABSTRACT

The purpose of this study was to investigate the antibacterial activity of orange peel extracts from five different orange varieties in North Sumatera against resistant *Escherichia coli* bacteria and compare it to the effectiveness of vancomycin and erythromycin. To assess antibacterial activity, orange peel extracts were prepared from five different orange varieties, namely *Citrus aurantifolia*, *Citrus microcarpa* Bunge, *Citrus limon* L., *Citrus sinensis* L., and *Citrus hystrix*. Antibacterial activities were evaluated using the microdilution method to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The best antibacterial activity was determined using the smallest MIC and MBC values. The research found that all five orange peel extracts exhibited significant antibacterial activity compared to the standard antibiotics vancomycin and erythromycin. The most potent inhibitory effect was observed with the *Citrus hystrix* peel extract, with the lowest MIC and MBC (125 and 250 µg/mL), followed by *Citrus aurantifolia* (250 and 500 µg/mL), and *Citrus sinensis* L. (500 and 500 µg/mL). The results showed significant antibacterial activity of orange peel extracts from five different orange varieties in North Sumatera against resistant *Escherichia coli* bacteria.

Keywords: Antibiotic resistance, citrus peel extracts, *Escherichia coli*, MIC, MBC.

INTRODUCTION

Antibiotic resistance is one of the most pressing global health issues of the 21st century. This phenomenon occurs when bacteria change and become resistant to the antibiotics used to kill them [1]. As a result, infections that were previously easily treatable become more difficult to overcome, increasing the risk of disease spread, higher morbidity and mortality [2]. The main cause of antibiotic resistance is the overuse and inappropriate use of antibiotics, both in human medicine and in animal husbandry practices [3]. When antibiotics are used inappropriately, resistant bacteria can survive and multiply, while antibiotic-sensitive ones die, creating selection pressure that strengthens resistant populations [4]. This is compounded by the lack of development of new antibiotics, largely due to economic and regulatory challenges in drug development [5]. Therefore, global efforts to use antibiotics more wisely, strengthen health systems, and encourage innovation in new drug development are essential to address this problem. one of the bacteria that has experienced a lot of resistance is *E. coli* bacteria.

Multi Drug Resistance (MDR) of *E. coli* occurs through several mechanisms [6]. One of them is by producing extended-spectrum β-lactamase enzymes (ESBLs) which are horizontally transferred to other non-resistant bacteria through plasmid transfer, and cause an increase in the number of resistant bacterial colonies, resulting in a significant surge in resistance cases in the world [7]. Efforts to explore natural materials should focus on agricultural waste, which if not optimally utilized can cause problems for the environment [8]. *Citrus aurantifolia* includes horticultural waste whose utilization is not optimal. *C. aurantifolia* accounts for 50-65% of residue by weight [9]. Unutilized *C. aurantifolia* waste can cause foul odor to cause environmental pollution [10].

C. aurantifolia contains active compounds in the form of flavonoids such as naringin, hesperidin, naringenin, hespiritin, rutin, nobiletin, and tangeretin. And in Dewi's research, ethanol extract of *Citrus sinensis* peel from the maceration method can be used as an antibacterial has antibacterial activity on *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi* bacteria and is included in the activity whose inhibitory power is strong because it is in the range (10-20 mm) [11]. Medicinal plants that can be utilized as antibacterial on *E. coli* and *S. thypi* are *Citrus limon* peel which contains many bioactive compounds such as flavonoids, karetenoids, limonoids, tannins, and phenols found in *Citrus limon* peel [12]. The main components in *Citrus hystrix* peel essential oil are limorene (29.2%) and Beta pinene (30.6%) [13]. In addition to essential oils, *Citrus hystrix* peel also contains saponins and secondary metabolites such as flavonoids, coumarins and steroidal tritreponoids [14]. Based on the antibacterial potential possessed by Citrus species plants of the *Rutaceae* family, further research was carried out using *Citrus aurantifolia* peel ethanol extract, *Citrus microcarpa* Bunge peel ethanol extract, *Citrus limon* L. peel ethanol extract, *Citrus sinensis* L. peel ethanol extract, and *Citrus hystrix* peel ethanol extract by analyzing their activity against resistant bacteria *E. coli*.

MATERIALS & METHODS

Materials

Materials such as *Citrus aurantifolia*, *Citrus microcarpa* Bunge, *Citrus limon* L., *Citrus sinensis* L, *Citrus hystrix*, antimicrobial erythromycin, NaCl 0.9%, Brain Heart Infusion (BHI) Broth, BHI Agar, Separate the *Escherichia coli* bacteria using the following methods: distilled water, 1% DMSO, 99% ethanol, 70% alcohol, and Ethidium Bromide.

Tools

The tools that will be used in the research include Measurement cups (Iwaki), erlenmeyer (Duran), beaker glass (Pyrex), measuring cup (Pyrex), maceration bottle, evaporator dish, watch glass, slide, petri dish (Petriq), test tube (Iwaki), test tube rack, funnel, eppendorf tube, microtip, micropipette, 96-well microplate, vacuum rotary evaporator, UV-Vis spectrophotometer, shaker incubator, vortex, digital microscope, autoclave, digital microscope, ELISA plate reader, oven, hot plate, analytical balance, spatula, spirit lamp, tweezers

Bacterial Strains

The bacterial resistance was obtained by the MERO Foundation, Bali, Indonesia.

Plant Identification

The *Rutaceae* family of plants includes *Citrus aurantifolia*, *Citrus microcarpa* Bunge, *Citrus limon* L., *Citrus sinensis* L., and *Citrus hystrix*, according to the results of plant identification conducted at the Medanense Herbarium Plant Systematics

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Extracts Preparation

In a glass container, 2500 mL of 96% ethanol was macerated with 250 g of simplicia powder. First, the macerated container was filled with 75% solvent to the brim. The mixture was allowed to sit for 24 hours while stirring from time to time. This procedure was continued. The macerate was then collected in a container and the remaining 25% of the solvent was added. Maceration was performed up to the seventh day. To obtain a more concentrated ethanol extract, the macerate products were collected and concentrated using rotary evaporators.

Phytochemical Screening

Qualitative Phytochemical Identification was conducted to determine the chemical constituents of *Citrus hystrix* peel extracts, including flavonoids, alkaloids, saponins, tannins, glycosides, and steroids/ terpenoids.[11,12,13]

Preparation of bacteria

Resistant *E. coli* was acquired from the Marine Education & Research Organization (MERO), which supports marine education and research. To rejuvenate the bacteria for metabolic activity, a 24-hour incubation at 37°C after culturing in pure culture is conducted, following initial sterilization of the Bio Hazard Safety Cabinet (BHSC) with UV light and a 70% ethanol spray. A bacterial suspension preparation involves adding 500 µL of multidrug resistant *E. coli* to BHIB media, vortexing, and incubating for 16-24 hours at 37°C. For testing, a Mc Farland 0.5 standard equivalent to 1.5 x 10^8 CFU/ml is prepared, and bacterial suspensions are matched to this standard to estimate colony numbers. To achieve a colony count of 10^6 CFU, a dilution from a 10^8 CFU concentration is made in a sterilized BHSC environment, leading to a suspension with 1.5 x 10^6 CFU after homogenization.[15].

Antibacterial Activity Test

The method of microdilution was employed to assess antimicrobial properties and to establish the values for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The guidelines from the Clinical Laboratory Standard Institute M7-A6 (2014) were followed to ascertain MIC values. The procedure involved using a 96-well microplate and the standard broth microdilution method, starting with a bacterial inoculum of 1 × 10^6 CFU/mL. Each well was initially filled with BHI media, followed by the addition and two-fold dilution of the test sample. Afterwards, 100 µL of bacterial suspension was dispensed into each well, and the microplates were then incubated at 37°C for 24 hours. MIC is defined as the concentration at which bacterial growth is visibly inhibited, while MBC is the lowest antimicrobial concentration that prevents bacterial growth in BHI medium. To determine MBC, colonies from the MIC level were transferred to a petri dish with agar and incubated for another 24 hours at 37°C, with the lowest concentration that halted microbial growth in the dish being identified as the MBC. [16]

RESULT AND DISCUSION

Extraction Results

Table 1 displays the results of the computation of the extract yield from the five collected ethanol extracts. Based on the Indonesian Herbal Pharmacopoeia (FHI), the five *Rutaceae* ethanol extracts had a percentage yield of at least 15%. These include *Citrus aurantifolia*, which is 15.6%; *Citrus microcarpa*, which is 15.7%; *Citrus limon*, which is 15.14%; *Citrus sinensis*, 15.76%; and *Citrus hystrix*, which is 15.72 percent. Thus, these five extraction findings satisfied the Indonesian Herbal Pharmacopoeia (FHI) standards.

Table 1. Extraction rendement

Sample	Weight of Dried Sample (g)	Weight of Extract (g)	% Yield	FHI Requirements (%)
Citrus aurantifolia	500	78.5	15.7	Not less than 15
Citrus macrocarpa	350	52.99	15,14	Not less than 15
Citrus limon	250	39.42	15.76	Not less than 15
Citrus sinensis	350	55.02	15.72	Not less than 15
Citrus hystrix	300	46.8	15.6	Not less than 15

Phytochemical Screening

The *Rutaceae* family ethanol extract includes secondary metabolite compounds such as flavonoids, tannins, glycosides, steroids/triterpenoids, alkaloids, and saponins, which are part of the five *Rutaceae* family ethanol extracts, according to the results of the phytochemical screening examination shown in Table 2.

Table 2. Results of phytochemical screening of ethanol extracts from the *Rutaceae* Family

Group	*Citrus aurantifolia*	*Citrus microcarpa*	*Citrus limon*	*Citrus sinensis*	*Citrus hystrix*
Alkaloids	+	+	+	+	+
Flavonoids	+	+	+	+	+
Tannin	+	+	+	+	+
Saponins	+	+	+	+	+
Glycosides	+	+	+	+	+
Steroids/triterpenoids	+	+	+	+	+

Antibacterial activity of *Rutaceae* family extracts

The minimum bactericidal concentration (MBC) value was determined by subculturing the MIC and 2 MIC concentrations from the microdilution plate onto Brain Heart Infusion Agar (BHIA). MBC of ethanol extract from citrus fruits: *Citrus microcarpa*, *Citrus limon* L., *Citrus sinensis* L., and *Citrus hystrix*. Table 3 shows the concentrations of vancomycin and erythromycin, which were 7.8 µg/mL, as well as the concentrations against *Escherichia coli*, which were 250 µg/mL, 1000 µg/mL, 1000 µg/mL, 500 µg/mL, and 125 µg/mL. The results of MIC and MBC can be seen on table 3 and 4, moreover visual observation showed in figure 1.

Table 3. Minimum inhibitory concentration (MIC) of *Rutaceae* family extract, positive control and negative control of bacteria *Escherichia coli*

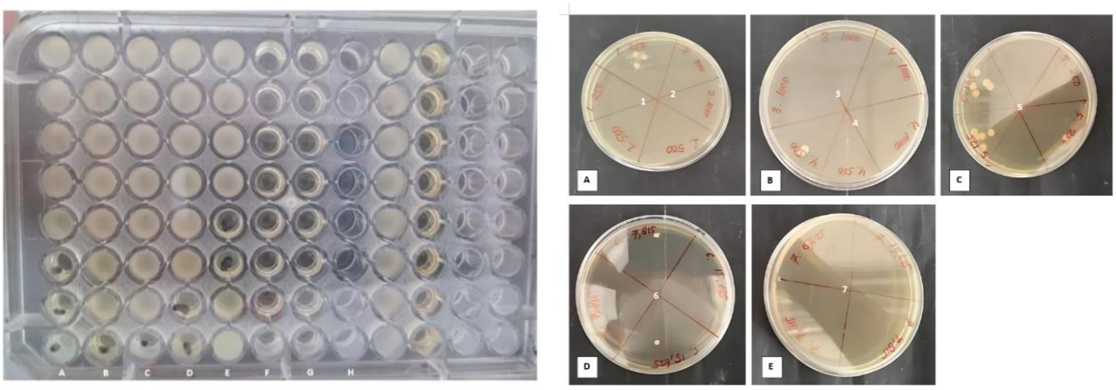
Sample	MIC (µg/mL) ± SD
Citrus aurantifolia	250
Citrus macrocarpa	1000
Citrus limon	1000
Citrus sinensis	500
Citrus hystrix	125
Vancomycin	7.8125
Erythromycin	7.8125

Table 3 shows the Minimum Inhibitory Concentration (MIC) values, which represent the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The lower the MIC value, the more effective the compound is considered to be at inhibiting bacterial growth.

Table 4. Minimum bactericidal concentration values (MBC) of *Rutaceae* family extract, positive control and negative control of bacteria *Escherichia coli*

Sample	MBC (µg/mL) ± SD
Citrus aurantifolia	500
Citrus macrocarpa	1000
Citrus limon	1000

Citrus sinensis	500
Citrus hystrix	250
Vancomycin	7.8125
Erythromycin	7.8125



I II

Figure 1. I = Microdilution MIC result. A: *Citrus aurantifolia* 250 mcg/ml, B: *Citrus macrocarpa* 1000 mcg/ml, C: *Citrus limon* L 1000 mcg/ml, D: *Citrus sinensis* L 500 mcg/ml; E: *Citrus hystrix* 125 mcg/ml, F: Erythromycin 7.8125 mcg/ml, G: Vancomycin 7.8125 mcg/ml, H: Negative Control (DMSO 1%).
II: Result of MBC examination A: 1. *Citrus aurantifolia* 250 and 500 mcg/ml 2. *Citrus macrocarpa* 1000 mcg/ml, B: 3. *Citrus limon* 1000 mcg/ml 4. *Citrus sinensis* 500 and 1000 mcg/ml, C: 5. *Citrus hystrix* 125 and 250 mcg/ml, D: 6. Erythromycin 7.8125 and 15.625 mcg/ml, E: 7. Vancomycin 7.8125 and 15.625 mcg/ml

These results suggest that while all tested extracts have some inhibitory and bactericidal effects on *E. coli*, *Citrus hystrix* extract is the most potent of the plant-derived substances. However, the standard antibiotics vancomycin and erythromycin are significantly more potent than the plant extracts. The exploration of natural antibacterial agents is more pertinent than ever. The research table provided in Table 3 demonstrates a concerted effort to evaluate the antibacterial properties of various citrus peel extracts of the *Rutaceae* family against *Escherichia coli*, a common bacterial pathogen. The results indicate that *Citrus hystrix* peel extract is the most effective among the tested citrus extracts, with the lowest MIC and MBC values. The potency of this extract could be attributed to specific phytochemicals known for their antimicrobial activity. In contrast, the extracts from *C. aurantifolia*, *C. sinensis* L., *C. microcarpa*, and *Citrus limon* L. showed varying degrees of efficacy, with *C. aurantifolia* and *C. hystrix* requiring the highest concentrations to inhibit and kill *E. coli*. These differences underscore the diversity within the *Rutaceae* family's phytochemical composition and the need for targeted selection of species and extraction methods for antibacterial use [17].

Comparing the MIC and MBC values of the citrus extracts with those of antibiotics vancomycin and erythromycin presents a stark difference in efficacy. The antibiotics are far more potent, which reflects their refined and targeted mechanisms of action against bacterial cells. However, the emergence of antibiotic resistance challenges the sustainability of relying solely on such drugs. In this regard, citrus extracts, despite their lower potency, hold promise as alternative or adjunctive therapies [18]. They could be particularly useful in applications where lower antibacterial strength is sufficient or where the use of natural products is preferred, such as in food preservation or as antiseptics in personal care products [19].

The mechanism of action behind the antibacterial properties of citrus peel extracts may involve the disruption of bacterial cell walls, interference with enzyme activity within the bacterial cells, or the impairment of bacterial communication systems, known as quorum sensing [20]. The exact mechanisms are likely as diverse as the phytochemicals present in the peels, and further research into these areas could unlock new antibacterial strategies and compounds [21].

When contextualizing the present research within the wider scientific literature, it is clear that there is a degree of variability in reported results for citrus-based antibacterial agents. Factors contributing to this variability include the method of extraction, the part of the plant used, the ripeness of the fruit at the time of extraction, the solvent used, and even the geographical origin of the plant material [22]. Moreover, different studies often target different bacterial strains, each with its own specific susceptibilities and resistances, making direct comparisons complex.

Despite these variations, the practical applications of citrus peel extracts in inhibiting bacterial growth are evident. Beyond their potential use in medicine, these extracts could serve in food preservation, where their natural origin and broad acceptance by consumers could provide a competitive advantage over synthetic preservatives [23]. In the cosmetic industry, their antibacterial properties could contribute to the formulation of natural skin care products, especially in products aimed at combating acne, which is often caused by bacterial infections [24].

Looking forward, it is imperative that the research not only continues to evaluate the efficacy of these extracts against a wider range of bacteria but also moves towards in vivo studies to confirm their safety and effectiveness within living organisms. The exploration of synergistic effects between different extracts or between extracts and antibiotics could reveal combinations that are more effective than their individual components. Additionally, assessing the potential development of resistance to these natural agents is essential, as is the case with any antibacterial compound.

CONCLUSION

Orange peel extracts from five distinct orange varieties from North Sumatera were shown to have strong antibacterial efficacy against resistant Escherichia coli bacteria.

Declaration by Authors

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