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EXTRACTION AND IDENTIFICATION OF COMPOUNDS FROM BIOETHANOL PRODUCTION WASTE WITH POTENTIAL AS PEST CONTROL AGENTS BY LC-MS/MS

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Abstract

Bioethanol production generates waste by-products that may contain various valuable chemical compounds, many of which have not yet been fully explored or utilized. Among these by-products, liquid waste, commonly known as vinasse, is one of the most frequently processed and reused forms. This study aimed to extract and identify compounds from bioethanol production waste, particularly those with potential as pest control agents. Chemical profiling was conducted using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Analysis of samples extracted with n-hexane, ethyl acetate, ethanol, and water revealed several putative bioactive compounds with pesticidal activity. Notable identified compounds included DEET (N,N-diethyl-m-toluamide) (m/z = 192.1388), loliolide (m/z = 197.1178), (S)-reticuline (m/z = 330.1705), 3,5,6-trihydroxy-5-(hydroxymethyl)-2-methoxy-2-cyclohexen-1-one (m/z = 206.0712), and cuscohygrine (m/z = 225.1983). These compounds are known to exert various biological activities, including antibacterial, antifungal, and insect-repellent effects, each operating through distinct mechanisms. However, further functional validation through field implementation studies is necessary to evaluate the effectiveness and long-term sustainability of their application, particularly regarding the presence of DEET in the samples.

Keywords: *biological control, bioactive compounds, bioethanol waste, extraction, LC-MS/MS*

Introduction

The global demand for alternative energy sources continues to increase, driving a significant rise in bioethanol production. In 2018, global bioethanol production reached approximately 103 billion liters per year, led by the United States, Brazil, and European Union member states [1]. Indonesia, ranked 21st among bioethanol-exporting countries, has also experienced production growth, resulting in substantial volumes of liquid waste, particularly vinasse. For every liter of ethanol produced, approximately 9–15 liters of vinasse are released [2],[3]. This condition poses environmental challenges due to its high biological oxygen demand, low pH, and elevated concentrations of salts and organic compounds, which may contaminate soil and water bodies [4], [5].

On the other hand, vinasse is rich in organic matter and secondary metabolites, including organic acids, polyphenols, flavonoids, and terpenoids, which exhibit high bioactivity [6],[7]. Vinasse treatment has gained increasing attention because of its flocculating properties and its ability to bind organic contaminants [8]. Several of these compounds have been reported to possess antimicrobial and natural pesticidal properties, suggesting their potential application as environmentally friendly pest control agents compared with synthetic pesticides or mineral fertilizers [9].

However, detailed information regarding the chemical composition of vinasse and the mechanisms of action of its constituent compounds as pest control agents remains limited. Therefore, this study aims to identify and characterize the chemical composition of bioethanol production waste using liquid chromatography–tandem mass spectrometry (LC–MS/MS), with particular attention to its potential as a pest control agent. The findings of this study are expected to support circular-economy-based bioethanol waste management and contribute to the development of sustainable and environmentally friendly agricultural inputs.

Experimental Section

Materials

The materials used in this study consisted of 50 mL of liquid waste collected as a by-product of bioethanol production on July 12, 2024. Analytical-grade solvents and reagents included ethanol (Merck), *n*-hexane (Merck), ethyl acetate (Merck), distilled water, formic acid (Merck), and acetonitrile (Merck).

Instruments

The instruments used in this study included a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system comprising an ACQUITY UPLC® H-Class System (Waters, USA) equipped with an ACQUITY UPLC® HSS C18 column (1.8 μm, 2.1 × 100 mm; Waters, USA) and a Xevo G2-S QTof mass spectrometer (Waters, USA) (see **Figure 1**). Data acquisition and processing were performed using MassLynx™ software version 4.1. Additional laboratory equipment included a rotary evaporator, syringes, a 100 mL volumetric flask, a 100 mL volumetric pipette with bulb, a 250 mL beaker, a 250 mL separatory funnel, a 50 mL graduated cylinder, a spatula, a retort stand, a funnel, Whatman No. 42 filter paper, a glass stirring rod, and eight sample containers.

Procedure

A total of 50 mL of liquid waste generated as a by-product of bioethanol production was filtered using filter paper prior to fractionation to remove suspended solids and other impurities present in the sample. This filtration step was also performed to enhance sample purity and ensure that the resulting fractions were free from contaminants and suitable for further analysis.

Sample Fractionation Process

A 50 mL sample of liquid waste obtained as a by-product of bioethanol production was fractionated using four solvents with different polarities. Fractionation was conducted in a separatory funnel, and the mixture was gently shaken until it separated into two distinct phases. The solvents were added sequentially from non-polar to polar, starting with *n*-hexane as the non-polar solvent, followed by ethyl acetate as the semi-polar solvent, ethanol, and finally water as the polar solvent.

The sample was transferred into a separatory funnel and mixed with 50 mL of *n*-hexane, resulting in a 1:1 (v/v) ratio. The separatory funnel was gently shaken for 20 minutes in three intervals, with periodic venting to release any generated gas, and then allowed to stand until two phases formed. The *n*-hexane fraction was subsequently separated.

The remaining sample in the separatory funnel was then mixed with 50 mL of ethyl acetate and treated using the same procedure to obtain the ethyl acetate fraction. The same procedure was repeated using ethanol and water, yielding ethanol and aqueous fractions, respectively. All fractions were concentrated using a rotary evaporator at 40°C and subsequently analyzed by LC–MS/MS.

Sample Identification by LC–MS/MS

The *n*-hexane, ethyl acetate, ethanol, and aqueous fractions were analyzed using an LC–MS/MS instrument (see **Figure 1**) at the Forensic Science Center of the Criminal Investigation Agency (*Puslabfor Bareskrim*), Sentul, Bogor. A 1 mL aliquot of each sample was collected using a syringe, filtered through a 0.2 µm syringe filter, and injected into the LC–MS/MS system with an injection volume of 5 µL (see **Table 1**).

Figure 1. LC–MS/MS instrumentation.

Table 1. Operating Conditions of the LC–MS/MS Instrument

Name	Condition
MS system: Xevo G2-S QTof (Waters, USA)	1. Ionization source: ESI (electrospray ionization) 2. Mass analyzer: Quadrupole time-of-flight mass spectrometry 3. Ionization mode: Positive mode 4. Source temperature: 100°C 5. Desolvation temperature: 350°C 6. Mass analysis range: 50–1200 m/z 7. Collision energy: 4 V, low energy 8. Collision energy ramp: 25–60 V, high energy 9. Cone gas flow: 0 L/h 10. Desolvation gas flow: 793 L/h
LC system: ACQUITY UPLC® H-Class System (Waters, USA)	11. Column: ACQUITY UPLC® HSS C18 column, 1.8 µm, 2.1 × 100 mm 12. Mobile phase: water containing 5 mM ammonium formate and acetonitrile containing 0.05% formic acid 13. Temperature: 50°C column temperature; 25°C ambient temperature 14. Flow rate: 0.2 mL/min 15. Run time: 23 min 16. Injection volume: 5 µL after filtration through a 0.2 µm syringe filter
Software	MassLynx™ version 4.1

Data Analysis

LC–MS/MS analysis generated chromatographic and spectral data for each investigated sample. The resulting chromatograms and mass spectra were further processed using MassLynx™ version 4.1 software to obtain retention times, peak areas, ion mass-to-charge ratios (m/z), and molecular formulas of the identified compounds.

The obtained data were subsequently used to tentatively identify compounds through comparison with reference databases, including MassBank, ChemSpider, and the Human Metabolome Database (HMDB). The compounds identified through database matching were further verified by an extensive literature review to determine their characteristics and functions.

Results and Discussion

Fractionation of Bioethanol Waste

The fractionation process yielded four distinct fractions based on the solvents used, namely *n*-hexane, ethyl acetate, ethanol, and aqueous fractions. Fractionation of bioethanol waste using *n*-hexane and ethyl acetate showed optimal results, as indicated by the complete separation into two distinct phases after the process was completed. In contrast, fractionation using ethanol and water did not produce optimal separation because the bioethanol waste was completely soluble in both solvents (see **Figure 2**).

The obtained *n*-hexane, ethyl acetate, ethanol, and aqueous fractions were subsequently concentrated using a rotary evaporator at 40°C. This temperature was selected to ensure stability and prevent the degradation of bioactive compounds present in the samples. The evaporation process was conducted to remove solvents from the extracts, resulting in more concentrated and purified fractions with enhanced biological activity [12].

LC–MS/MS Analysis

LC–MS/MS analysis successfully detected a wide variety of compounds in each fraction, with the results varying according to the polarity of the solvents used. The compounds were identified by matching their mass spectra with reference data from HMDB, ChemSpider, and MassBank (see **Table 2** and **Table 3**). This technique works by generating a unique fragmentation pattern from parent ions and fragmenting them into smaller daughter ions. These patterns are essential for identifying and quantifying the compounds present [13]. In this study, a mass analyzer combining quadrupole and time-of-flight (QToF) technologies was used, allowing accurate determination of the mass-to-charge (*m/z*) ratios of each ion.

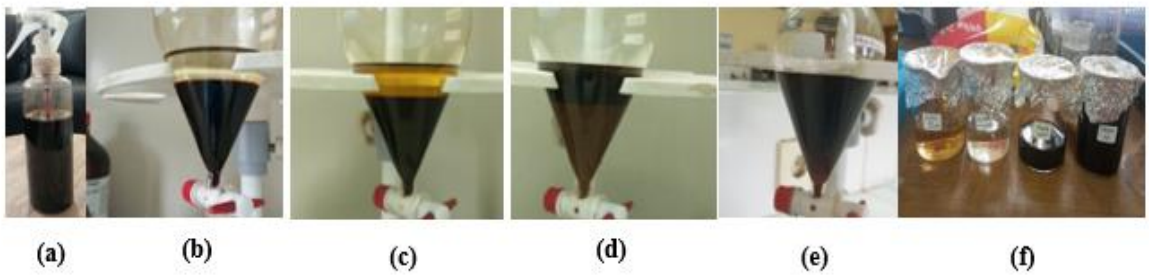


Figure 2. (a) Initial sample, (b) *n*-hexane fraction, (c) ethyl acetate fraction, (d) ethanol fraction, (e) aqueous fraction, and (f) sequentially separated fractions.

During the chromatographic process, molecules were separated based on the strength of their interactions with the stationary and mobile phases. Initially, the analytes bind to the stationary phase; however, as the mobile phase interacts more strongly with them, the compounds

are gradually eluted through the column [14].

The mobile phase consisted of water containing 5 mM ammonium formate and acetonitrile containing 0.05% formic acid. These additives helped maintain the protonated state of the compounds and reduced issues such as signal tailing or unwanted residues during separation. The system used a C18 column with an octadecyl-bonded silica surface for separation, installed in the ACQUITY UPLC® H-Class System. This UHPLC column has smaller particle sizes, making it more efficient and capable of faster and more precise analysis [15].

The samples were optimized through direct infusion into the mass spectrometer at a flow rate of 5 µL/min. The instrument was operated in positive ionization mode using electrospray ionization (ESI). It first detected the parent ion $[M+H]^+$ in MS1 and then fragmented it in MS2 to generate daughter ions. This quasi-molecular ion detection method improves identification accuracy [16].

Because of its high-resolution capability, the LC–MS/MS system can distinguish compounds with the same molecular weight but different chemical structures, known as isobaric compounds [17]. The system works by ionizing molecules and then detecting the resulting mass spectra. The ion signal is measured and plotted as a graph showing ion abundance against the mass-to-charge ratio, allowing the molecular structures of unknown compounds to be interpreted [17].

Each solvent extract was analyzed separately. The resulting chromatograms showed various peaks at different retention times, each representing different compounds. These retention times indicate how long a compound takes to pass through the column, thereby helping identify and compare the chemical profiles of the extracts [18].

Each chromatogram showed distinct peaks that varied in height and width (see **Figure 3**) because each compound had a specific structure and retention time. The mass spectrometer scanned the ionized compounds, recorded their mass-to-charge ratios (*m/z*), and measured the intensity of each signal (see **Table 2** and **Table 3**). As the compounds passed through the column at different times, the system detected and displayed separate peaks for each compound [19].

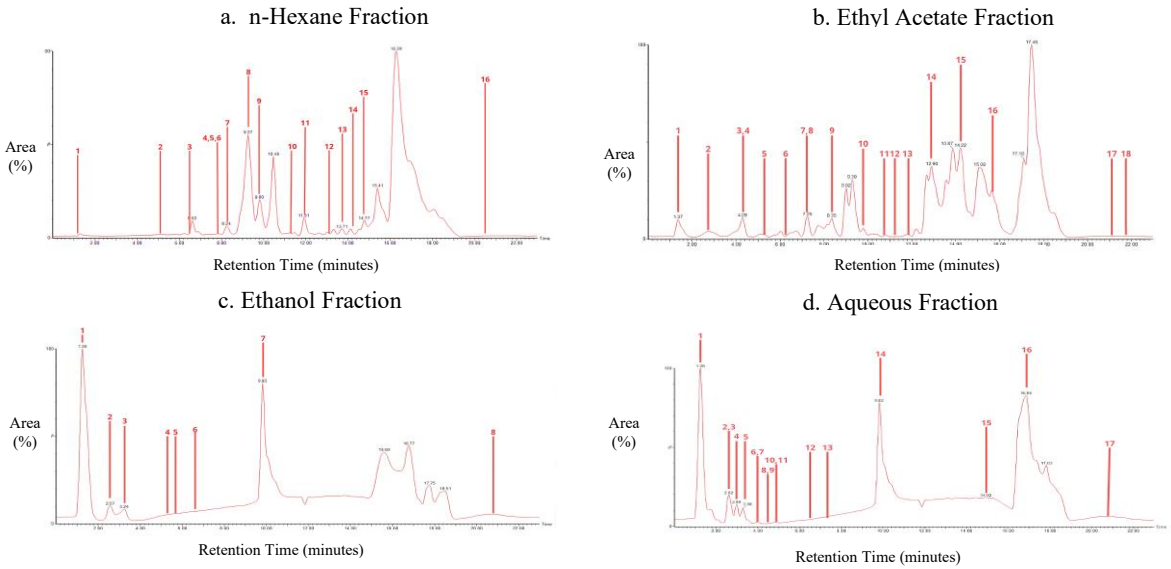


Figure 3. Chromatogram obtained from LC–MS/MS analysis.

LC–MS/MS was used to identify the compounds in each fraction without quantifying them. This technique enabled the detection of compounds, even at low concentrations, in complex mixtures. The ion masses were matched with reference data from HMDB, ChemSpider, and MassBank to identify each compound [20]. Even when some chromatographic peaks were not well resolved, compounds could still be identified based on their fragmentation patterns and mass-to-charge ratios [21].

Analysis of Bioactive Components as Pest Control Agents

The identification results revealed the presence of 16 potential compounds in the *n*-hexane fraction (see **Figure 3a** and **Table 2**). Among these, cuscohygrine (Compounds Nos. 9 and 16) (see **Figure 4b**) and DEET (Compound No. 7) (see **Figure 4a**) were identified as compounds with potential pest control activity. The compounds extracted using *n*-hexane were predominantly non-polar in nature, although several exhibited low polarity. As a non-polar solvent, *n*-hexane preferentially separates non-polar compounds according to the principle of "like dissolves like."

Compound identification was performed by comparing the retention times and mass spectra of the detected compounds with those of reference standards in the databases. The observed chromatographic peaks were relatively low in intensity, indicating that the detected compounds were present at low concentrations in the analyzed sample (see **Figure 3**).

In the ethyl acetate fraction, the observed peaks appeared at retention times corresponding to peak number 6, identified as loliolide, and peak numbers 10, 17, and 18, identified as cuscohygrine. Both compounds have been reported to exhibit potential activity as pest control agents.

Table 2. Identification Data for the *n*-Hexane and Ethyl Acetate Fractions

n-Hexane fraction						Ethyl acetate fraction					
No	RT	% Area	Measured mass (*m*)	Calculated mass (*z*)	Source	No	RT	% Area	Measured mass (*m*)	Calculated mass (*z*)	Source
1.	1.01	0.01	225.1983	225.1967	HMDB0030290	1.	1.37	2.88	182.0836	182.0817	HMDB0250803
2.	5.08	0.39	206.0840	206.0817	HMDB0004096	2.	2.75	0.85	144.0667	144.0661	HMDB014491
3.	6.24	0.10	207.1409	207.1385	HMDB0036386	3.	4.29	1.63	251.1051	251.1072	HMDB0028983
4.	7.89	0.11	177.0575	177.0585	HMDB0040003	4.	4.29	1.63	251.1051	251.1032	HMDB0240342
5.	7.89	0.11	177.0575	177.0552	HMDB0029758	5.	5.30	0.32	193.0529	193.0501	HMDB0303562
6.	7.89	0.11	177.0575	177.0624	HMDB0001209	6.	6.03	0.39	197.1203	197.1178	HMDB0302428
7.	8.24	0.75	192.1417	192.1388	MSBNK-BAFG-CSL2311094697	7.	7.26	1.16	368.1514	368.1516	HMDB0250479
8.	9.27	12.79	342.1701	342.1705	HMDB0031998	8.	7.26	1.16	368.1514	368.1557	HMDB0005817
9.	9.80	2.19	225.1984	225.1967	HMDB0030290	9.	8.35	1.80	328.1564	328.1549	HMDB0304001
10.	11.25	0.22	297.2451	297.2430	HMDB0253281	10.	9.87	0.51	225.1985	225.1967	HMDB0030290
11.	11.91	1.52	302.3076	302.3059	MSBNK-LCSB-LU100203	11.	10.79	0.10	263.2398	263.2375	HMDB0034495
12.	13.01	0.14	263.2403	263.2375	HMDB0034495	12.	11.30	0.02	346.2233	346.2210	HMDB0252929
13.	13.71	0.46	291.2345	291.2324	HMDB0000546	13.	11.81	0.15	437.3424	437.3420	HMDB0035983
14.	14.13	0.41	293.2497	293.2481	HMDB0000412	14.	12.90	10.86	629.4596	629.4629	HMDB0032086
15.	14.77	1.53	263.2396	263.2375	HMDB0034495	15.	14.22	9.37	613.4669	613.4679	HMDB0041428
16.	20.39	0.00	225.1999	225.1967	HMDB0030290	16.	15.64	4.35	597.4722	597.4730	HMDB0033327
						17.	21.09	0.47	225.1976	225.1967	HMDB0030290
						18.	21.85	0.10	225.1984	225.1967	HMDB0030290

The chromatographic peaks detected as pest control-related compounds in the chromatogram (see **Figure 3b**) showed relatively low intensities, indicating that the concentrations of these analytes in the sample were also relatively low (see **Table 2**).

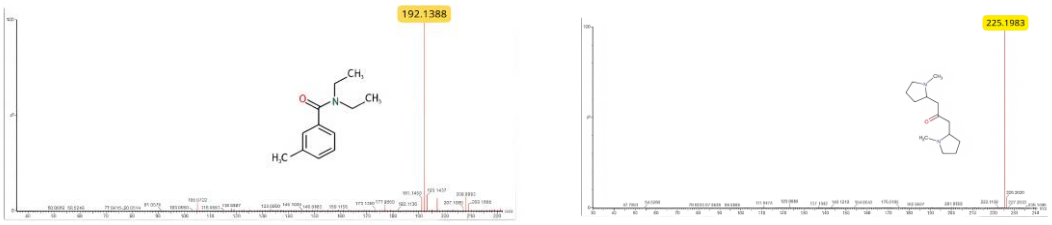
In the ethanol fraction, the chromatographic peak associated with a compound exhibiting potential pest control activity was observed at the retention time corresponding to peak number 1 and was identified as 3,5,6-trihydroxy-5-(hydroxymethyl)-2-methoxycyclohexan-1-one, which showed high signal intensity. Other detected compounds included cuscohygrine, corresponding to peak numbers 4, 5, 6, 7, and 8.

In the aqueous fraction, chromatographic analysis similarly revealed a prominent peak at retention time number 1, identified as 3,5,6-trihydroxy-5-(hydroxymethyl)-2-methoxycyclohexan-1-one, with high intensity. Additional compounds detected included (*S*)-reticuline (see **Figure 5c**) at peak number 10 and cuscohygrine at peak numbers 12, 13, 14, 15, and 17, all of which exhibited relatively low intensities in the chromatogram. Overall, the chromatographic profile of the aqueous fraction was comparable to that of the ethanol fraction.

Table 3. Identification Data for the Ethanol and Aqueous Fractions

Ethanol fraction						Aqueous fraction					
No	RT	% Area	Measured mass (*m*)	Calculated mass (*z*)	Source	No	RT	% Area	Measured mass (*m*)	Calculated mass (*z*)	Source
1.	1.26	20.36	205.0701	205.0712	HMDB0041031	1.	1.26	15.53	205.0710	205.0712	HMDB0041031
2.	2.57	1.55	174.1512	174.1494	HMDB0243489	2.	2.62	2.38	199.1096	199.1083	HMDB0014606
3.	3.24	1.66	158.0833	158.0817	HMDB0094701	3.	2.62	2.38	199.1096	199.1923	HMDB0302735
4.	5.27	1.55	225.1985	225.1967	HMDB0030290	4.	2.99	1.28	144.0680	144.0661	HMDB0014491
5.	5.63	0.02	225.1983	225.1967	HMDB0030290	5.	3.30	1.21	158.0839	158.0817	HMDB0341241
6.	6.50	0.12	225.1982	225.1967	HMDB0030290	6.	4.00	0.09	245.0617	245.0661	HMDB0039177
7.	9.82	18.68	225.1983	225.1967	HMDB0030290	7.	4.00	0.09	245.0617	245.0596	HMDB0248479
8.	20.72	2.05	225.1986	225.1967	HMDB0030290	8.	4.66	0.02	301.1420	301.1375	HMDB0013938
						9.	4.66	0.02	301.1420	301.1440	HMDB0038325
						10.	4.88	0.08	330.1720	330.1705	HMDB0003601
						11.	4.88	0.08	330.1720	330.1719	HMDB 0251466
						12.	6.55	0.02	225.1984	225.1967	HMDB0030290
						13.	7.03	0.07	225.1990	225.1967	HMDB0030290
						14.	9.82	20.09	225.1984	225.1967	HMDB0030290
						15.	14.92	12.12	225.1987	225.1967	HMDB0030290
						16.	16.85	33.49	419.3535	419.3525	HMDB0011644
						17.	20.88	1.83	225.1984	225.1967	HMDB0030290

Notes: The green line indicates compounds with potential pest control activity. RT: retention time



(a) (b)

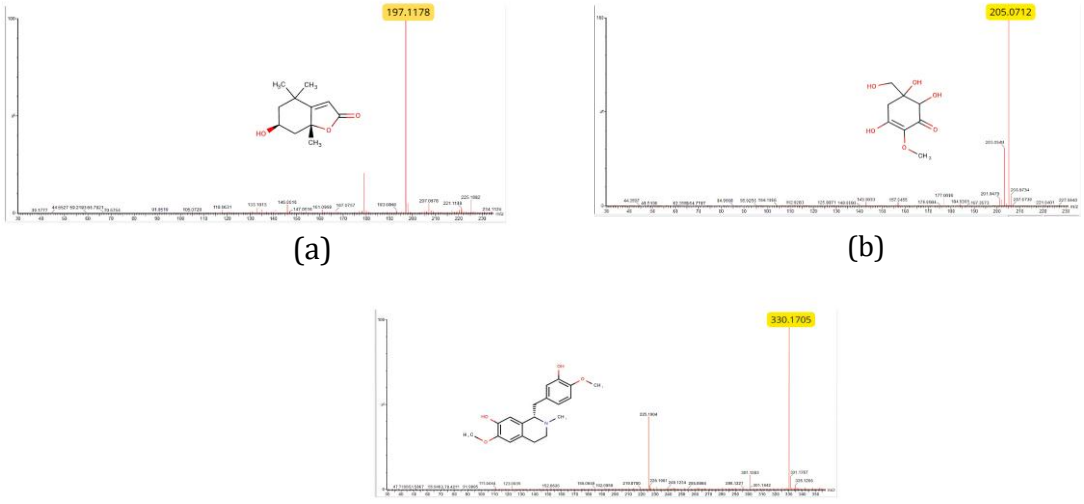
Figure 4. LC–MS/MS spectra of (a) DEET and (b) cuscohygrine.

The chromatograms obtained from the ethanol and aqueous fractions displayed broader peak shapes (see **Figure 3c** and **Figure 3d**) than those of the *n*-hexane and ethyl acetate fractions. Peak broadening may be attributed to several factors, including the presence of impurities resulting from suboptimal extraction

processes. The greater the amount of co-extracted impurities present in the sample, the more pronounced their effect on the resulting chromatographic profile [22].

The fractionation process using ethanol and water was less effective, likely because bioethanol waste is predominantly polar in nature. Consequently, extraction with polar solvents such as ethanol and water may reduce process selectivity, leading to less efficient separation.

Plants are rich sources of natural compounds that help defend them against threats such as fungi, bacteria, viruses, and insects. These bioactive compounds, such as alkaloids, flavonoids, and terpenoids, not only protect plants but also have useful applications in agriculture. They can act as natural pesticides, reduce pest resistance, and, in some cases, support plant growth and immunity [23].



(a) (b)

(c)

Figure 5. LC–MS/MS spectra of (a) loliolide, (b) 3,5,6-trihydroxy-5-(hydroxymethyl)-2-methoxycyclohexan-1-one, and (c) (*S*)-reticuline.

Using MassLynx™ version 4.1 software, four major compounds that may function as pest control agents were identified in the fractions. These compounds likely interfere with pest systems through several mechanisms. For example, alkaloids can block nerve signals by disrupting neurotransmitters, causing paralysis or death in pests [24]. Terpenoids damage cell membranes and disrupt metabolism, whereas phenolic compounds degrade microbial cell walls or inhibit their communication signals, thereby reducing their ability to infect plants [25],[26].

This study revealed that several compounds detected in the *n*-hexane, ethyl acetate, ethanol, and aqueous fractions likely act as bioactive agents (see **Table 4**). These compounds interact with living tissues and trigger beneficial biological responses in humans or other organisms [27].

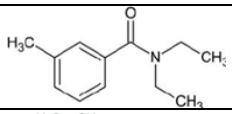
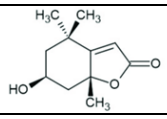
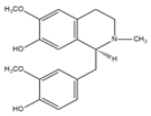
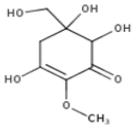
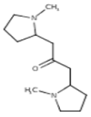
Mechanism of DEET

DEET (*N,N*-diethyl-*meta*-toluamide) acts through multiple sensory pathways, serving as both a spatial repellent and a contact irritant by interfering with insect olfactory and gustatory receptors [33] (see **Figure 6**).

These odorants serve as sensory cues and are detected by olfactory sensory neurons (OSNs) located in the antennae (see **Figure 6**). Although the exact molecular targets of DEET remain under debate, behavioral and electrophysiological studies confirm that it acts as a sensory stimulus affecting both olfactory and gustatory receptors [34] (see **Figure 7**).

In behavioral assays, DEET-contaminated sucrose deterred egg laying by female flies, indicating its role in modifying feeding and reproductive behaviors [33],[35]. This mechanism was investigated through studies using synthetic DEET

Table 4. Bioactive Compounds for Biocontrol

Compound	Chemical Structure	Function	Source Fraction(s)	Compound No.*
DEET		Insect/mosquito repellent [28]	*n*-Hexane	7
Loliolide		Systemic signaling molecule for plant defense [29]	Ethyl acetate	6
(*S*)-Reticuline		Antibacterial and antifungal activities [30]	Aqueous	10
3,5,6-Trihydroxy-5-(hydroxymethyl)-2-methoxy-2-cyclohexene-1-one		Antifungal activity [31]	Ethanol and aqueous	1 and 1
Cuscohygrine		Antifungal and antibacterial activities [32]	*n*-Hexane, ethyl acetate, ethanol, and aqueous	9 and 16; 10, 17, and 18; 4, 5, 6, 7, and 8; and 12, 13, 14, 15, and 17

Notes:
* These data refer to the identified components highlighted in green in Table 2 and Table 3.

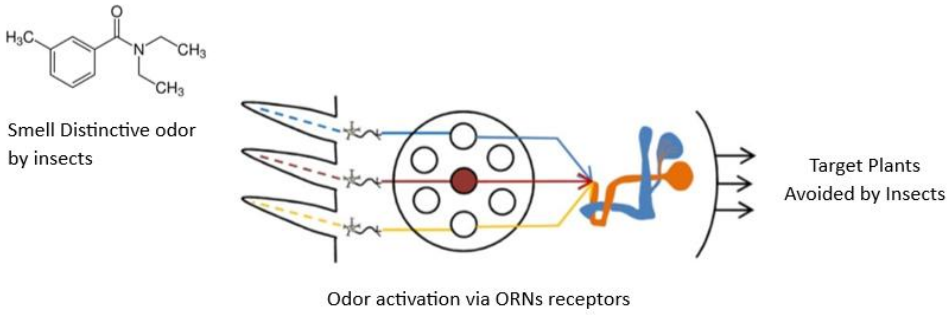


Figure 6. Detection of DEET compounds through olfactory receptors [35].

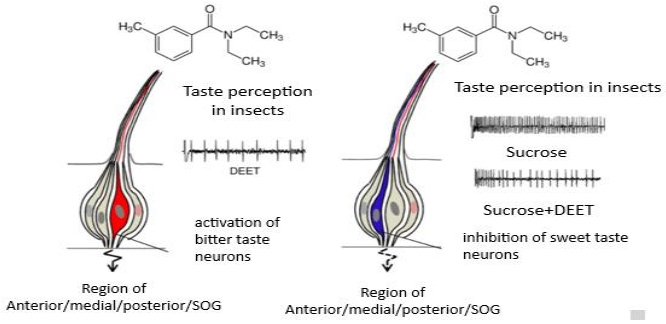


Figure 7. Detection of DEET and sucrose compounds through gustatory receptors [35].

The presence of DEET compounds may be due to environmental contamination, which can enter the environment directly or be transported through rainwater and soil runoff. Substances released into the environment are distributed among different environmental compartments. Ongoing agricultural and domestic activities in the area may also contribute to this contamination. Wastewater plays a significant role in water complexity and may affect the presence of DEET compounds [36]. DEET compounds can decompose in water by approximately 80%, while some may accumulate in wastewater sludge, soil, and sediment. Biodegradation studies have shown that DEET is biodegradable [37]. However, the consistent presence of DEET compounds in the samples requires further analysis before further implementation.

Mechanism of Loliolide

Loliolide, a naturally occurring benzofuran derivative, was originally isolated from plants and marine organisms. This compound has demonstrated antibacterial and antifungal activities [38]. Loliolide is believed to function as a systemic signal in plants by activating chemical defenses and mediating responses to herbivores, pathogens, and environmental stress [39].

Loliolide plays a role in underground chemical communication, as shown in wheat plants that secrete loliolide and jasmonic acid through their roots (see **Figure 8**) to trigger the production of defense-related compounds such as DIMBOA [40].

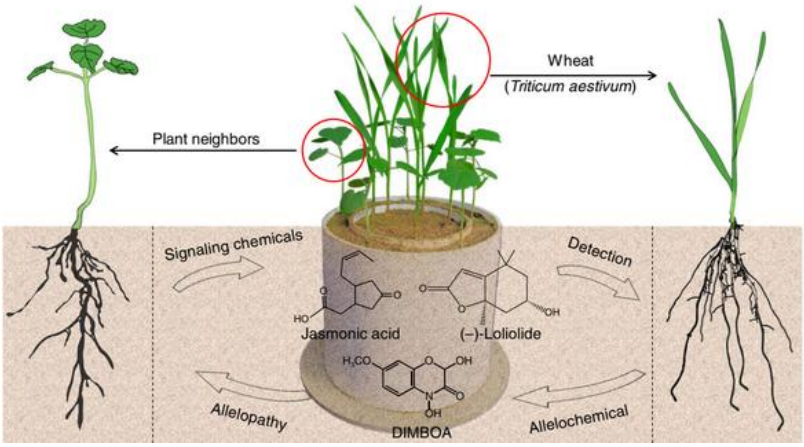


Figure 8. Underground chemical interaction of loliolide in wheat plants [40].

Loliolide application has been shown to reduce mite and caterpillar survival and egg laying on tomato leaves (see **Figure 9**) without causing toxicity to the plants. Gene expression analysis indicated that loliolide activates genes related to cell wall defense [41]. As a benzofuran compound, its antifeedant and antimicrobial activities are enhanced by specific substituents, such as methoxy and hydroxyl groups [42].

The described mechanism involved the use of 100 plant species selected based on their occurrence and distribution in the local wheat industry or their ecological relevance as weeds and crops in agricultural ecosystems. In addition, Tobacco mosaic virus-infected tobacco (*Nicotiana tabacum*) leaves were also used in the study [40].

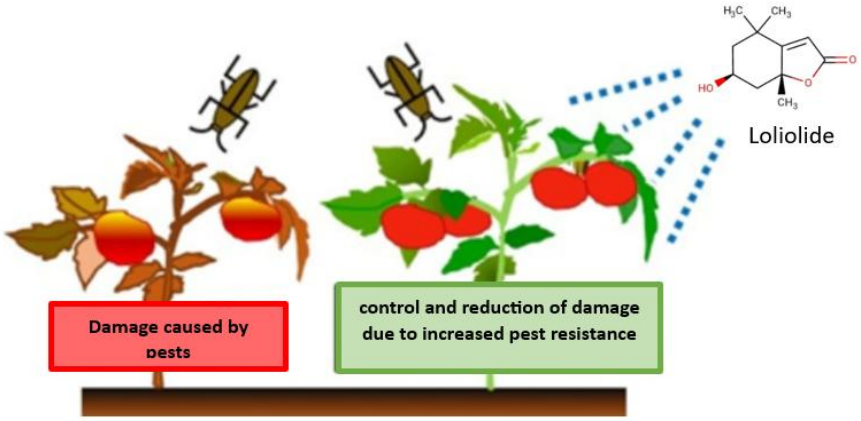


Figure 9. The role of loliolide in biocontrol [41].

Mechanism of (S)-Reticuline

(*S*)-Reticuline is a benzylisoquinoline alkaloid with demonstrated antibacterial and antifungal properties [43]. It serves as a key intermediate in the biosynthesis of various isoquinoline alkaloids, which are nitrogen-containing metabolites with diverse biological functions [44] (see **Figure 10**).

Plants store these alkaloids in tissues such as leaves, roots, and seeds as a defense mechanism against predators and pathogens, as reported, for example, in alkaloids isolated from the stem bark of *Annona cherimola* Mill. [43]. These compounds target bacterial DNA and enzymes, such as topoisomerases, and disrupt cytoplasmic membranes [45,46]. Structurally similar to protoberberines, (*S*)-reticuline contains nitrogen and methoxy groups that enhance its antimicrobial properties (see **Figure 11**). These structural features allow it to interfere with DNA replication, transcription, and protein synthesis [47,48].

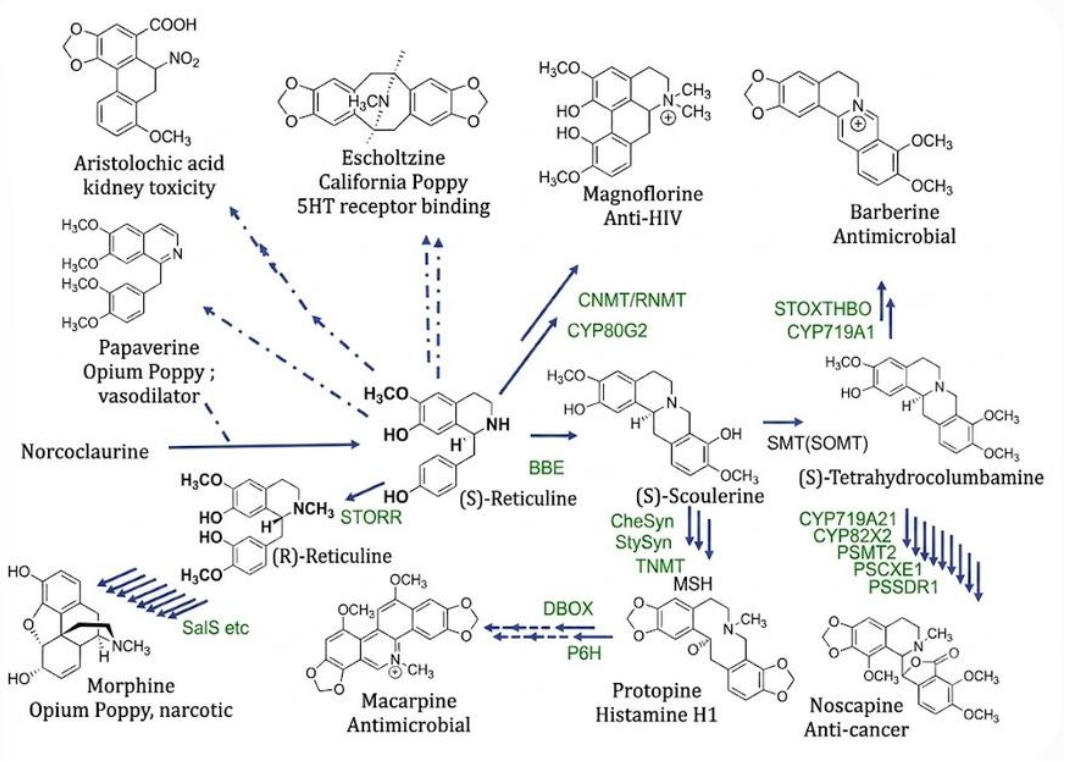


Figure 10. Biosynthetic pathway of (*S*)-reticuline [44].

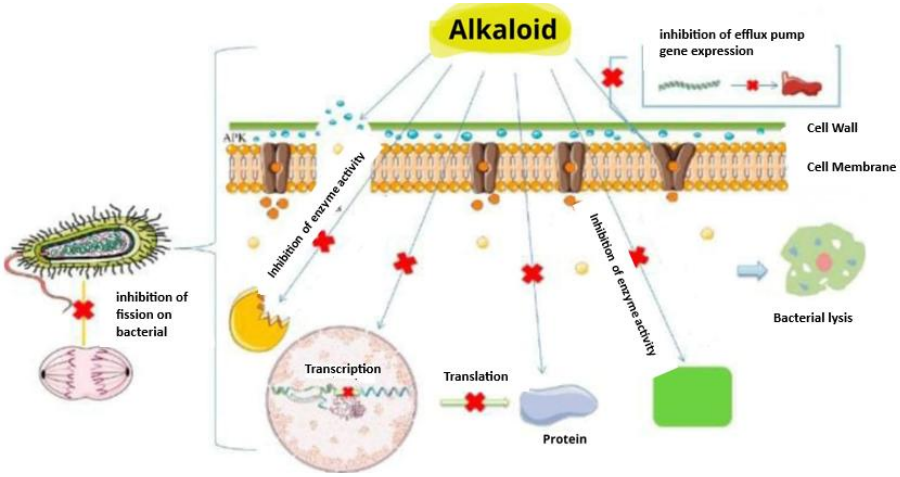


Figure 11. Antibacterial mechanism of natural alkaloids [52].

Mechanism of 3,5,6-Trihydroxy-5-(hydroxymethyl)-2-methoxy-2-cyclohexan-1-one

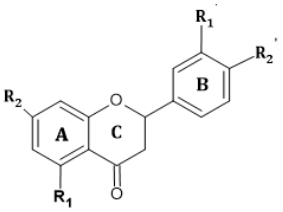
This compound belongs to the cyclohexanone group, which features a six-membered aliphatic ring with a ketone group and an endocyclic double bond. It has demonstrated antifungal activity, although it remains poorly studied in the literature [49].

Structurally related compounds have demonstrated inhibitory effects against fungi such as *Candida albicans* and *Fusarium* species. These effects are typically mediated by the disruption of fungal cell membranes and cell walls [50]. The presence and position of hydroxyl groups play a significant role in antibacterial activity by destabilizing bacterial membranes and inducing leakage of intracellular contents [51] (see **Figure 12**).

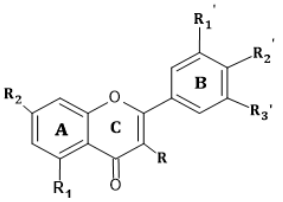
Mechanism of Cuscohygrine

As a pyrrolidine derivative, cuscohygrine exhibits notable antibacterial and antifungal activities [32]. Pyrrolidine rings, which are nitrogen-containing heterocycles, interfere with bacterial functions such as DNA replication and protein synthesis. Cuscohygrine's dual pyrrolidine rings contribute to its ability to inhibit enzymes such as DNA gyrase and topoisomerase IV, which are key targets in antibacterial strategies [53,54]. Other pyrrolidine-based compounds exhibit similar biological activities, and their stereochemistry may affect specific biological functions [55].

In summary, these identified compounds each possess distinct chemical structures and biological mechanisms that contribute to their potential as biopesticides.



Flavanones	R_1	R_2	R_1'	R_2'
Narigenin	OH	OH	H	OH
Pinocembrin	OH	OH	H	H
7-*O*-Methyleriodictyol	OH	OCH_3	OH	OH



Flavones	R	R_1	R_2	R_1'	R_2'	R_3'
Quercetin	OH	OH	OH	H	OH	OH
Galangin	OH	OH	OH	H	H	H
3-*O*-Methylisorhamnetin	OCH_3	OH	OH	OCH_3	OH	H
3-*O*-Methylgalangin	OCH_3	OH	OH	H	H	H
3,7-*O*-Dimethylgalangin	OCH_3	OH	OCH_3	H	H	H

Figure 12. Structure–activity relationship of galangin [51].

Conclusion

Chemical profiling was conducted using LC–MS/MS. Analysis of the samples extracted with *n*-hexane, ethyl acetate, ethanol, and water revealed the presence of several putative bioactive compounds with pesticidal activity. The notable identified compounds included DEET (*m/z* = 192.1388), loliolide (*m/z* = 197.1178), (*S*)-reticuline (*m/z* = 330.1705), 3,5,6-trihydroxy-5-(hydroxymethyl)-2-methoxy-2-cyclohexene-1-one (*m/z* = 206.0712), and cuscohygrine (*m/z* = 225.1983). These compounds are known to exert a range of biological activities, including antibacterial, antifungal, and insect-repellent effects, each operating through distinct mechanisms. However, further functional validation through field implementation studies is necessary to evaluate the effectiveness and long-term sustainability of their application, particularly considering the presence of DEET in the samples.

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