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Antibacterial activity of ethanol extract of kingkit orange (*Triphasia trifolia*) fruit and leaves against *Escherichia coli*

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Abstract

Escherichia coli is a gram-negative bacteria known to cause diarrhea and infection and is frequently found in foodstuffs. These bacteria may be susceptible to the microbial activity of kingkit orange due to the presence of active compounds, such as flavonoids, tannins, terpenoids, and alkaloids. Antibiotics are commonly used to treat infections, but inappropriate dose levels can cause resistance. Therefore, this study aims to determine the antibacterial effectiveness of kingkit orange fruit and leaf extracts against *E. coli* infection by in vitro. The materials used included fruit and leaf extract of the kingkit oranges with five concentration levels, i.e., 5%, 10%, 15%, 20%, and 25%. Observational data were analyzed using the complete randomized block design method and the least significant different follow-up test at the 5% level. The best concentration of kingkit orange fruit extract to reduce the growth of *E. coli* was 25%, and the diameter of the inhibition area was 6.65 mm with moderate inhibition strength. The best concentrations of Kingkit orange leaf extract to reduce the growth of *E. coli* were 20% and 25% with diameters of inhibition areas of 6.82 mm and 7.15 mm, respectively, with medium inhibition strength. It can be concluded that extracts from kingkit orange fruit and leaf have antibacterial activity, a potential substitute for antibiotics.

1. Introduction

Diseases caused by microbes, such as bacteria, viruses, fungi, and parasites, are commonly referred to as infections (Jawetz *et al.,* 2005). The bacteria that most often infect humans is *E. coli*, and it causes urinary tract infections, diarrhea, sepsis, and meningitis (Grafton-Cardwell *et al.,* 2013). Diarrhea is a digestive disorder characterized by the consistency of liquid stools with a frequency of defecation of more than 3 times a day.

According to WHO, diarrheal infections that occur in developing countries such as Indonesia are caused by *Escherichia coli*, with a mortality rate of 200,000 (Chandra *et al.,* 2022). It is an example of gram-negative bacteria in the form of bacilli, which do not form spores and are facultatively anaerobic. *Escherichia coli* is also present in the human large intestine with the function of preventing colonization of pathogenic bacteria (WHO, 2013). The development of bacteria in the body that exceeds the normal threshold can cause illness with symptoms of diarrhea, decreased appetite, and body weakness. Untreated symptoms of a disease can led to death, and infections caused by *E. coli* can be dangerous if not properly managed (Owusu-Asiedu *et al.,* 2003; Fikri *et al.,* 2018).

The primary step taken to cure an infection involves providing antibacterial agents that can inhibit the growth and development of microorganisms, ultimately leading to their destruction. One of the most widely used steps to treat infections is antibiotics but using them at inappropriate dose levels can cause resistance (Fatisa, 2013; Fikri *et al.,* 2019). The development of this resistance can be detrimental to patients, both in terms of health and economy. To address this issue, natural antibacterials found in plants can be used. Active ingredients derived from natural sources are highly effective in inhibiting the growth of microbes that cause food-borne illnesses, even at relatively low concentrations (Paramita *et al.,* 2014).

One of the plants that has an antibacterial active ingredient is the kingfruit. According to Theanphong and Mingvanish (2018), alkaloids, flavonoids, tannins, and triterpenoids in kingkit orange *(Triphasia trifolia)* have antibacterial activity. A previous study found that the essential oils of 10 µL and 15 µL of kingkit orange leaves have a strong inhibitory ability against *E. coli*

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(ATCC 25922) (Hardisto and Tjandra, 2019). Theanphong and Mingvanish (2018) reported that extracts of hexane, ethanol, and dichloromethane from the leaves and stems of kingkit oranges have antibacterial activity against *E. coli* with moderate to strong inhibitory power. However, there are limited studies on fruit extract as an antibacterial. On this basis, this study aimed to determine the benefits of the fruit and leaf extracts of the kingkit as a natural antimicrobial that can reduce *E. coli* contamination.

2. Materials and methods

2.1 Materials

The materials used in this study include kingkit oranges obtained from Krui, Pesisir Barat District, *E. coli* bacterial culture obtained from the Bacteriology Laboratory of Veterinary Association Lampung, 70% alcohol (Emsure®) 96% ethanol ((Emsure®), distilled water, aluminum foil, blank paper disks, cotton, physiological NaCl (Emsure®), and Mullen Hilton Agar (MHA) (Merck©).

2.2 Sample preparation

The sample preparation process was carried out by preparing 2 kg each of fruit and kingkit leaf samples. The preparation process begins with sorting to obtain good quality fruit and orange leaves, then calculating their moisture content. Afterwards, the fruit and kingkit orange leaves were dried under direct sunlight for 8 hrs, followed by drying in an oven at 50°C for 72-93 hrs for fruit and 24 hrs for leaves. This was followed by reducing the size of the fruit and leaf samples using a chopper and smoothing the sample texture using a power grinder. After the fine powder of kingkit orange fruit and leaves was produced, the water content was calculated. Fine powder of fruit and lime leaves are ready for the maceration process (Sahoo *et al.,* 2014).

2.3 Extraction process

The process of making kingkit orange fruit and leaf extracts uses the maceration method. A total of 450 g of fruit powder and 300 g of kingkit orange leaf powder were dissolved using 96% ethanol (750 mL for fruit simplicia and 500 mL for leaf simplicial). Subsequently, the dissolved simplicial was placed in a closed container, and stored by placing the container containing the extract on a shaker (120 rpm). The maceration process lasted for 3 days with three times filtering. After the storage process was complete, filtrates were produced by filtering, and then the extract was evaporated using a vacuum rotary evaporator at 30°C to produce concentrated kingkit orange fruit and leaf extracts. This was followed by calculating the water content of the concentrated extract of orange kingkit fruit and leaves (Sahoo *et al.,* 2014).

2.4 Gas chromatography-mass spectroscopy analysis

Concentrated extracts of kingkit orange fruit and leaves were analyzed to determine the compounds or ingredients present in kingkit orange using an Agilent 8890 Gas Chromatography-Mass Spectroscopy (GC/MS). HP-5 MS column used in the experiment had a glass length of 30 m, diameter of 250 µm, and thickness of 0.25 µm. The injection and detector temperature were both set at 280°C, while the oven temperature was programmed to increase from 60°C, at the beginning to 280°C at the end of the experiment, with an increased rate of 5°C/mins. The flow rate was 1 mL/mins and the split ratio was 100:1. The MS source temperature used was 230°C, and the MS squad temperature was 150°C. The results of the analysis listed on the tool are then recorded (Salvatore *et al.,* 2017).

2.5 Preparation of extract concentrations

This study used five concentration levels, i.e., 5%, 10%, 15%, 20%, and 25%. Preparation of extract preparations at these concentrations was carried out by taking the volume of the fruit and leaf extract of the kingkit orange, and then calculating the extract using the dilution formula.

2.6 Preparation of the test bacterial suspension

A total of two loopfuls of pure *Escherichia coli* bacteria culture were taken using an ose needle that has been sterilized on a Bunsen flame, then evenly and aseptically streaking the pure culture on the surface of the Mullen Hilton agar and incubated at 37°C for 24 hrs. Preparation of the test bacterial suspension was carried out by taking 2 loops of *Escherichia coli* bacterial culture and suspending the culture in 9 mL of 0.9% sterile NaCl aseptically, and vortexing until homogeneous. Subsequently, the turbidity level of the test bacterial suspension was measured using a Biomerieux Densichek Plus Instrument until the number 0.5 McFarland was visible. Turbidity was compared by inserting the tube containing the inoculum into the measurement hole on the Biomerieux Densichek Plus Instrument with a 360°rotation for 2 s. After that, the measurement results that appear on the Densichek tool were compared with the McFarland 0.5 standard. The 0.5 McFarland standard is the comparator commonly used to compare the turbidity of liquid mediums with a density equivalent to 1×10^8 CFU/mL (Muharni *et al*., 2017).

2.7 Antibacterial activity test

Escherichia coli cultures were used to test the

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antibacterial activity of the fruit and leaf extracts of the kingkit oranges. Distilled water was used as a control. The antibacterial activity test was carried out by dipping the paper discs into each concentration for 5 seconds until all parts were submerged in the extract. After that, 200 µm of the test bacterial suspension was taken and poured over the prepared petri dish, and then the Mullen Hilton Agar liquid was added into the petri dish which has the test bacteria. The paper discs that had been dipped in extracts with different concentrations (5%, 10%, 15%, 20%, and 25%) were placed on the agar medium, which contained *E. coli* cultures, and then incubated at 37°C for 24 hrs. The diameter of the inhibition zone formed on the media was measured using a caliper (Muharni *et al*., 2017).

3. Results and discussion

3.1 Samples of kingkit orange fruits

Fresh kingkit oranges with 3.5 kg weight produce simplicia weighing 428 g, with a yield of 12%. Fresh fruit has a water content of 77.63%, while simplicia or dry powder of orange fruit contains 5.16%. The reduced water content was affected by the drying process, resulting in samples that are not easily damaged and not easy for microorganisms to grow. According to Handoyo and Pranoto (2020), drying reduced the water content in the sample to avoid easy damage and ensure a longer shelf life. The extraction was made using a maceration method using 96% ethanol. The solvent is used because it is semi-polar, thus considered more effective for extracting active compounds. This method is consistent with the opinion of Marandingsih *et al.* (2014), who find that ethanol solvent is good for extracting phenolic and flavonoid compounds.

The organoleptic results of the kingkit orange fruit extract are liquid with a thick texture, a blackish-green color, and a distinctive smell of kingkit oranges.

3.2 Samples of kingkit orange leaves

A total of 2 kg of Fresh kingkit orange leaves produced simplicia with a weight of 1.2 g and yield of 60%. Fresh fruit contains % water content of 60.18%, while simplicial or dry powder of orange fruit contains 8%. The drying process affects reduced water content, thereby restraining microbial growth in simplicia (Rachmawati *et al.,* 2006). Samples that are already dry are followed by the refining process, which will reduce the size of the sample to powder form. With a small powder form, it is beneficial in expanding the surface area, thereby accelerating the process of penetration of the solvent into the sample to be extracted, resulting in easy extraction of the active compounds (Tambun *et al.,* 2016). The simplicia of kingkit orange leaves was macerated using 96% ethanol, which is in the form of a liquid with a thick texture, a greenish-black color, and a distinctive aroma of kingkit oranges. Kingkit orange leaves have an aroma that is slightly similar to the smell of lemons, but the orange has a sweeter aroma.

3.3 Gas chromatography-mass spectrum analysis of kingkit orange ethanol extract

The ethanol extract of kingkit oranges was analyzed using the GC-MS tool. This analysis produced chromatograms and mass spectra that displayed the components contained in the extract. Based on the data obtained from the GC-MS readings, the chromatography results on the ethanol extract of kingkit oranges are presented in Table 1.

Based on the GC-MS readings in Table 1, it was found that several compounds contained antibacterial activity. Acetic acid compound content in the ethanol extract of kingkit oranges was seen at a peak of 1.755. It functions as an antimicrobial by lowering the pH in bacterial cells, causing the cell membrane to become unstable (Miskiyah and Juniawati, 2014). Peak 4.983 on the list corresponds to Compound 15, identified as [1,3,4] Thiadiazol, 2-amino-5-(2-piperidine-1-ylethyl). This compound contains piperidine, a natural alkaloid group that can disrupt the constituent components of peptidoglycan in bacterial cells. This disruption prevents the bacterial cell walls from forming completely, ultimately leading to their death (Mangga *et al.*, 2022).

Table 1. Data on GC/MS results of ethanol extract of kingkit orange.

Cpd	Name	Formula	RT
3	*Acetic acid*	$C_2H_4O_2$	1.755
15	*[1,3,4]Thiadiazol, 2-amino-5-(2-piperidin-1-ylethyl)*	$C_9H_{16}N_4$ S	4.983
16	*4H-Pyran-4-one, 2,3-dihydro-3,5 dihydroxy-6-methyl*	$C_6H_8O_4$	5.998
21	*1,1'-Methylenebis(3-methylpiperidine)*	$C_{13}H_{26}N_2$	8.358
26	*terpinen-4-ol*	$C_{10}H_{18}O$	11.011
35	*Caryophyllene*	$C_{15}H_{24}$	17.348
49	*n-Hexadecanoic acid*	$C_{16}H_{32}O_2$	29.280
58	*cis—Vaccenic acid*	$C_{18}H_{32}O_2$	32.648
60	*7H-Furo[3,2-g][1]benzopyran-7-one,4,9-dimethoxy*	$C_{11}H_{10}O_5$	34.391

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At peak 5.998, Compound 16 was identified as 4H-Pyran -4-one, 2,3-dihydro-3,5 dihydroxy-6-methyl, belonging to the class of flavonoid compounds which are capable of forming complex compounds with extracellular proteins. It can also dissolve proteins which causes the release of intracellular compounds, such that the bacterial cell membrane becomes damaged (Maharani and Fernandes, 2021). Compound 21 at peak 8.358 contains the compound 1,1'-Methylenebis(3-methylpiperidine), which has the same piperidine component as compound 15, a class of alkaloids that can inhibit or kill bacterial growth.

Subsequent chromatographic analysis revealed the presence of Caryophyllenein compound 35, with a corresponding peak at 17.348. Caryophylleneis a sesquiterpenoid compound known for its antibacterial properties with a mechanism of inhibiting or killing bacteria by changing the constituent components of the bacterial cell. This process damages the cell membrane, and ultimately disrupts the process of bacterial growth (Jasmansyah *et al.,* 2020). Compound 49 identified as n-Hexadecanoic acid (palmitic acid)and compound 53 identified as cis-vaccenic acid, both of which belong to the class of fatty acids, were observed at peaks 29.280 and 32.648, respectively. Fatty acids can inhibit microbial growth by damaging the structure of bacterial cell walls and membranes (Karunia *et al*., 2017). At peak 34.391, compound 66 was identified as 7H-Furo[3,2-g] [1]benzopyran-7-one,4,9-dimethoxy, belonging to the group of flavonoids. According to Manik *et al.* (2014), flavonoid compounds exhibit antibacterial activity through a mechanism that involves inhibiting nucleic acid synthesis, thereby inhibiting the function of the cytoplasmic membrane, and the energy metabolism of bacteria.

3.4 Gas chromatography-mass spectrum analysis of kingkit orange leaf ethanol extract

GC-MS analysis was also carried out on the ethanol extract of kingkit orange leaves to determine the compounds that are present. Based on the data obtained from the GC-MS readings, the chromatography results on the ethanol extract of kingkit orange leaves are presented in Table 2.

Based on the GC-MS readings in Table 2, it was found that several compounds contained or had antibacterial activity. In compound 8, Cycloserine content was identified in the ethanol extract of kingkit oranges at a peak of 4.135. Cycloserine belongs to the β-lactam class of antibiotic compounds and is commonly used to treat bacterial infections due to its antibacterial activity, which causes the bacterial cell wall to be brittle and easy to lyse (Aziz, 2010). Compound 12, namely 4H -Pyran-4-one, 2,3-dihydro-3,5 dihydroxy-6-methyl, was detected at peak 4.983 and belongs to the flavonoid groups of compounds, which is known to possess antibacterial properties (Maharani and Fernandes, 2021). Compound 22 Phenol, 4-ethenyl-2,6-dimethoxy- which is listed at peak 20,891, is a group of phenol compounds. At high concentrations, it can penetrate and disrupt bacterial cell walls (Sitorus *et al.,* 2020). At peak 29.237, compound 31 identified as n-Hexadecanoic acid, was detected in the ethanol extract of kingkit oranges and can inhibit bacterial growth. Compound 41, identified as Isoaraptene*,* was observed at a peak of 34.496. It is a monoterpene coumarin ether commonly found in the citrus genus.

The results of the chromatographic analysis are compound 62 at peak 41.737, namely Propanamide, 2,2-dimethyl-N-(1,4,6 trimethyl-1H-pyrazolo[3,4-b]pyridine-3-yl). The pyridine compound belonging to the class of alkaloids has the ability to inhibit and kill bacteria. Compound 66 at peak 43.263 obtained the compound (S) -7-Hydroxy-8,8-dimethyl-7,8 dihydropyrano(3,2-g) chromen-2(6H)-one, containing pyrano belonging to the alkaloid group (Warandi, 2015). The last compound, namely compound 68 at peak 44.033, obtained compound (R)-9-(2,3-Dihydroxy-3-methylbutoxy)-4 methoxy-7H-furo(3,2-g)(1)benzopyran-7-one. This compound contains the same pyran group as compound 66 and is also classified as an alkaloid, thus it has the ability as an antibacterial.

3.5 Antimicrobial activity test

3.5.1 Inhibitory power of kingkit orange fruit ethanol extract

The clear zone was measured by adding up the diameters of its 2 sides, which averaged to obtain the diameter of the inhibition area (mm). The measurement

Table 2. Data on GC/MS results of ethanol extract of kingkit orange leaves.

Cpd	Name	Formula	RT
8	*Cycloserine*	$C_3H_6N_2O_2$	4.135
12	*4H-Pyran-4-one, 2,3-dihydro-3,5 dihydroxy-6-methyl*	$C_6H_8O_4$	4.983
22	*Phenol, 4-ethenyl-2,6-dimethoxy-*	$C_{10}H_{12}O_3$	20.891
31	*n-Hexadecanoic acid*	$C_{16}H_{32}O_2$	29.237
41	*Isoauraptene*	$C_{15}H_{16}O_4$	34.496
62	*Propanamide, 2,2-dimethyl-N-(1,4,6 trimethyl-1H-pyrazolo[3,4-b]pyridin-3-yl)-*	$C_{14}H_{20}N_4O$	41.737
66	*(S)-7-Hydroxy-8,8-dimethyl-7,8 dihydropyrano(3,2-g)chromen-2(6H)-one*	$C_{14}H_{14}O_4$	43.263
68	*(R)-9-(2,3-Dihydroxy-3-methylbutoxy)-4 methoxy-7H-furo(3,2-g)(1)benzopyran-7-one*	$C_{16}H_{28}O$	44.033

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results of the inhibitory diameter of the ethanol extract of kingkit oranges are shown in Table 3 and Figure 1.

Table 3. Diameter of inhibition zone (clear zone) of kingkit orange fruit against *Escherichia coli.*

Concentration extract (%)	Average inhibition area diameter ± SD (mm)
B1 (5%)	6.05±0.02[a]
B2 (10%)	6.08±0.02[ab]
B3 (15%)	6.22±0.07[c]
B4 (20%)	6.50±0.08[d]
B5 (25%)	6.65±0.04[e]
Distilled water (Control)	-

Values with different superscripts are statistically significantly different ($p<0.05$).

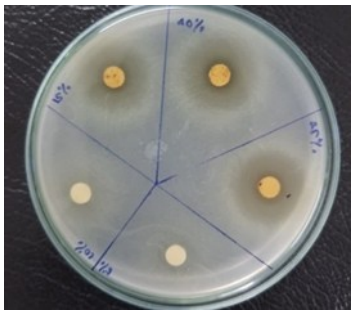

Figure 1. Diameter of inhibition zone (clear zone) of kingkit orange fruit against *Escherichia coli.*

This table showed that the diameter of the resulting inhibition area is even more significant when a more considerable extract is used. According to Surjowardojo *et al.* (2015), the diameter zones formed in the media are grouped into several groups, i.e. >20 mm, 11 - 20 mm, 6 - 10 mm, and <5 mm, belonging to the group "very strong", "strong", "moderate", and "low" inhibition areas. The results showed that treatment B5 with a concentration of 25% had the largest diameter of the inhibition area compared to the others, with an average diameter of 6.65 mm. For concentrations of 20%, 15%, 10%, and 5% the diameters of the inhibition areas were 6.50 mm, 6.22 mm, 6.08 mm, and 6.05 mm, respectively. Based on the average diameter of the inhibition area, treatments B1, B2, B3, B4, and B5 fall into the category of moderate inhibition.

Based on the results obtained, there was an increase in the diameter of the inhibition area as the concentration of the extract increased. This statement is consistent with the opinion of Rahmawati (2014) that there is an increase in the diameter of the large inhibition area along with the greater concentration. This is due to the presence of more bioactive components in the extract at higher concentrations. The secondary bioactive components contained in the extract of cassava led to the formation of the diameter of the inhibition area. Theanphong and Mingvanish (2018) find that the ethanol extract of the leaves and stems of the kingkit orange contains active compounds in the form of alkaloids, flavonoids, tannins, and triterpenoids. These compounds have the ability to inhibit and kill bacteria.

3.5.2 Inhibitory power of kingkit orange leaf ethanol extract

The results of the diameter test of the inhibition area of the kingkit orange leaf extract are shown in Table 4 and Figure 2.

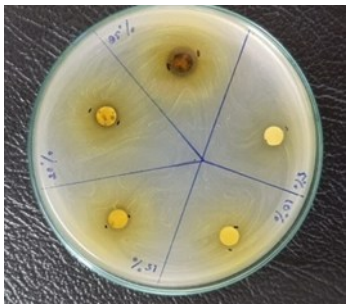

Figure 2. Diameter of inhibition zone (clear zone) of kingkit orange leaves against *Escherichia coli.*

Table 4. Diameter of inhibition zone (clear zone) of kingkit orange fruit leaves against *Escherichia coli.*

Concentration extract (%)	Average inhibition area diameter ± SD (mm)
B1 (5%)	0.00±0.00
B2 (10%)	0.00±0.00
B3 (15%)	0.00±0.00
B4 (20%)	6.82±1.78[a]
B5 (25%)	7.15±0.14[b]
Distilled water (Control)	-

Values with different superscripts are statistically significantly different ($p<0.05$).

Table 4 shows that only treatments D4 and D5 showed inhibition area diameters, while D1, D2, and D3 did not show inhibition area diameters. D4 produces an average diameter of inhibition area of 6.82, and D5 produces 7.15 which is categorized as moderate inhibition. This is in accordance with the research of Theanphong and Mingvanish (2018) proving that ethanol extract from kaffir lime leaves has antibacterial activity against *Escherichia coli* with a minimum inhibitory concentration of 125 mg/mL, and ethanol extract from kingkit orange leaves and stems can inhibit the growth of *E. coli* at a concentration of 500 mg/mL. The difference in the diameter of the inhibition area observed with previous studies is thought to be due to differences in the growing location or planting conditions of kingkit orange used in this study.

4. Conclusion

The fruit and leaf extracts of kingkit orange (*Triphasia trifolia*) have antibacterial activity against *E. coli* bacteria as indicated by the presence of inhibition in the bacterial inhibition test at a concentration of 25%

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(kingkit orange fruit extract) and concentrations of 20% and 25% (kingkit orange leaf extract) with moderate inhibition strength.

Conflict of interest

The authors declare no conflict of interest.

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