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METABOLOMIC ANALYSIS TO DETERMINE THE ANTIBACTERIAL BIOMARKERS OF CITRONELLA (*Cymbopogon nardus* L.) ESSENTIAL OIL AND EXTRACT

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ABSTRACT

This study aims to find the biomarkers for antibacterial activity in Mahapengiri citronella grass (*Cymbopogon nardus* L.). They were determined by comparing the compounds contained between each sample of essential oil and extract thoroughly using a Gas Chromatography-Mass Spectrometry (GC-MS)-based metabolomic approach. Essential oil was obtained using hydrodistillation with and without sonication pre-treatment, and steam distillation. Citronella extract was obtained through maceration using n-hexane and ethyl acetate. The samples' antibacterial activity was tested by the broth microdilution method against *E. coli* and *S. aureus*, chemical component analysis using GC-MS, and determination of biomarker compounds using MetaboAnalyst application. Principal Component Analysis successfully differentiates essential oils and extracts into three groups based on their compound similarity, namely hydrodistillation, steam distillation, and extracts. Based on Orthogonal Partial Least Squares-Discriminant Analysis, the biomarkers of citronella essential oil that have potential as antibacterial agents were citronellal, citronellol, geraniol, and limonene; while the potential biomarkers in the extract were ethylbenzene, bis(2-ethylhexyl) phthalate, and geranyl acetate.

Keywords: Antibacterial, citronella grass, metabolomics, Orthogonal Partial Least Square-Discriminant Analysis, Principal Component Analysis.

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INTRODUCTION

Indonesia has a very high plant diversity with over 30,000 plant varieties, of which about 7,500 are indicated to be potent as medicine, and around 500 species have been manufactured on an industrial scale.[1,2] One of them is the citronella grass (*Cymbopogon nardus* L.). This plant has green, flat, elongated leaves resembling reeds and a pungent aroma like orange, with the main product being citronella oil, which is commonly used in industry as a mosquito repellent, pesticide, or fragrance agent in food, soap, and cosmetics.[3] Citronella grass has potential as an antibacterial agent due to most of its secondary metabolites being terpenoid compounds and their three main components being citronellal, citronellol, and geraniol.[4,5] Antibacterial activity determination of citronella grass has been done previously by Verma *et al.* (2020)[6]; however, they didn't specify which compounds in the samples possibly influence the antibacterial activity of citronella grass. Metabolomics approach in this study was carried out to find the correlation between the components of citronella essential oils and extracts with their bioactivity to determine biomarkers that have a specific influence on this bioactivity. Metabolomics is a comprehensive study of small molecules called metabolites to quantitatively identify overall changes in large numbers of metabolites in a sample. Multivariate data

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analysis in metabolomics can be used to identify biomarkers from each sample and their correlation to bioactivity, so the compounds that influence its bioactivity can be identified.[7] Prior research by Santos *et al*.[8] showed that metabolomic analysis using Gas Chromatography-Mass Spectrometry (GC-MS) could be done to identify and correlate biomarkers responsible for the antibacterial activity of various essential oils using the Orthogonal Partial Least Square–Discriminant Analysis (OPLS-DA) model. Metabolomics studies have been widely applied to identify biomarkers from a sample and their bioactivity. Meanwhile, metabolomics studies of citronella grass of the Mahapengiri variety have never been conducted to determine the specific secondary metabolites that influence antibacterial activity. Therefore, this research aims to identify the chemical components responsible for the antibacterial activity of citronella (*C. nardus* L.) essential oils and extracts of the Mahapengiri variety obtained from various extraction methods through a GC-MS-based metabolomics approach, so it can be a basis of consideration to develop citronella essential oils and extracts as antibacterial drugs.

EXPERIMENTAL

Material

Citronella grass (*C. nardus* L.) of the Mahapengiri variety from Balai Penelitian Tanaman Obat dan Aromatik (Balittro) experimental garden Cimanggu, Bogor. *Escherichia coli* ATCC 11229 and *Staphylococcus aureus* ATCC 6538 bacteria, obtained from laboratorium central Universitas padjadjaran. Chemicals include ethyl acetate and n-hexane technical grade solvent, distilled water, ciprofloxacin (Sigma-Aldrich), DMSO 2% (Sigma-Aldrich), Mueller Hinton Broth medium (Himedia), and Nutrient Broth medium (Himedia).

Isolation of Citronella Oil and Extract

Citronella leaves were prepared by air-drying without direct sunlight for ± 7 days and then cut homogeneously to a size of 1–1.5 cm. The essential oils from the hydrodistillation process were acquired by introducing a total of 80 g of the citronella leaves with 1600 mL of distilled water in the boiling flask of the hydrodistillation unit. They were distilled for 6 hours at a temperature of 116-120 ℃. Sonication pre-treatment before the hydrodistillation process was done at 90% amplitude and a temperature of 20 ºC for 15 minutes. Meanwhile, the steam distillation processes were carried out using 500 g samples of dried citronella leaves for 6 hours using hot steam. The collected essential oils were dried using sodium sulfate crystals (Na_2SO_4). Citronella extract was obtained using a maceration process by soaking 100 g dried citronella leaves of 6 mesh size in 1000 mL of n-hexane or ethyl acetate for 3x24 hours in an incubator shaker with constant stirring at 100 rpm and a temperature of 27 ºC, as well as solvent replacement every 24 hours. The extracts were then concentrated using a rotary evaporator until they were free from solvents.

Citronella Oil and Extract Antibacterial Activity Determination

E. coli and *S. aureus* bacteria were cultured in 5 mL of Nutrient Broth and Mueller Hinton Broth respectively, and incubated at 37 ºC for 24 hours to obtain bacterial suspension. The suspension was adjusted according to 0.5 McFarland Standard (0.1 absorbance at a wavelength of 600 nm) and diluted 20x to gain 10^5 CFU/mL concentration of bacteria. The sample solution was made by dissolving them in 2% DMSO to obtain a 50% concentration of the sample. The minimum inhibitory concentration (MIC) of the samples was determined by using the broth microdilution method. This test starts by inserting 100 µl of bacterial growth medium into all the wells in a 96-well plate. A total of 100 µl sample solution was then incorporated into the first column, and serial dilutions were carried out up to column 9 to obtain varying sample solution concentrations (25 to 0.098% w/v for *E. coli* and 3.125 to 0.012% w/v for *S. aureus*). Column 10 was added with 100 µl of bacterial medium as a media control, column 11 was added with 50 µl of bacterial medium and 50 µl of 2% DMSO solvent as a solvent control, and column 12 was added with ciprofloxacin and diluted up to row 8 to obtain a concentration of 5 to 0.039 ppm as a positive control.
Bacterial suspension with the amount of 10 µl was filled to each well except column 10 and then incubated at 37 ºC for 24 hours. The MIC value of each sample can be determined by measuring the absorbance before and after incubation, where wells that had a higher absorbance after incubation than before incubation indicate bacterial growth. The MIC value chosen was the well with the lowest sample concentration that can inhibit bacterial growth.

Chemical Component Analysis and Multivariate Analysis Using Metabolomics Approach

GC-MS analysis was used to determine the chemical components of citronella oil and extract. The components were determined by comparing the mass spectra produced from GC-MS to the National Institutes of Standards and Technology (NIST) 20 database. The chromatogram data from all the samples was processed further using MS-Dial software by aligning secondary metabolites for each citronella extraction method based on the area and retention time of each peak. The data alignment was then analyzed further using multivariate data analysis, such as Principal Component Analysis (PCA) and OPLS-DA using MetaboAnalyst 5.0 software. These multivariate analyses will give us all the possible biomarkers that can influence the antibacterial activity of the samples. Pearson Correlation analysis between the abundance of a specific biomarker in all the samples and the MIC value of all the samples will then be conducted. It is done to find out whether this specific biomarker has a positive or negative influence on the samples' antibacterial activity and whether they have the capability as antibacterial agents or not.

RESULTS AND DISCUSSION

Analysis of Citronella Leaves Chemical Compounds

GC-MS analysis is important in this study to determine all the compounds in the sample along with their abundance, which will be used later on for metabolomic analysis. The identified compounds from GC-MS analysis were sorted by carrying out a tracing process on compounds that had an area above 0.1% and a level of similarity to NIST 20 above 85%.[9] There were 31 secondary metabolites identified in the essential oils obtained from the hydrodistillation processes with sonication pre-treatment (HS) and without sonication pre-treatment (HTS), while steam-distilled (DU) samples only had 30 secondary metabolites. GC-MS analysis of citronella leaf extract succeeded in identifying 51 secondary metabolites. Between the 51 secondary metabolites, ethyl acetate (MEA) extract had a total of 44 compounds, while n-hexane (MNH) extract had 43 compounds, of which 37 (72.5%) were present in both extracts. Most of the compounds identified in citronella leaves belong to the monoterpenoid and sesquiterpenoid groups, while a fraction of them belong to the diterpenoid, triterpenoid, aromatic, ester, ether, and ketone groups. The chemical compositions identified in the citronella essential oils and extracts can be seen in Table-1. Table-1 shows that all the samples have the same main components in the form of *citronellal*, *geraniol*, and *citronellol*. The main components of each sample have different average concentrations compared to each other, where the concentration of the main components in citronella extracts tends to be less than the essential oils. This is due to the greater number of compounds obtained through the maceration method compared to distillation, and so the percent area of the main components became smaller. The maceration process involves immersing the sample in a solvent to extract a wider range of compounds, in contrast to the distillation process, where most of the compounds extracted are volatile compounds.[10]

Antibacterial Activity of Citronella Essential Oils and Extracts

The essential oils and extracts of citronella grass were evaluated for antibacterial activity by determining the Minimum Inhibitory Concentration (MIC) value using the broth microdilution method against Gram-negative and Gram-positive bacteria, *E. coli* and *S. aureus* respectively. The tests were carried out using a concentration range of 0.098% - 25% for *E. coli* bacteria and 0.012% - 3.125% for *S. aureus* bacteria. According to Aligiannis *et al*.[11], the antibacterial activity of extracts based on the MIC value can be classified into three categories, namely strong (MIC value < 0.5%), moderate (MIC value 0.6-1.5%), and weak (MIC value > 1.6%). The results of the broth microdilution method can be seen in Table-2.

The MIC test results of citronella products obtained by various methods had varying MIC values against *E. coli* and *S. aureus*. Based on Table-2, the highest antibacterial activity against *E. coli* of all samples was found in MEA, which was classified as medium-weak with an MIC value of 0.781–3.125%. The MNH sample, on the other hand, had the lowest activity with an MIC value of 25%. Meanwhile, the highest antibacterial activity against *S. aureus* bacteria was found in the HS sample, which was classified as strong with a MIC value of 0.195-0.391%. Extracts obtained from the maceration process, in contrast, tend to have lower antibacterial activity against *S. aureus*, with the MNH sample having the lowest activity with an MIC value of 6.25-25%. The result reveals that most of the citronella essential oil and extract samples in this study were more effective as antibacterial agents against *S. aureus* bacteria than *E. coli*. This can be caused by the biochemical composition of the Gram-negative bacteria (*E. coli*) outer membrane.

This outer membrane consists of lipoproteins and lipopolysaccharides which are selectively permeable; therefore, they can regulate the absorption of antibacterial compounds into the cells.[12] The antibacterial activity of each sample is much lower than the positive control, ciprofloxacin. This is because ciprofloxacin is a pure compound, whereas the samples in this study contain various compounds that can interact synergistically, causing the antibacterial activity to be greater. They can also interact antagonistically, causing the activity to decrease.[13] The difference in antibacterial activity of each sample can be explained by the differences in the concentration and variations of active compounds contained in each sample.[14] The result also shows that extract samples have relatively lower antibacterial activity than essential oils. This is because the maceration process can also extract long-chain fatty acid compounds such as geranyl linoleate, citronellyl linoleate, geranyl palmitate, and citronellyl myristate, which can act as carbon and energy sources for bacteria.[15]

Table-1: Secondary Metabolite Components of Citronella Essential Oils and Extracts

Component	Area (%)														
	DU1	DU2	DU3	HTS 1	HTS 2	HTS 3	HS1	HS2	HS3	MEA1	MEA2	MEA3	MNH1	MNH2	MNH3
D-Limonene	2.39	2.28	2.28	2.82	2.89	2.79	2.4	2.9	2.93	0.24	0.2	0.2	0.42	0.32	0.22
Linalool	0.43	0.42	0.41	0.67	0.73	0.72	0.68	0.69	0.74	0.37	0.36	0.36	0.4	0.33	0.41
Isopulegol	0.28	0.27	0.37	1.32	1.67	1.71	1.6	1.65	1.96	0.54	0.64	0.54	0.29	0.2	0.21
Citronellal	46.81	48.23	48.32	41.76	44.13	42.05	40.94	44	44.1	33.21	28.07	32.98	30.74	29.97	26.81
Neo-isoisopulegol	0.18	0.18	0.24	0.41	0.51	0.51	0.49	0.51	0.59	0.32	0.15	0.17	0.16	0.11	0.13
Nerol	-	-	-	0.19	0.18	0.19	0,18	0,15	0,11	-	-	-	-	-	-
Citronellol	12.31	12.67	12.98	12.78	13.25	13.29	12.97	13.5	14	10.61	7.77	10.66	9.57	8.71	9
Neral	0.21	0.18	0.17	0.3	0.27	0.23	0.3	0.24	0.22	0.32	0.56	0.31	0.11	0.15	0.1
Geraniol	13.84	13.23	13.78	15.99	15.24	15.88	16.06	15.6	16.1	13.46	9.83	12.89	11.46	10.51	10.71
Citral	0.31	0.27	0.25	0.4	0.34	0.28	1.5	0.29	0.27	0,2	0,17	0,19	0.18	0.24	0.16
Citronellyl acetate	1.41	1.28	1.35	1.12	1.3	1.2	1.23	1.19	1.06	2.02	4.35	1.76	0.88	1.44	0.83
Geranyl acetate	1.03	0.97	1.31	0.73	0.91	0.84	0.81	0.8	0.72	2.08	4.46	2.04	0.94	1.56	0.87
β-Elemene	0.41	0.4	0.37	0.58	0.54	0.58	0.58	0.5	0.49	0.15	0.18	0.13	0.13	0.07	0.12
Caryophyllene	4.11	4.43	4.03	3.82	3.77	4.03	3.88	3.81	3.56	3.13	2.83	3.22	3.26	3.34	2.95
Isoeugenol	0.7	0.82	0.45	0.52	0.62	0.67	0.55	0.72	0.64	1.7	1.31	1.45	1.08	1.39	1.08
Humulene	0.6	0.63	0.59	0.57	0.54	0.58	0.56	0.55	0.52	0.47	0.42	0.5	0.46	0.49	0.44
Germacrene-D	1.62	1.59	1.38	1.18	1.16	1.29	1.17	1.2	1.09	1.47	1.09	2.02	1.24	1.46	1.13
α-Muurolene	0.47	0.44	0.43	0.41	0.35	0.42	0.38	0.39	0.32	0.29	0.15	0.11	0.09	0.11	0.09
Germacrene-B	0.34	0.33	0.31	0.27	0.23	0.26	0.23	0.28	0.22	0.45	0.76	0.65	0.35	0.42	0.32
γ-Muurolene	0.39	0.36	0.36	0.25	0.22	0.25	0.23	0.24	0.22	-	-	-	-	-	-
δ-cadinene	1.23	1.24	1.28	1.49	1.36	1.47	1.44	1.4	1.32	0.68	0.28	0.32	0.29	0.3	0.25
Citronellyl butyrate	0.57	0.58	0.61	0.6	0.54	0.6	0.54	0.54	0.52	0.47	0.43	0.5	0.51	0.51	0.48
Elemol	1.37	1.29	1.56	2.24	2.13	2.36	2.08	2.09	2.18	0.66	0.71	0.78	0.84	0.77	0.79
Geranyl isobutyrate	0.64	0.64	0.61	0.72	0.62	0.53	0.62	0.56	0.56	0.47	0.46	0.51	0.54	0.55	0.57
Germacrene D-4-ol	2.09	2.02	1.78	0.84	0.47	0.53	0.48	0.44	0.34	3.4	4.03	4.23	4.8	6	5.22
Cubenol	0,1	0,1	0,1	0.15	0.14	0.15	0.16	0.14	0.14	-	-	-	-	-	-
γ-Eudesmol	0.47	0.4	0.4	0.69	0.59	0.58	0.55	0.58	0.51	-	-	-	-	-	-
α-Cadinol	1.26	1.08	0.62	1.69	1.38	1.41	1.4	1.38	1.3	0.63	0.62	0.23	0.39	0.3	1
T-Muurolol	2.08	1.97	2.09	3.05	2.63	2.83	2.74	2,72	2,58	1.11	0.96	0.77	0.81	0.57	1.41
Citronellyl hexanoate	0.24	0.24	0.23	0.24	0.17	0.21	0.18	0.18	0.14	0.33	0.28	0.31	0.31	0.3	0.28
Geranyl hexanoate	0.2	0.19	0.17	0.19	0.14	0.17	0.16	0.14	0.11	0.31	0.21	0.38	0.29	0.26	0.23
Butyl acetate	-	-	-	-	-	-	-	-	-	1.19	1.17	0.87	-	-	-
Ethylbenzene	-	-	-	-	-	-	-	-	-	0.4	0.68	0.52	0.16	0.17	0.22
m-Xylene	-	-	-	-	-	-	-	-	-	0.08	0.37	0.26	-	-	-
o-Xylene	-	-	-	-	-	-	-	-	-	0.18	0.27	0.21	-	-	-
Cyclohexanone	-	-	-	-	-	-	-	-	-	0.16	0.4	0.26	-	-	-
2-Butoxyethanol	-	-	-	-	-	-	-	-	-	1.01	2.13	2.46	0.27	0.21	0.42
Butoxyethyl acetate	-	-	-	-	-	-	-	-	-	0.62	3.7	2.46	-	-	-
Citronellic acid	-	-	-	-	-	-	-	-	-	0.2	0.22	0.22	0.5	0.41	0.5
Neomenthoglycol	-	-	-	-	-	-	-	-	-	1.81	0.75	1.61	1.82	1.69	1.6
4-methylcyclohexane	-	-	-	-	-	-	-	-	-	0.91	0.75	0.82	0.87	0.8	0.77
Vanillin	-	-	-	-	-	-	-	-	-	0.39	0.49	0.33	-	-	-
2,4-Di-tert-butylphenol	-	-	-	-	-	-	-	-	-	0.19	0.44	0.32	-	-	-
Proximal	-	-	-	-	-	-	-	-	-	0.4	0.37	0.37	0.41	0.26	0.37
Neophytadiene	-	-	-	-	-	-	-	-	-	0.67	0.43	0.41	0.22	0.33	0.25
Citronellyl myristate	-	-	-	-	-	-	-	-	-	0.32	0.52	0.43	2.18	2.79	1.58
Cycloartenol	-	-	-	-	-	-	-	-	-	3.6	3.99	3.56	3.89	4.73	4.66
2-Hexanone	-	-	-	-	-	-	-	-	-	-	-	-	0.17	0.12	0.24

Component	Area (%)														
	DU1	DU2	DU3	HTS 1	HTS 2	HTS 3	HS1	HS2	HS3	MEA1	MEA2	MEA3	MNH1	MNH2	MNH3
Cyclohexane	-	-	-	-	-	-	-	-	-	-	-	-	1.63	0.82	3.03
1-Methylcyclo-pentanol	-	-	-	-	-	-	-	-	-	-	-	-	0.17	0.15	0.36
Geranyl palmitate	-	-	-	-	-	-	-	-	-	-	-	-	2.68	3.75	1.81
Citronellyl linoleate	-	-	-	-	-	-	-	-	-	-	-	-	1.02	1.7	0.78
Bis(2-ethylhexyl) phthalate	-	-	-	-	-	-	-	-	-	0.27	0.96	0.56	-	-	-
Geranyl linoleate	-	-	-	-	-	-	-	-	-	-	-	-	1.2	1.96	1.07
Glutaric acid, tridec-2-yn-1-yl geranyl ester	-	-	-	-	-	-	-	-	-	-	-	-	1.33	2.39	1.07
Total Components	30	30	30	31	31	31	31	31	31	44	44	44	43	43	43

Chemical Components Distribution and Biomarkers Determination of Citronella Products

Principal component analysis (PCA) was done in this study to find out the distribution of chemical components found in citronella extracts and essential oils. PCA works by identifying the distribution patterns in the data that have been reduced efficiently to find the similarities and differences in each sample.[16] The results are displayed as a score plot that describes the distribution and grouping of each sample based on the extraction methods. The grouping of samples obtained from various extraction methods based on PCA can be seen in Fig.-1.

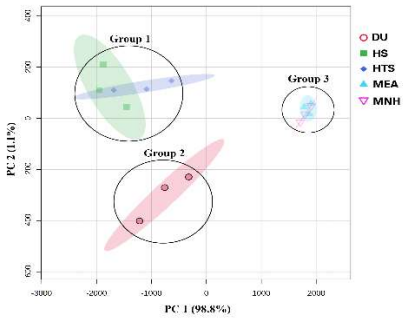

Fig.-1: PCA Scores Plot of Citronella Essential Oils and Extracts

The PCA score plot reveals that differences in the methods of obtaining citronella samples influence the distribution of secondary metabolites contained in the samples. Based on their secondary metabolites' distribution, citronella samples can be divided into three groups. Group 1 consists of essential oils produced using hydrodistillation, either with or without sonication (HS or HTS). Group 2 consists of steam-distilled essential oils (DU). Group 3 consists of extracts obtained from the maceration process using n-hexane (MNH) or ethyl acetate (MEA) as solvent. Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) in this study aims to identify the biomarkers of each citronella sample. OPLS-DA is a multivariate analysis suitable for diagnosing differences between two groups by showing what variables have the greatest discriminatory power and how these variables correlate with each group.[17] Two plots can be obtained from OPLS-DA, which are the scores plot and S-plot. The visual representation of the separation of two sample groups into two blocks is provided by the scores plot. The S-plot, on the other hand, indicates the relative content of each metabolite detected in each sample group.[18] Citronella leaf samples were successfully divided into two blocks by OPLS-DA: extract samples and essential oil samples (Fig.-2a). The OPLS-DA S-plot (Fig.-2b) shows that the X+ axis represents the dominant compounds in essential oil samples, while the X-axis represents the compounds in extract samples.

Biomarkers must meet the criteria of variable importance in projection (VIP) value exceeding 1, indicating that the compound has a significant effect on the differences between samples in the OPLS-DA model.[19] Based on the S-plot in Fig.-2b, the biomarkers for essential oils (HS, HTS, and DU) that meet the VIP value criteria are citronellal **(50)**, citronellol **(60)**, limonene **(32)**, and geraniol **(64)**, which have structures that can be seen in Fig.-3. The extract samples, on the other hand, don't have biomarkers based on this OPLS-DA model, because all variables (compounds) that represent the extract samples (X-axis) have covariance

values close to 0. Therefore, it is necessary to carry out an OPLS-DA analysis between MEA and MNH extracts to determine the biomarkers for each extract.

Table-2: MIC value of citronella essential oils and extracts

Sample	MIC (%)		Mean (%)	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
HTS 1	6.25	0.391	8.33 ± 3.61	0.78 ± 0.68
HTS 2	6.25	0.391		
HTS 3	12.5	1.563		
HS 1	6.25	0.391	4.17 ± 1.8	0.33 ± 0.11
HS 2	3.125	0.391		
HS 3	3.125	0.195		
DU 3	3.125	0.391	3.13 ± 0.0	0.39 ± 0.0
DU 4	3.125	0.391		
DU 5	3.125	0.391		
MEA 1	0.781	3.125	2.34 ± 1.35	7.29 ± 4.77
MEA 2	3.125	12.5		
MEA 3	3.125	6.25		
MNH 1	25	6.25	25 ± 0.0	14.58 ± 9.55
MNH 2	25	25		
MNH 3	25	12.5		
Ciprofloxacin	0.0000625	0.00003125	0.0000625	0.00003125

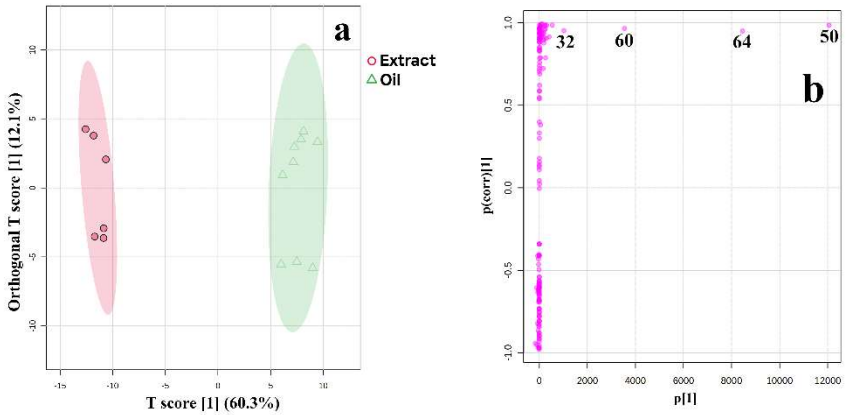

Fig.-2: OPLS-DA Scores Plot (a) and S-plot (b) between Citronella Essential Oils and Extracts

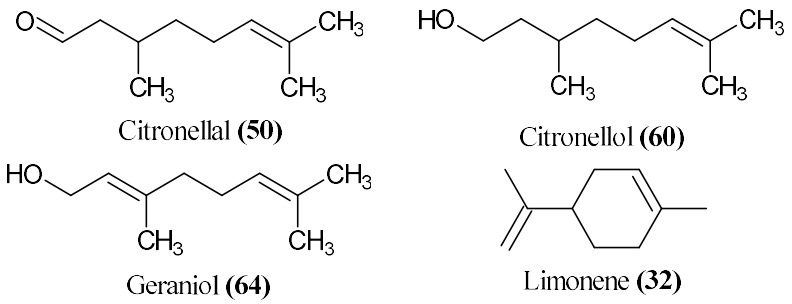

Fig.-3: Citronella Oil Biomarkers: Citronellal **(50)**, Citronellol **(60)**, Geraniol **(64)**, and Limonene **(32)**

The scores plot and S-plot between the MEA and MNH groups are shown in Fig.-4. OPLS-DA succeeded in separating the citronella leaf extract samples into two groups, which are MEA and MNH (Fig.-4a), with the X-axis representing the dominant compounds in MEA samples, while the X+ axis represents the compounds in MNH samples (Fig.-4b).

The S-plot in Fig.-4b reveals that the biomarkers from the MEA samples that met the VIP value criteria are ethylbenzene (8), geranyl acetate (76), and bis(2-ethylhexyl) phthalate (138); while cyclohexane (20), citronellyl myristate (142), and geranyl palmitate (143) are biomarkers of MNH samples, which structures can be seen in Fig.-5.

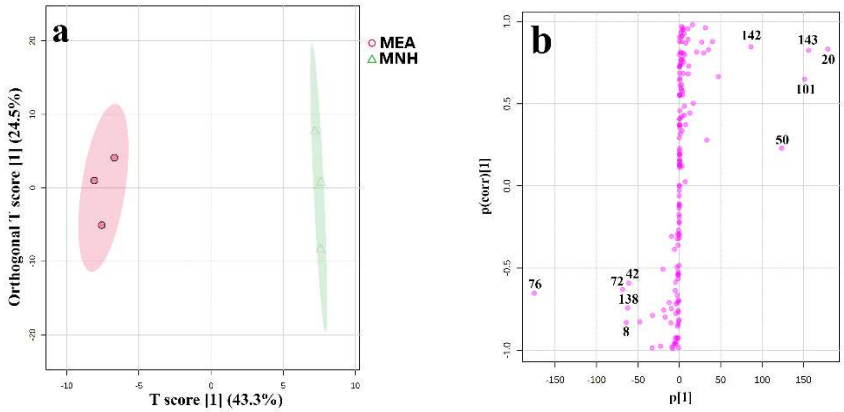

Fig.-4: OPLS-DA Scores Plot (a) and S-plot (b) between MEA and MNH Extracts

Prediction of Antibacterial Active Compound Components in Citronella Grass

Biomarkers that have been found in each sample by OPLS-DA were analyzed further using the Pearson correlation coefficient by correlating their abundance with the antibacterial activity (MIC value) of the samples. Previously, authors have reported that citronella grass (*C. nardus* L.) essential oil has good antibacterial activity.[6,20] However, no specific compounds have been reported to be responsible for this bioactivity; hence, this analysis and to extend, this study aimed to determine these specific compounds from citronella grass for the first time. In Pearson correlation analysis, a correlation coefficient (r) with values of 0 shows that there is no correlation between the two variables. A coefficient value between 0 and +1 shows that there is a positive correlation, where if one variable changes, the other variable also changes in the same direction. Coefficient values between 0 and -1, on the other hand, have the opposite effect. The relationship between the two variables becomes stronger with the coefficient value getting closer to -1 or 1.[21] The calculation of the Pearson correlation coefficient for essential oil biomarkers can be seen in Table-3.

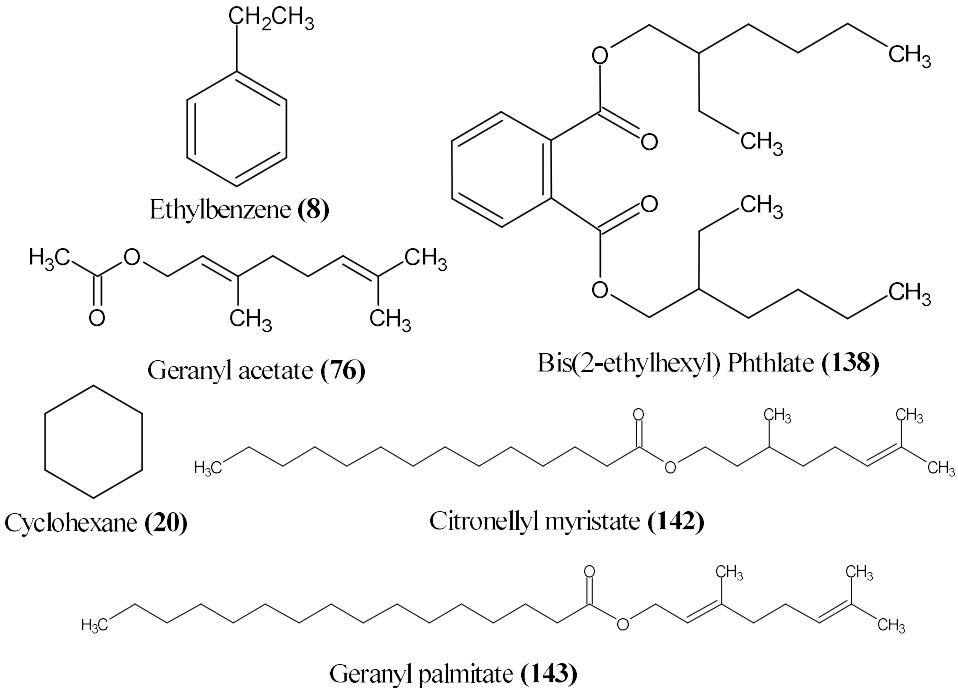

Fig.-5: MEA Biomarkers: Ethylbenzene (8), Geranyl Acetate (76), and Bis(2-ethylhexyl) Phthalate (138); MNH Biomarkers: Cyclohexane (20), Citronellyl Myristate (142), and Geranyl Palmitate (143)

Table-3: Correlation Coefficient of Essential Oil Biomarkers Against MIC Values of *E. coli* and *S. aureus*

Compounds	Pearson Correlation Coefficient			
	MIC *E. coli*	Significance value (α=0,05)	MIC *S. aureus*	Significance value (α=0,05)
Citronellal (50)	-0.3747	0.084	-0.6689	0.003
Citronellol (60)	-0.3793	0.082	-0.6596	0.004
Geraniol (64)	-0.3789	0.082	-0.6291	0.006
Limonene (32)	-0.4915	0.031	-0.6959	0.004

Based on the Pearson correlation analysis in Table-3, all four biomarkers of citronella oil correlate with negative values against the MIC value. This shows that these biomarkers are active antibacterial compounds against *E. coli* and *S. aureus* bacteria. Literature studies also show that citronellal (50), citronellol (60), geraniol (64), and limonene (32) have potential as antibacterial agents.[22,23] These four compounds are terpenoid compounds, which work as antibacterial substances by binding to the surface constituents of bacterial cells essential for their growth and survival. The compounds can build a monolayer surrounding the cell, which alters their electrostatic potential and hydrophobicity. This compromises the stability of membrane integrity and ultimately causes the internal bacterial cell components to be released.[24]

Table-4: Correlation Coefficient of Extract Biomarkers Against MIC Values of *E. coli* and *S. aureus*

Compounds	Pearson Correlation Coefficient			
	MIC *E. coli*	Significance value (α=0,05)	MIC *S. aureus*	Significance value (α=0,05)
Ethylbenzene (8)	-0.6419	0.085	-0.12	0.410
Geranyl acetate (76)	-0.3861	0.225	0.3422	0.253
Bis(2-ethylhexyl) phthalate (138)	-0.6822	0.068	-0.1126	0.416
Cyclohexane (20)	0.8247	0.022	0.1741	0.371
Citronellyl myristate (142)	0.9371	0.003	0.6523	0.080
Geranyl palmitate (143)	0.9099	0.006	0.6776	0.070

Pearson correlation analysis in Table-4 reveals that the biomarker compounds from the MEA extracts, ethylbenzene (8) and bis(2-ethylhexyl) phthalate (138), are active compounds against *E. coli* and *S. aureus* bacteria. The other biomarker, geranyl acetate (76), is active as an antibacterial agent only for *E. coli* bacteria. Biomarker compounds of MNH extract, on the other hand, don't work as antibacterial agents against both *E. coli* and *S. aureus* bacteria. Literature studies on MEA biomarker compounds show that ethylbenzene, geranyl acetate, and bis(2-ethylhexyl) phthalate have good activity as antibacterial agents.[23,25,26] Geranyl acetate works as an antibacterial agent like terpenoid compounds in general.[24] Ethylbenzene works as an antibacterial agent by penetrating the cytoplasmic membrane, resulting in membrane swelling and an increase in membrane fluidity. This causes the loss of membrane function and damage to bacterial cells.[26] However, no research explains the antibacterial mechanism of bis(2-ethylhexyl) phthalate compound. Biomarkers of MNH samples in the form of cyclohexane (20), citronellyl myristate (142), and geranyl palmitate (143), on the other hand, did not show good activity as antibacterial agents. Cyclohexane can promote bacterial growth; this is because cyclohexane can be a carbon and energy source for bacterial growth if added to the culture medium. The same reason applies to citronellyl myristate and geranyl palmitate, as they are unsaturated long-chain fatty acids, which can be broken down to become carbon and energy sources. Therefore, they can stimulate the growth of microorganisms in low concentrations.[15,27]

CONCLUSION

Citronella leaves of the Mahapengiri variety's samples showed varying antibacterial activity based on the method of obtaining them. In general, the essential oils (HTS, HS, and DU) were classified to be weakly active against Gram-negative bacteria (*E. coli*) and had strong activity against Gram-positive bacteria (*S. aureus*). The MEA extracts had moderate-weak activity against Gram-negative bacteria and weak activity against Gram-positive bacteria. Meanwhile, the MNH extract's antibacterial activity was weak, both against Gram-negative and Gram-positive bacteria. The results suggested that the antibacterial activity of

citronella essential oils and extracts depends on the concentration and variations of active compounds contained in the samples. Biomarkers of citronella essential oil were citronellal (50), citronellol (60), limonene (32), and geraniol (64). MEA biomarkers were ethylbenzene (8), geranyl acetate (76), and bis(2-ethylhexyl) phthalate (138), while the biomarkers of MNH were cyclohexane (20), citronellyl myristate (142), and geranyl palmitate (143). Based on Pearson correlation analysis, the biomarkers that have antibacterial activity against both *E. coli* and *S. aureus* bacteria were citronellal, citronellol, limonene, geraniol, ethylbenzene, and bis(2-ethylhexyl) phthalate, while geranyl acetate can only work against *E. coli*. The outcomes indicate that these specific compounds that were isolated from citronella grass have potential as an antibacterial agent; therefore, they can be analyzed further to be developed into antibacterial drugs.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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