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UHPLC-HRMS-Based Metabolomics To Evaluate the Antibacterial Compounds of *Coix lacryma-jobi* Seeds With Different Extraction Solvent Concentrations

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ABSTRACT

Coix lacryma-Jobi L., commonly known as hanjeli, has traditional medicinal properties and potential antibacterial efficacy. The antibacterial potential of hanjeli seeds is attributed to their inherent metabolites. The composition and concentration of these metabolites in hanjeli seed extracts vary depending on the concentration of the extraction solvent used. Thus, selecting the optimal extraction solvent is crucial. This study employed a metabolomics approach using ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS) to assess variations in metabolite profiles and antibacterial activities of hanjeli seeds under different extraction solvent concentrations (25%, 50%, and 75% ethanol). Extraction was conducted using the maceration method for 2 × 24 hours. In addition, metabolite profiles were analyzed using principal component analysis (PCA) and hierarchical clustering analysis (HCA) to distinguish the samples and correlate them with their antibacterial activity against *Escherichia coli* (*E. coli*). A total of 22 metabolites were putatively identified using UHPLC-HRMS. PCA effectively distinguished extracts based on extraction solvent concentration, whereas the HCA heatmap demonstrated variations in metabolite composition among the samples. The antibacterial activity of hanjeli seeds ranged from 33.16 ± 0.00% to 40.03 ± 2.14%. These findings indicate a significant influence of the extraction solvent concentration on the metabolite profile and antibacterial activity of hanjeli seeds.

***Keywords*:** *Coix lacryma-Jobi* L., Antibacterial, Metabolomics, Extraction solvent concentration

Introduction

Coix lacryma-jobi L., commonly known as hanjeli, is a member of the herbaceous plant family Poaceae. The dried and ripe seeds of hanjeli, called Biji Lame in Indonesia, have long been utilized in traditional medicine for various purposes, including fever reduction, lung function enhancement, diuretic effects, and the alleviation of stomachache, diarrhea, dysentery, and arthritis.[1,2] Beyond traditional medicine, hanjeli seeds have applications in multiple industries, such as health, pharmaceuticals, and food.[3,4] Numerous studies have highlighted the diverse bioactivities of hanjeli seeds, including melanogenesis inhibition,[5] antioxidant properties,[6,7] antimicrobial effects,[8] anticancer activity,[9,10] antiproliferative activity,[11] antidiabetic effects,[4] antifungal properties,[12] antigout activity,[13] anti-inflammatory effects,[14] and antibacterial properties.[15]

Despite the broad-spectrum bioactivity reported for hanjeli seeds, their potential as antibacterial agents has not been extensively studied. The antibacterial efficacy of hanjeli seeds may be attributed to their metabolites.

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Previous studies by Yu *et al*.[3] and Devaraj *et al*.[16] have identified fatty acids, phenolics, and flavonoids as major constituents of hanjeli seeds - compounds known to possess antibacterial properties.[17,18] However, the composition of the extracted metabolites can be influenced by various factors, including the concentration of the extracting solvent. These differences in metabolite composition can consequently affect the biological activities of hanjeli seeds, particularly their antibacterial efficacy.[19] Additionally, synergistic or antagonistic effects among the metabolites in the extracts can further modulate the effectiveness of hanjeli seeds as antibacterial agents.[20] Therefore, it is imperative to evaluate the variances in the metabolite composition of hanjeli extracts under different extraction solvent concentrations and assess their antibacterial activities to identify the most effective extraction solvent.

To achieve this, a UHPLC-HRMS-based non-target metabolomics approach was employed to evaluate metabolite compositions and predict active compounds in hanjeli seed extracts.[21] This approach, which is widely utilized in fields such as food, health, and agriculture, facilitates the characterization of metabolite distribution in the studied samples.[22–26] However, analysis using UHPLC-HRMS generates large datasets that require complementary chemometric techniques for effective interpretation, maximizing the extraction of information from experiments.[27] This integration of metabolomic and chemometric approaches presents an efficient technique for predicting active compounds compared to the more laborious and costly bioassay-guided fractionation method.[28]

While previous research has explored the antibacterial potential of hanjeli seed extracts against *Escherichia coli* [29] and hanjeli seed oils against various bacterial strains, including *E. coli*,[15] the influence of the extraction solvent concentration on the antibacterial activity of hanjeli seeds remains unexplored. In addition, Diningrat *et al*. (2020) reported the potential of *C. lacryma-jobi* oil as an antibacterial agent against *E.*

coli, *Staphylococcus aureus*, and *Bacillus subtilis*. However, no research has yet reported the effect of the extraction solvent concentration on the antibacterial activity of hanjeli seeds. This study used a metabolomics approach to evaluate the differences in metabolite profiles and antibacterial activities of hanjeli seed extracts with varying concentrations of the extraction solvent. Furthermore, the potential of the compounds to act as an antibacterial agent will be predicted using chemometrics. Overall, this study provides new insights into the phytochemical composition of job's tears seed extracts with different extraction solvent concentrations as well as a prediction of antibacterial active compounds that have so far been little explored.

Materials and Methods

Material and apparatus

Ethanol, DMSO, LC-MS-grade acetonitrile, methanol, and water (all from Merck, Germany), nutrient agar, and *Escherichia coli* ATCC 25922 were obtained from the Tropical Biopharmaca Research Center, IPB University. Metabolite profile identification was conducted using a Thermo Scientific Vanquish Flex UHPLC coupled with a Q Exactive Plus Orbitrap High-Resolution Mass Spectrometer (Thermo Scientific, Germany).

Plant collection and identification

The materials used included hanjeli seeds (*C. lacryma-jobi*), which were obtained from the Medicinal Plant Conservation and Cultivation Unit at the Tropical Biopharmaca Research Center, IPB University (Latitude: -6.550674052803142, Longitude: 106.72549002111798). The sample was identified at the Tropical Biopharmaca Research Center, IPB University, with specimen voucher number BMK0336122016.

Extraction of hanjeli seeds

Hanjeli seeds were ground into a fine powder, and 50 g of this powder was subjected to extraction using the maceration method. The extraction was performed with 500 mL of ethanol at varying concentrations—75% ethanol (EtOH75), 50% ethanol (EtOH50), and 25% ethanol (EtOH25)—over a period of 48 hours, with the solvent refreshed every 24 hours. The resulting extracts were then concentrated using a rotary evaporator. These concentrated extracts were subsequently analyzed using Ultra-High Performance Liquid Chromatography coupled with High-Resolution Mass Spectrometry (UHPLC-HRMS) and tested for their antibacterial activity.

Evaluation of antibacterial activities

A 100 µL bacterial suspension of *E. coli* was added to 100 mL of NA medium. After mixing, the medium was poured into different Petri dishes (20 mL each). Once the NA medium and Tryptone Soya Agar (TSA) had solidified, a paper disc was placed on the media, and 10 µL of sample was added dropwise to each Petri dish. The Petri dishes were incubated at 37°C for 24 h in an inverted position. The zone of inhibition on each disc was measured using a caliper. Antibacterial activity was expressed as percent inhibition, with chloramphenicol as the positive control and 20% DMSO as the negative control. The formula calculates the percent inhibition:

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

Metabolites profiling using UHPLC-HRMS

The metabolites in the hanjeli seed extracts were separated and detected using UHPLC-HRMS. Five milligrams of extract was dissolved in 1 mL of LC-MS-grade methanol, and then 2.5 µL of the sample solution was injected into the instrument. The mobile phase used was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a flow rate of 0.2 mL per minute. The elution system was a gradient with the following mobile phase composition: 0-1 minute (5% B), 1-25 minutes (5-25% B), 25-28 minutes (95% B), and 28-33 minutes (5% B). Metabolites were separated using an Accucore Column™ C18 (100×2.1 mm, 1.5 µm). The ionization source used was ESI (-) with a range of m/z 150-2000 and ionization energies of 18, 35, and 53 eV. Other MS system parameters were set as follows: capillary temperature 320°C; spray voltage 3.8 kV; sheath and auxiliary 15 and 3 arbitrary units, respectively; and resolving power of 70000 FWHM.

Data from the UHPLC-HRMS analysis were processed using Compound Discoverer 3.2 software (Thermo Scientific, Germany) with online databases from Chemspider and mzCloud. Metabolite identification stages included spectral stage selection, retention time alignment, unknown compound detection, grouping of unknown compounds, composition prediction, mass list search, gap filling, area normalization, and marking of background compounds. Metabolites were detected with a maximum mass error limit of 10 ppm, a minimum intensity of 1,000,000, and an S/N ratio of 3. The MS^2 spectrum of the detected metabolites was confirmed using the CFM-ID database to identify putative metabolites.[30]

Statistical and multivariate analysis

The extraction yield and antibacterial activity were determined in three replicates and expressed as mean ± standard deviation. To determine any significant differences, one-way analysis of variance (ANOVA) was performed, followed by Duncan's test if $p < 0.05$. The correlation between the identified metabolites and antibacterial activity was assessed using Pearson's correlation in SPSS (Version 25.0).

Multivariate principal component analysis (PCA) was used to differentiate each sample based on the extraction solvent concentration and the variable peak areas of the metabolites detected. Differences in metabolite distribution in hanjeli extracts are visualized as a hierarchical cluster analysis (HCA) heatmap. PCA and HCA models were generated using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/).

Results and Discussion

Extraction yield

Hanjeli seed powder was extracted using ethanol at three different concentrations: 25% ethanol, 50% ethanol, and 75% ethanol. The extraction yield of Hanjeli seeds ranged from 4.21 ± 1.04 to 6.00 ± 1.81% (Table 1). The results of the variance analysis showed that differences in the extraction solvent concentrations did not significantly affect the extraction yields at the 95% confidence level (*p*-value> 0.05). Extraction by maceration was chosen because it has several advantages, such as being simple, efficient, and using a long soaking time to dissolve more metabolites in the sample. In addition, maceration is carried out at room temperature, which reduces compound degradation at high temperatures.[31] The extraction yield shows that the three solvent concentrations can extract metabolites in amounts that are not significantly different. However, despite the solvents having different ethanol concentrations of 25%, 50%, and 75%, the resulting extracts were not significantly different in terms of concentrations and contents, suggesting that other factors might have contributed to the similarity in extraction yield.

Antibacterial activity

E. coli is a pathogenic bacterium found in human and animal environments, food, and intestines. *E. coli* often causes food spoilage, which, if consumed, can cause digestive disorders.[32,33] This study evaluated the potential of hanjeli seed extract as an antibacterial agent against *E. coli*, expressed as percent inhibition. The antibacterial activity of hanjeli seed extracts ranged from 33.16 ± 0.00% to 40.03 ± 2.14% and was significantly different at the 95% confidence level ($p < 0.05$). The 25% ethanol extract had the highest percent inhibition, while the 50% ethanol extract had a percent inhibition that was not significantly different from that of the 75% ethanol extract (Table 1). This shows that the bioactive metabolites in hanjeli seeds are more soluble in 25% ethanol, although the extraction yields from the three solvents were not significantly different.

Metabolites profile of hanjeli seeds extract

The metabolite profiles of the hanjeli seed extract with varying extraction solvent concentrations were analyzed using UHPLC-HRMS in negative ionization mode. The results of the analysis of the base peak chromatograms are shown in Figure 1. The samples generally had

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similar chromatogram patterns but differed in peak intensities. The differences in intensities are most apparent at retention times of 1–26 min. The similarities in chromatogram patterns and differences in peak intensities indicate that each extract has a similar metabolite content, but they differ in the levels of each metabolite.

Next, the raw data from UHPLC-HRMS analysis were processed using Compound Discoverer 3.2 software. Data processing yielded 399 detected metabolites. Of the 399 metabolites detected, 22 were putatively identified by matching the experimental MS/MS spectrum with the reference MS/MS spectrum in the Competitive Fragmentation Modeling for Metabolite Identification (CFM-ID) database. The identified metabolites mostly comprised phenolic and fatty acids (Table 2). Phenolic acids are bioactive plant metabolites with one or more hydroxyl groups attached to an aromatic ring.[34] Phenolic acids generally exhibit a fragmentation pattern by releasing CO_2 molecules $[M–H–44]^-$.[35] Wang *et al*. [36] and Yin *et al*. [37] reported that hanjeli seeds mainly contain phenolics and fatty acids. Xi *et al*. [38] also reported that the main fatty acids in hanjeli seeds were linoleic and palmitic acids.

Table 1: Extraction yields and antibacterial activities of hanjeli seed extracts

Solvent	Extraction yield (%)	Inhibition *E. coli* (%)
EtOH25	4.48±0.40	40.03±2.14[b]
EtOH50	4.21±1.04	33.52±0.00[a]
EtOH75	6.00±1.81	33.16±0.00[a]

Mean values followed by different alphabets are significantly different by Duncan's post hoc test at $p < 0.05$

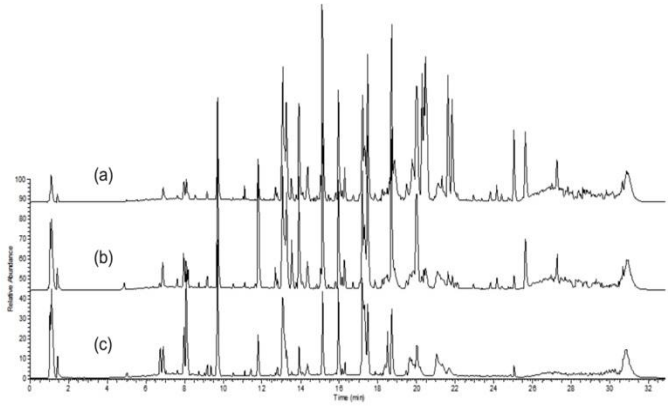

Figure 1: Base peak chromatogram of hanjeli seeds extracts using (a) ethanol 75%, (b) ethanol 50%, and (c) ethanol 25% as extraction solvent

Metabolite distribution in hanjeli seeds extract

The variances in metabolite distribution within hanjeli seed extracts were assessed using principal component analysis (PCA) and hierarchical cluster analysis (HCA) for multivariate data analysis. PCA, known for elucidating relationships among samples, such as grouping patterns, enables the differentiation of samples based on the concentration of the extraction solvent.[39] Furthermore, the metabolite composition differences of the extracts were visualized using an HCA heatmap. The heatmap, generated using the identified metabolite peak area variables and employing the Euclidean distance measure algorithm and Ward's clustering method, illustrates variations in metabolite abundance across samples.[40] This visualization provides insights into the clustering patterns of metabolites and highlights any trends or similarities within a the dataset.

A PCA model was created using the peak areas of the detected metabolites. Based on the resulting score plot, each sample could be differentiated based on the extraction solvent concentration using PC1 and PC2, with a total PC of 78.2% (Figure 2). The PC value showed that the PCA model can explain as much as 78.2% of the data variability. Furthermore, based on the classification pattern obtained, it can be inferred that there are differences in the distribution of metabolites in each extraction solvent, and each sample can be grouped according to the extraction solvent.

The HCA heatmap illustrates the metabolite variations in each sample (Figure 3). Fatty acids dominated the metabolite content in the 75% ethanol extract, whereas phenolic acids dominated the 25% ethanol extract. The variations in the metabolites contained in each extract indicate that differences in the concentration of the extraction solvent influenced the composition of the extracted metabolites.

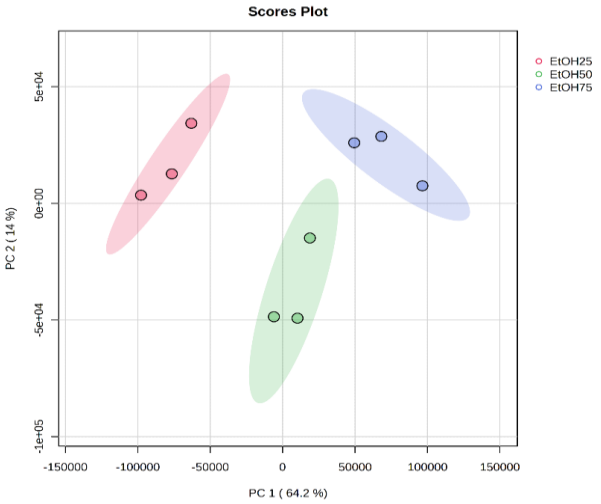

Figure 2: Plot score PCA of hanjeli seed extracts

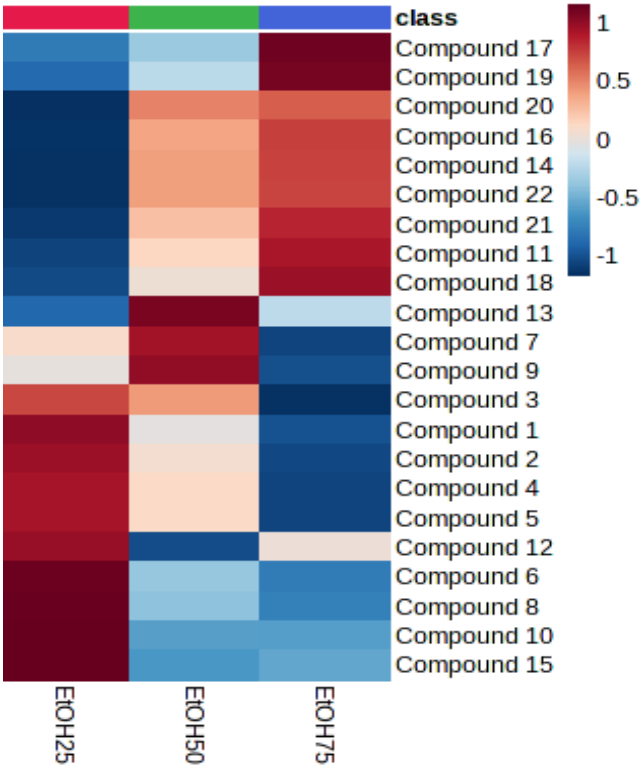

Figure 3: Heatmap visualization for each identified metabolite. Compounds 1-22, see in Table 2.

Prediction of potential metabolites as antibacterial agents in hanjeli seed extracts

Our study aimed to predict the potential metabolites within hanjeli seed extracts that exhibit antibacterial properties against *E. coli* using Pearson correlation. Metabolites exhibiting a positive correlation with percent inhibition (antibacterial activity), with correlation coefficients

near 1, were identified as potential antibacterial agents. Pearson correlation analysis revealed several phenolic acid compounds, including gluconic acid, citric acid, caffeic acid, *p*-coumaric acid, and hexadecanedioic acid, which displayed significant positive correlations with the percent inhibition value of *E. coli* (Figure 4). Previous studies support the antibacterial potential of gluconic acid produced by *Gluconacetobacter diazotrophicus* against bacterial pathogens.[41] Additionally, research by Masoura *et al*. indicated that a combination of gluconic acid and H_2O_2 effectively inhibits *E. coli* growth through membrane depolarization and cell wall damage.[42] Citric acid, recognized for its role in plant metabolism, has been reported to exhibit antibacterial activity against *E. coli* by lowering the environmental pH and inducing structural damage to bacterial cells.[43,44] Furthermore, studies by Kang *et al*. highlighted citric acid's ability to inactivate superoxide dismutase in *E. coli*, leading to reactive oxygen species induction and subsequent cell membrane damage.[45] Caffeic acid and *p*-coumarin acid, natural antibacterial compounds, have been shown to inhibit *E. coli* growth.[46–49] Caffeic acid alters bacterial cell membrane structure and function, whereas p-coumarin acid disrupts cell membranes and binds to bacterial genomic DNA, inhibiting cellular functions. Additionally, our study identified hexadecanedioic acid, a fatty acid compound, as positively and significantly correlated with antibacterial activity. Fatty acids inhibit bacterial growth through various mechanisms, including DNA/RNA replication inhibition, cell wall synthesis disruption, and cytoplasmic membrane damage.[18] Although previous research supports the antibacterial activity of organic acids, phenolic acids, and fatty acids, the potential antibacterial role of hexadecanedioic acid in *E. coli* requires further investigation. Further studies are required to confirm the efficacy of hexadecanedioic acid as an antibacterial agent.

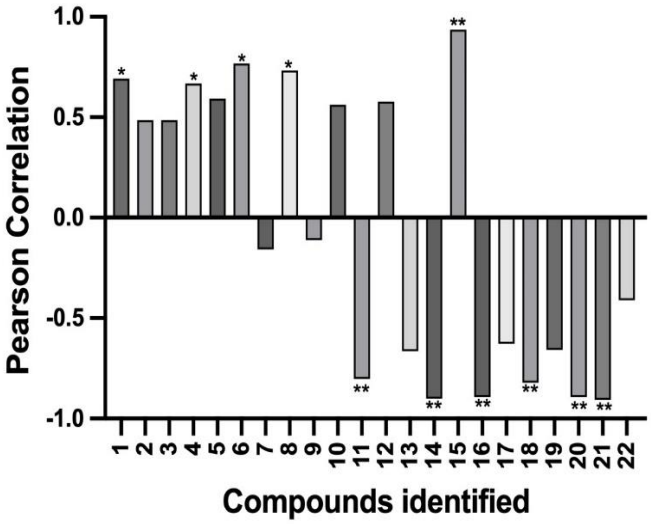

Figure 4: Pearson correlation value between antibacterial activity and compounds identified in hanjeli seed extracts. Compound 1-22, see Table 2. * Significant at $p < 0.05$, ** significant at $p < 0.01$.

Table 2: Putative identified metabolites in hanjeli seeds extract

No	Name	Molecular formula	Error (ppm)	Molecular weight	RT (min)
1	Gluconic acid	$C_6H_{12}O_7$	-3.4	196.0576	1.101
2	*D*-(-)-Quinic acid	$C_7H_{12}O_6$	-3.69	192.0627	1.113
3	*DL*-Malic acid	$C_4H_6O_5$	-7.24	134.0206	1.124
4	Citric acid	$C_6H_8O_7$	-3.05	192.0264	1.124
5	Isocitric acid	$C_6H_8O_7$	-3.12	192.0264	1.437
6	Caffeic acid	$C_9H_8O_4$	-3.48	180.0416	6.76
7	3-*O*-Feruloylquinic acid	$C_{17}H_{20}O_9$	0.17	368.1108	7.977
8	*p*-coumaric acid	$C_9H_8O_3$	-4.65	164.0466	8.102
9	4'-Hydroxy-5,6,7-trimethoxyflavone	$C_{18}H_{16}O_6$	0.89	328.095	8.397
10	Hydrocinnamic acid	$C_9H_{10}O_2$	-5.05	150.0673	11.438
11	(15*Z*)-9,12,13-Trihydroxy-15-octadecenoic acid	$C_{18}H_{34}O_5$	-0.14	330.2406	13.097
12	Isorhamnet	$C_{16}H_{12}O_7$	0.63	316.0585	13.121
13	*p*-Coumaric acid ethyl ester	$C_{11}H_{12}O_3$	-3.42	192.078	13.551
14	(+/-)9,10-dihydroxy-12*Z*-octadecenoic acid	$C_{18}H_{34}O_4$	-0.24	314.2456	17.48
15	Hexadecanedioic acid	$C_{16}H_{30}O_4$	0.06	286.2144	18.513
16	9,10-Dihydroxystearic acid	$C_{18}H_{36}O_4$	-0.82	316.2611	18.725
17	(10E,12*Z*)-9-Hydroperoxy-10,12-octadecadienoic acid	$C_{18}H_{32}O_4$	-0.29	312.23	20.475
18	9-Oxooctadecanoic acid	$C_{18}H_{34}O_3$	-0.25	298.2507	21.311
19	13*S*-hydroxyoctadecadienoic acid	$C_{18}H_{32}O_3$	-0.85	296.2349	21.875
20	2-Hydroxypalmitic Acid	$C_{16}H_{32}O_3$	0.07	272.2352	24.166
21	Linoleic acid	$C_{18}H_{32}O_2$	-0.61	280.2401	25.649
22	13-Hydroxydocosanoic acid	$C_{22}H_{44}O_3$	0.01	356.3291	26.998

Conclusion

This study, utilizing a UHPLC-HRMS-based metabolomics approach, demonstrated that the extraction solvent concentration significantly affects the metabolite profile and antibacterial activity of hanjeli seeds. Notably, 22 metabolites were identified, with the 25% ethanol extract showing the highest antibacterial activity, predominantly due to its phenolic acid content. These findings underscore the importance of solvent selection in optimizing the bioactive potential of Hanjeli seed extracts and in identifying promising metabolites for further antibacterial research.

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 tothe content of this article will be borne by them.

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