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Original Research Article

Screening of Antibacterial Compounds Against *Escherichia coli* from Hanjeli Seeds (*Coix lacryma-jobi*) Based on Metabolomics

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ABSTRACT

Hanjeli seed (*Coix lacryma-jobi* L.) is one of the important plants used as raw materials for food and traditional medicine. In addition, hanjeli seed also has several biological activities, one of which is antibacterial activity comes from the activity of the metabolites contained in the extract of the seed. This study aims to determine the antibacterial activity of hanjeli seed extract and identify the metabolites contained in some selected extracts using a metabolomics approach. Hanjeli seed was extracted with distilled water, 50% ethanol, 96% ethanol, and n-hexane. The crude extracts of hanjeli seed were evaluated for the activity against *Escherichia coli* ATCC 25922 and the metabolites were identified using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Hanjeli seed extracts had antibacterial activity ranging from 7.85±4.48 to 27.99±6.02%, with 50% ethanol extract had the highest antibacterial activity and n-hexane extract had the lowest. LC-MS/MS analysis of the 50% ethanol extract obtained 22 metabolites which were putatively identified. The antibacterial activity of the hanjeli seed extract is predicted to be caused by the phenolic compounds, such as gluconic acid, quinic acid, malic acid, citric acid, p-coumaric acid, and ferulic acid. The phenolic compounds contained in the hanjeli seed extract can inhibit the growth of *E. coli*, so that the extract can be applied as an antibacterial against *E. coli*.

Keywords: antibacterial, *Coix lacryma-jobi*, *E. coli*, LC-MS/MS, PCA

Introduction

The use of plants as traditional medicine has been used for a long time to treat health problems. Apart from being caused by the tendency of people in the current era to adopt a back-to-nature lifestyle, people realize that plants as medicines have advantages. Namely, they have few side effects compared to chemical treatments, are easy to find, economical, and can be simply made.[1]

Herbal medicines have been widely used worldwide, both in developed and developing countries. Specifically for developing countries, the use of herbal medicines will reduce dependence on imported drugs which are expensive and have side effects.[2] About 63% of the world's population uses traditional medicine.[3] Regarding biodiversity, Indonesia ranks second after the Amazon forest, having many native plants with high potential for medicinal and pharmaceutical opportunities.[4] Therefore, according to Batubara and Prastya (2020), further research on Indonesian medicinal plants must be carried out to provide holistic knowledge about the development of cosmetic and health products, for example, the human's oral health.[5]

One of the medicinal plants that can be used as traditional medicine is hanjeli (*Coix lacryma-jobi*). Besides being used as food, animal feed, and handicrafts, people in Asian countries also use hanjeli for traditional medicine.[6,7]

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This plant have been traditionally applied for treating diuretic, anti-rheumatic, antispasmodic, anti-inflammatory, antidiarrheal, anthelmintic, antipyretic, antispasmodic, diuretic, hypoglycaemic, antioxidant, antifungal, and anti-cancer.[8–12] The part of the hanjeli plant that has antibacterial properties is the seeds. Compounds with antibacterial activity can inhibit bacterial growth by interfering with their metabolism.[13] Zhu (2017) and Devaraj *et al*. (2020) reported that the chemical components identified in hanjeli seeds were alkaloids, tannins, saponins, flavonoids, triterpenoids, and phenols.[14,15] According to Seukep *et al*. (2023), these compounds can function as antibacterials.[16]

Hanjeli is one of the antibacterial plants that has not been studied scientifically, so the information on the content that has antibacterial pharmacological activity in hanjeli seeds has not been known. Given that hanjeli contains antibacterial compounds and can be used as medicine, it is necessary to do the measurement of the antibacterial of hanjeli extract. In this study, we evaluated the activity of hanjeli seed extract against *E. coli* bacteria because *E. coli* is a pathogenic bacterium that often causes food spoilage and when consumed can cause mouth ulcers and gastroenteritis.[17] Therefore, information about the activity of hanjeli seed extract against *E. coli* can be used as a reference in medical science, food safety, and environmental protection.

In general, the bioactive components of natural products are identified using the bioassay-guided isolation method, which is an identification method by repeatedly isolating compounds based on bioactivity until pure compounds are obtained. That method is carried out repeatedly so that it is less effective in terms of time and cost. An alternative method is needed to identify bioactive components using a metabolomics approach. The metabolomics approach is carried out by comprehensively extracting samples using a combination of solvents at different polarity levels.[18] Then, the chemical content in the sample was identified using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

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The data obtained from the LC-MS/MS analysis is a complex data set, so the interpretation requires the chemometric methods. Chemometrics is a branch of science that derives data by applying mathematical and statistical methods to extract useful information from physical and chemical phenomena involved in a manufacturing process. Chemometrics is used for multivariate data collection and analysis protocols, calibration, process modeling, pattern recognition and classification, signal correction and compression, and statistical process control.[19] The chemometric pattern recognition technique used in this study is principal component analysis (PCA) because it can facilitate visualization in grouping data, evaluating similarities between groups or classes, and finding factors or reasons behind observed patterns through correlations based on the chemical or physicochemical properties of the samples.[20] In this study, the antibacterial compounds in hanjeli seed extract were predicted using LC-MS/MS-based metabolomic. LC-MS/MS results were further analyzed by PCA to determine the grouping pattern between the selected extracts with and without the addition of *E. coli* bacteria.

Materials and Methods

The materials used were hanjeli seeds (*Coix lacryma-jobi*), ethanol (Merck, Germany), n-hexane (Merck, Germany), DMSO (Merck, Germany), tetracycline (Darya-Varia, Indonesia), filter paper, methanol LC-MS grade (Merck, Darmstadt, Germany), acetone, distilled water LC-MS grade (Merck, Darmstadt, Germany), acetonitrile LC grade (Merck, Darmstadt, Germany), formic acid, distilled water, nutrient agar media, nutrient broth media, and *E. coli* ATCC 25922 bacteria obtained from the Laboratory of the Center for Tropical Biopharmaceutical Studies, LPPM IPB.

Extraction of Hanjeli Seeds.
Hanjeli seeds were obtained from the Medicinal Plants Cultivation and Conservation Unit, Tropical Biopharmaca Research Center, IPB University on 20 February 2022. Identification of the sample was performed by Mr. Taopik Ridwan (Trop-BRC, IPB University) with voucher specimen number BMK0336122016. The seeds were crushed into powder and extracted using the maceration method.[21] A total of 50 g of hanjeli seed powder soaked in four different solvents at a ratio of 1:10 for 7x24. The solvents used were distilled water, 50% ethanol, 96% ethanol, and n-hexane. For solvent distilled water, maceration was carried out for 4 hours at 70 ºC. The mixture was filtered and the filtrate was concentrated using a rotary evaporator.

Bacterial Inoculation
Inoculation of *E. coli* ATCC 25922 was done using NB (Nutrient Broth) and NA (Nutrient Agar) media. One dose (10 µL) of bacteria was added to 50 mL of NB medium and stirred thoroughly. The NB medium was incubated at 37ºC for 24 hours. The turbidity of the NB medium indicated bacterial growth.

Antibacterial Activity Test with Disc Method
A total of 300 µL of bacterial suspension was added to 300 mL of NA medium. After mixing, the media was poured into 12 petri dishes. After becoming agar, five paper discs were added to each medium containing *E. coli* ATCC 25922. Test solutions with extract concentrations of 1%, positive control (tetracycline), and negative control (20% DMSO) was added to the disc paper. The plates were then incubated at 37 ºC for 24 hours. The clear zone around the disc paper indicates the antibacterial activity of the hanjeli seed extract. The antibacterial activity of the extract is expressed in % inhibition and is calculated using the following equation:

$$\% \text{ inhibition} = 1 - \left(\frac{\text{positive control diameter - diameter of extract}}{\text{positive control diameter}}\right) \times 100\%$$

Sample Preparation
The extract with the highest antibacterial activity was analyzed using LC-MS/MS. The samples were divided into 3 groups, i.e. (I) hanjeli seed extract, (II) hanjeli seed extract in NB medium, and (III) hanjeli seed extract in NB media with the addition of *E. coli*. Group III was prepared by adding 1 mL of hanjeli seed extract to 2.8 mL of NB medium and 0.2 mL of *E. coli* suspension. The mixture was incubated for 24 hours at 37 ºC. The mixture was then centrifuged at 7000 rpm for 5 minutes. Acetonitrile (threefold of the supernatant volume) was added to the mixture and centrifuged at 12000 rpm for 1 minute. The supernatant obtained was analyzed using LC-MS/MS. Group II was prepared using the same method, but without the addition of bacterial suspension. Each group was prepared in three replicates. The second and third groups were compared to predict which compounds had antibacterial activity.

Identification of compounds with LC-MS/MS
The chemical compounds of hanjeli seed extract which have the highest antibacterial activity were analyzed using LC-MS/MS referring to the previous method with modifications.[22] The extract solution was filtered with a 0.22 µm filter membrane, and 5.0 µL of the filtrate was injected in LC-MS/MS. Samples were analyzed using two mobile phases, 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a flow rate of 0.2 mL/min. The elution system used was a gradient, with the following composition of the mobile phase: 0-0.8 minutes (5%B), 0.8-12 minutes (5-95%B), 12-13 minutes (95%B), 13-15 minutes (5 %B). The column used was Accucore™ Phenyl Hexyl (100 × 2.1 mm, 2.6 µm). The MS ionization source is ESI(-) with a Q-Orbitrap mass analyzer. The m/z range is from 80-1200, with ionization energies of 18, 35 and 53 eV. Other MS system parameters are set as follows: capillary temperature 320 ºC; spray voltage 3.8 kV; sheath and auxiliary 15 and 3 arbitrary units, respectively; and resolving power of 70,000 FWHM.
Data from LC-MS/MS analysis were processed using Compound Discoverer 3.2 (Thermo Scientific, Waltham, USA) with online databases from ChemSpider and mzCloud. The steps for identifying metabolites include the selected spectra stage, alignment retention time, detected unknown compounds, grouping unknown compounds, predicting composition, filling gaps, normalizing areas, and marking background compounds. Metabolites were detected with a maximum mass error limit of 15 ppm and a minimum intensity of 1000000. Metabolites that were not identified using the database were not included in Table 1.

Statistical analysis
Extraction yield and antibacterial activity determined in three replicates and expressed as mean ± standard deviation. Principal component analysis (PCA) was used to differentiate between 50% ethanol extract in NB media with and without the addition of *E. coli* bacteria. The PCA model was performed using MetaboAnalyst ver 5.0 (https://www.metaboanalyst.ca/) with a variable peak area of all detected metabolites.

Results and Discussion

Hanjeli seed extraction aims to separate the bioactive compounds contained in the seeds. The solvent used in this extraction is distilled water, 96% ethanol, 50% ethanol, and n-hexane. The use of polar, semi-polar, and non-polar solvents aims to extract all the metabolites contained in hanjeli seeds which have different polarities. Extraction was carried out by maceration method. This method was chosen because the process is easier, more efficient, and does not damage the thermolabile compounds. In addition, Agustien *et al*.[23] also reported that the content of metabolites such as flavonoids obtained from the maceration method (1.39%) was higher compared to the soxhlet method (0.81%) which used heat.[23]
The extraction yield shows the number of compounds that extracted during the maceration process. The extraction yield of the four solvents is shown by Figure 1a. The 96% ethanol extract has the highest extraction yield, which is 2.59%. This is shows that the compounds in hanjeli seeds more extracted in 96% ethanol solvents. Ethanol has two groups associated with polarity, namely polar hydroxyl groups and nonpolar alkyl groups, causing polar and nonpolar compounds to be extracted and obtain high yield.[24]
Hanjeli seed extract has several biological activities, one of which is as an antibacterial. The antibacterial activity of hanjeli seed extract was measured using *E. coli* ATCC 25922 with various solvents. Hanjeli seed

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extract from the four solvents can inhibit *E. coli* bacteria with varying inhibition percentages (Figure 1b). Hanjeli extract with 50% ethanol solvent has more potential to inhibit *E. coli* activity compared to hanjeli extract using other solvents. This shows that the 50% ethanol extract contains bioactive compounds that have potential as antibacterial. In contrast, the 96% ethanol extract, which had the highest extraction yield produced a lower inhibition percentage. This can be predicted because the many metabolites contained in the 96% ethanol extract have an antagonistic effect between compounds which can reduce their antibacterial activity.

The metabolites contained in the 50% ethanol extract with and without the addition of *E. coli* were identified using an LC-MS/MS based metabolomics approach. The column used is Accucore™ Phenyl Hexyl, commonly used for aromatic analytes and relatively polar compounds. The column provides fast separation with high resolution without the high back pressure required by the particles and has good sensitivity. This column has a reversed phase so that nonpolar compounds will pass through the column for longer than more polar compounds. The polarity of the mobile phase will change systematically with time, resulting in compounds with different polarity levels being separated from each other so that a gradient elution system is chosen.

Basepeak chromatogram of 50% ethanol extract in NB medium with and without the addition of *E. coli* bacteria is shown in Figure 2. The chromatogram was processed and 369 metabolites were detected. The 369 metabolites were then identified using the ChemSpider and mzCloud databases. A total of 22 metabolites were putatively identified based on the database and confirmed using the MS2 spectrum (Table 1). Meanwhile, we did not include unknown metabolites in Table 1. Most of the putatively identified metabolites belong to the phenolic, flavonoid, and fatty acid groups. This is in accordance with the results reported by Huang *et al*.[25] that hanjeli seed extract contains phenolic and flavonoid compounds.

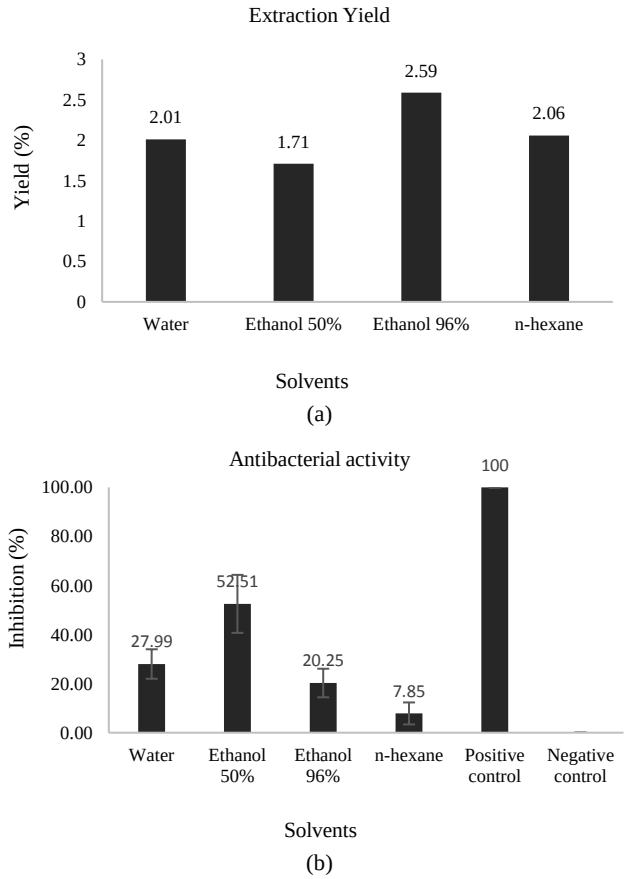

Figure 1: Extraction yield (a) and antibacterial activity (b) of hanjeli seeds

The 50% ethanol extract in NB media with and without the addition of *E. coli* had similar chromatograms and was difficult to distinguish. Therefore, to differentiate the two groups we used principal component analysis (PCA) with the peak areas of all detected metabolites as variables. PCA is a technique for reducing the dimensionality of a data set and improving interpretation by minimizing loss of information.[26] The PCA score plot showed that the samples could be divided into two groups, i.e. 50% ethanol extract in NB media with and without the addition of *E. coli* using PC1 and PC2 with a total PC of 83.7% (Figure 3). The PC value shows the variance that can be explained by the two main components is 83.7% of the total variance. The two groups shown on the score plot indicated differences in metabolites detected with and without the addition of *E. coli* bacteria.

Antibacterial compounds against *E. coli* from hanjeli seed extract predicted based on literature study. The potency of hanjeli seed extract as an antibacterial is caused by the bioactive metabolites contained in the extract. Inhibition of bacteria by bioactive metabolites in extracts generally occurs through the mechanism of cell wall damage in bacteria which causes the plasma membrane to rupture, cytoplasm to exit, substance transport to be disrupted, and metabolism to be inhibited so that bacterial growth is also inhibited and even die.

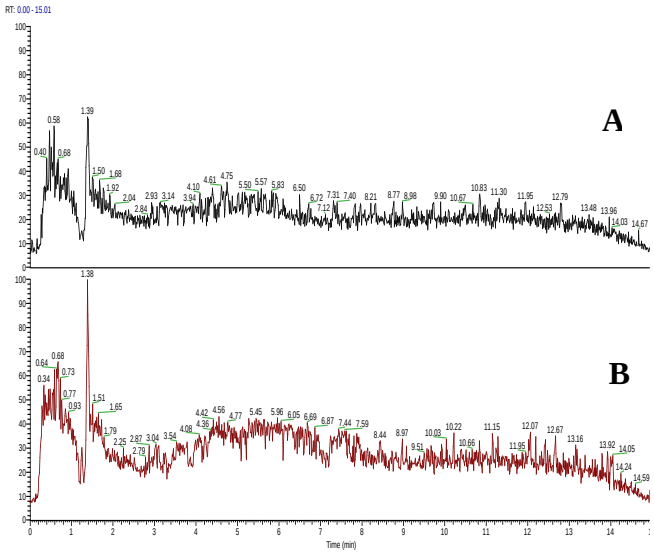

Figure 2: Basepeak chromatogram of hanjeli seed extract + NB media (A) and seed extract + NB media + *E. coli* bacteria (B) in negative ionization mode

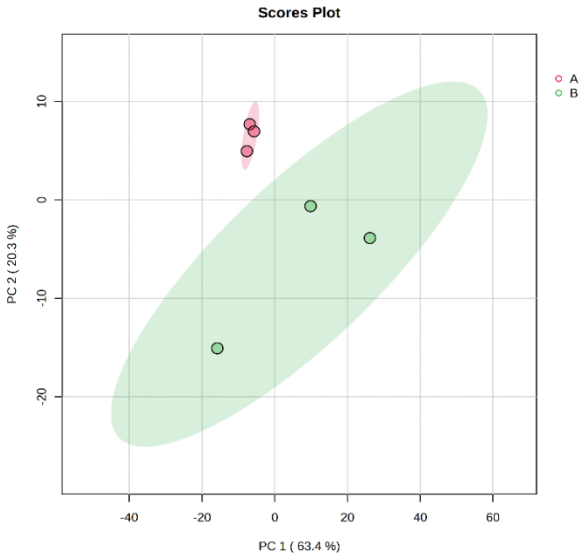

Figure 3: PCA score plot of hanjeli seed extract + NB media (A) and seed extract + NB media + *E. coli* bacteria (B)

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Table 1: Putative identified metabolites in 50% ethanol extract with media and *E. coli*

No.	Name	Formula	Error (ppm)	Calc. MW	RT (min)	MS2
1	Gluconic acid	$C_6H_{12}O_7$	-4.88	196.0574	1.304	195, 177, 87, 75
2	D-(-)-Quinic acid	$C_7H_{12}O_6$	-5.02	192.0624	1.32	191, 173, 131, 129
3	4-Hydroxy-L-threonine	$C_4H_9NO_4$	1.26	135.0533	1.34	134, 116
4	Isocitric acid	$C_6H_8O_7$	-5.07	192.026	1.405	191, 173, 129
5	D-(+)-Malic acid	$C_4H_6O_5$	-9.1	134.0203	1.407	133, 115, 71
6	L-gamma-Glutamyl-L-leucine	$C_{11}H_{20}N_2O_5$	-2.02	260.1367	1.489	259, 130, 114, 112
7	Citric acid	$C_6H_8O_7$	-5.16	192.026	1.493	191, 147, 129
8	Methylmalonic acid	$C_4H_6O_4$	-10.15	118.0254	1.499	117, 73
9	N(2)-succinyl-L-glutamic acid	$C_9H_{13}NO_7$	-3.03	247.0685	1.501	246, 128, 115
10	2-methylcitric acid	$C_7H_{10}O_7$	-4.56	206.0417	1.504	205, 187, 161
11	Pyroglutamylisoleucine	$C_{11}H_{18}N_2O_4$	-2.41	242.1261	5.894	241, 197, 130, 82
12	4-Hydroxybenzaldehyde	$C_7H_6O_2$	-10.2	122.0355	6.213	121, 93, 67
13	p-coumaric acid	$C_9H_8O_3$	-7	164.0462	6.515	163, 119, 93
14	2,3-Dihydro-1-benzofuran-2-carboxylic acid	$C_9H_8O_3$	-6.71	164.0462	6.686	163, 119
15	Ferulic acid	$C_{10}H_{10}O_4$	-5.43	194.0569	7.582	193, 147, 133
16	Corchorifatty acid F	$C_{18}H_{32}O_5$	-2.32	328.2242	8.28	327, 229, 171
17	Coniferyl ferulate	$C_{20}H_{20}O_6$	-2.4	356.1251	8.342	355, 337, 219, 175
18	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	$C_{18}H_{34}O_5$	-2.39	330.2398	8.536	329, 229, 171
19	(10E,12E)-9-hydroperoxyoctadeca-10,12-dienoic acid	$C_{18}H_{32}O_4$	-2.21	312.2294	9.859	311, 275, 235
20	9,10-dihydroxy-12Z-octadecenoic acid	$C_{18}H_{34}O_4$	-2.06	314.2451	10.249	313, 201, 171, 127
21	13S-hydroxyoctadecadienoic acid	$C_{18}H_{32}O_3$	-2.58	296.2344	11.865	295, 183
22	2,2'-Methylenebis(4-methyl-6-tert-butylphenol)	$C_{23}H_{32}O_2$	-1.92	340.2396	13.663	339, 163

Based on literature study, the antibacterial activity of hanjeli seed extract is caused by phenolic compounds, i.e. phenolic acids and flavonoids. This is in accordance with the research of Dzoyem *et al.*[27] who reported that in antibacterial activity (against Gram-positive and Gram-negative bacteria), DNA, RNA, and protein synthesis were inhibited by three flavonoids isolated from Dorstenia species. In addition, Shamsudin *et al.*[28] also reported that the hydroxylation of C5, C7, C3′, and C4′ in the flavonoid structure can increase bacterial inhibition.

Research from Wu *et al*.[29] reported the mechanism of action of five flavonoids against *E. coli*. These compounds were effective via rigidifying the membrane of liposomal. The molecular hydrophobicity (C log P) and charges on the C atom at position three might play a role in the intercalation of liposomal model membranes. He *et al.*[30] screened the antimicrobial mechanism of flavonoids (kaempferol, hesperetin) for inhibitory activity against *E. coli* through the cell membranes and liposomal model. The studies result that interaction between the group of polar heads of the membrane model and the hydrophobic regions may damage the *E. coli* membrane.

Furthermore, Liu *et al*.[31] reported the mechanism of bacterial inhibition by phenolic acid compounds. Phenolic acid can inhibit bacterial growth by destabilizing the bacterial cytoplasmic membrane, changing the permeability of the bacterial plasma membrane, inhibiting extracellular microbial enzymes, directly changing microbial metabolism, changing the physicochemical surface properties of bacteria, and removing microbes from the substrate needed for bacterial growth. In addition, phenolic acid can also change the polarity of bacteria by changing the surface electron acceptor of bacteria in gram-positive (increased acceptor component) and gram-negative (decreased acceptor component) strains.[32]

Several metabolites identified in hanjeli seed extract have been reported to have inhibitory activity against *E. coli* bacteria, such as gluconic acid, quinic acid, malic acid, citric acid, p-coumaric acid, and ferulic acid. Masoura *et al.*[33] reported that there was a synergistic effect between gluconic acid and H_2O_2 in inhibiting the growth of *E. coli* bacteria. This synergistic effect causes membrane depolarization, cell wall destruction, and finally inhibition of *E. coli* growth.

Quinic acid and citric acid are short-chain non-aromatic hydroxy acids which have important roles in plant metabolism. Both of these compounds have been reported to have antibacterial activity. Quinic acid and citric acid can inhibit the growth of *E. coli* bacteria with an MIC of 500 µg/mL.[34] Quinic acid has the potential as an antibacterial by damaging cell membranes and interfering with cell metabolic activity.[35] Bai *et al.*[36] reported that quinic acid can inhibit the growth of bacteria by interfering with the oxidative phosphorylation pathway, and changing levels of glycerophospholipids and fatty acids to disrupt membrane fluidity. After penetrating the cell membrane, quinic acid affects ribosome function and aminoacyl-tRNA synthesis, thereby interfering with protein synthesis to inhibit bacterial growth.

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Meanwhile, citric acid can inhibit bacterial growth by passing through the microbial membrane. Once inside the cytoplasm, it dissociates into citric acid anions and generates protons which cause acidification of the intracellular medium while causing functional and structural damage to the bacterial cell.[37] In addition, Kang *et al*.[38] reported that citric acid can pass through cell membranes and promote superoxide dismutase (SOD) inactivation in *E. coli* O157:H7. This induces reactive oxygen species (ROS) and promotes cell inactivation by activating lipid peroxidation, causing cell membrane damage. Al-Rousan *et al*.[39] also reported that citric acid could inhibit the growth of *E. coli* bacteria in salads, thereby reducing the risk of contamination by *E. coli* bacteria.[39]

Malic acid is included in organic acids that can act as an antibacterial caused by a decrease in pH below the growth range of microorganisms and inhibition of metabolism by con dissociated acid molecules. In addition, according to Massilia *et al*.[40] transmission electron microscopy showed that malic acid produced damage in the cell cytoplasm of pathogens without apparent changes in the cell membrane. Ballal *et al*.[41] found that 17% of malic acid showed antimicrobial activity on *Enterococcus faecalis*, *Candida albicans*, and *Staphylococcus aureus*. The presence of malic acid in trimethoprim can increase its solubility and antibacterial activity.[42]

Ferulic acid and p-coumaric acid are phenolic acids which have been reported to have activity against *E. coli* bacteria. Borges *et al*.[43] reported MIC and MBC ferulic acid for *E. coli* was 100 µg/mL and 2500 µg/mL, respectively. Ferulic acid can cause damage to cell membranes, thereby inhibiting bacterial growth. These compounds damage cell membranes by changing the properties of the membrane through hydrophobic changes and a decrease in negative surface charge.[44] While p-coumaric acid can inhibit bacterial growth by disrupting cell membranes and binding to bacterial genomic DNA to inhibit cellular functions.[45]

Conclusion

Hanjeli seed extract inhibit the activity of *E. coli* bacteria, with the highest percentage of inhibition found in the extract with 50% ethanol solvent. A total of 22 compounds were identified in hanjeli seed extract. The identified compounds belong to the phenolic and fatty acid groups. The identified phenolic compounds are predicted to influence the antimicrobial activity of hanjeli seed extract.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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