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Determination of antibacterial properties and chemical composition of pyrolysis liquid from feeding wastes of mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) adults and larvae

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Abstract: This study aimed to evaluate the antibacterial properties and phytochemical composition of pyrolysis liquids obtained from the biowaste of the mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) adults and larvae. Biowaste was subjected to pyrolysis at 295±5 °C for 7 h under a nitrogen atmosphere using a laboratory-scale system equipped with a dual condensation unit to separate light and heavy fractions. The biowaste pyrolysis liquid (BPL) were purified and analysed for their chemical content using LC–MS/MS, which identified seven key phenolic compounds, with cinnamic acid being the most abundant (358.05 μg/g), followed by salicylic acid, 4-hydroxybenzoic acid and naringenin. Notably, the most of flavonoid derivatives were either absent or below detection limits, indicating the extract's phenolic acid-dominated profile. The antibacterial activity of BPL was assessed against two gram-negative (*E. coli* ATCC-25922, *P. aeruginosa* ATCC-27853) and two gram-positive (*E. faecalis* ATCC-23212, *S. aureus* ATCC-25923) bacterial strains using the agar well diffusion method. The results demonstrated significant antibacterial effects, particularly against *S. aureus* (29.5±0.3 mm) and *P. aeruginosa* (23.5±0.2 mm), followed by *E. coli* (21.7±0.1 mm). These findings highlight the potential of insect-derived pyrolysis liquids as natural antibacterial agents, driven largely by their phenolic acid content. This study underscores the value of biowaste valorisation for developing bioactive compounds with potential applications in antimicrobial therapies.

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INTRODUCTION

The growing demand for sustainable and eco-friendly solutions to combat microbial infections has intensified the search for novel antibacterial agents from unconventional sources.[1] In this context, insect biowaste, particularly from species such as mealworm *Tenebrio molitor*, has emerged as a promising substrate due to its rich organic content.[2] Through pyrolysis, biowaste can be thermochemically converted into valuable bio-oils containing a diverse range of phytochemicals.[3] These bio-oils offer potential applications not only as fuels but also as bioactive materials with antioxidant and antimicrobial properties. Understanding the chemical composition and biological activities of these pyrolysis-derived liquids is crucial for unlocking their potential in pharmaceutical and environmental applications.[4]

The growing global concern over antibiotic resistance has prompted extensive research into alternative sources of antimicrobial agents. Natural and waste-derived compounds have emerged as promising candidates due to their bioactive properties and potential to combat resistant bacterial strains. One such source is the insect-derived biowaste, which has received increasing attention for its rich chemical profile and potential biological activities.[5]

Pyrolysis, a thermochemical decomposition process performed under an inert atmosphere, is widely used to convert organic waste materials into valuable products, including pyrolysis liquids rich in bioactive compounds.[6] Pyrolysis liquids derived from plant biomass, algae, and animal waste have been shown to possess antimicrobial, antioxidant, and anti-inflammatory properties, making them suitable for pharmaceutical and agricultural applications.[7] However, the use of insect-derived biowaste as a substrate for pyrolysis remains relatively unexplored, presenting a unique opportunity to valorize such materials.

Phenolic compounds, particularly phenolic acids and flavonoids, are key contributors to the biological activities of plant and waste-derived pyrolysis liquids.[8] These compounds are well known for their antioxidant capacities, free radical scavenging activities, and ability to modulate inflammatory responses. Their antibacterial mechanisms include disrupting bacterial membranes, chelating metal ions and inhibiting nucleic acid synthesis.[9] Identifying these compounds using advanced analytical techniques, such as liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS), allows for a detailed characterization of their potential therapeutic applications.

Several studies have demonstrated the antibacterial activity of pyrolysis products from different biomass sources. For example, Kim *et al.*[10] reported that the pyrolysis liquids derived from pine and oak wood exhibited significant antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Similarly,

Demirbas[11] highlighted that bio-oils produced from agricultural residues showed antimicrobial effects against various Gram-positive and Gram-negative bacteria.

Insects such as *T. molitor* (mealworms) and *Hermetia illucens* (black soldier fly) have been increasingly studied for waste management and biomass production, but few studies have investigated the chemical analysis and antibacterial potential of their waste by-products.[12] Exploring the biowaste of *T. molitor* through pyrolysis offers a novel approach to both waste valorisation and bioactive compound discovery. The anticipated presence of phenolic acids such as cinnamic acid, salicylic acid, and ferulic acid could contribute significantly to antibacterial and antioxidant activities.[13]

The antimicrobial resistance among common pathogens such as *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis* poses a severe threat to public health worldwide.[14] The search for novel antibacterial agents, particularly from natural and waste-derived sources, is therefore a critical research priority. Investigating the antibacterial activity of pyrolysis liquids from insect waste not only addresses this need but also aligns with sustainable waste management practices and circular bioeconomy principles.

The present study aims to determine the antibacterial properties of extracts obtained from the pyrolysis of feeding waste of *T. molitor* adults and larvae and to analyse their chemical composition using LC–MS/MS. By evaluating their effects against both gram-positive and gram-negative bacteria, this research contributes to the expanding field of bioactive compound discovery from unconventional sources, the potential of insect-derived biowaste as an alternative antimicrobial reservoir.

By combining chemical profiling with biological evaluation, this study provides a comprehensive assessment of BPL derived from *T. molitor* biowaste, highlighting its potential as a natural antibacterial agent. The integration of waste management, chemical valorisation and antimicrobial exploration presents an exciting frontier in the development of sustainable solutions for public health challenges.

EXPERIMENTAL

Mealworm T. molitor production and waste handling

T. molitor adults and larvae were reared in controlled laboratory conditions at 70 % relative humidity and 28±2 °C under appropriate conditions. The insects were monitored weekly by keeping their food in plastic containers and fresh medium was added as needed in order to maintain optimum rearing conditions. The mixtures of the food given to the insects were adjusted as 30 wt. % wheat bran, 60 wt. % wheat flour and 10 wt. % corn flour (Fig. 1). The Fig. 1 shows the initial state of the mixture given as food to *T. molitor* individuals (left) and the waste state of the mixture (right). The darker coloured waste was used in the studies. The waste material consisting of feces, exuvia and leftover feed was collected after a period of four weeks by carefully sieving the contents of the containers to separate the insects from the accumulated waste. The collected feed waste was then air dried at room temperature for 48 h to remove

moisture and then ground into fine powder using a laboratory mill. This powdered biowastes were stored in airtight containers until further use in pyrolysis experiments.

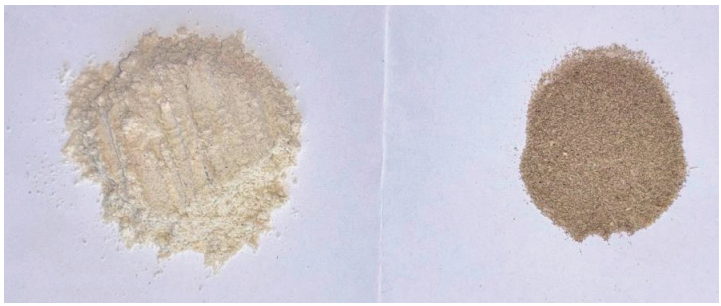

Fig. 1. Mixture used in the study (dark coloured waste was used).

Obtaining pyrolysis liquid from biowastes

In this study, the biowaste pyrolysis liquid (BPL) derived from mealworms was used as a primary feedstock for the pyrolysis experiments (Fig. 2). The biowaste materials were first collected and subjected to a drying process at 60 °C for 24 h to remove excess moisture. Once dried, the biowaste was ground using a mechanical grinder to achieve a uniform particle size, enhancing thermal homogeneity and improving reaction efficiency during pyrolysis. Prior to the thermal degradation process, all materials were stored in airtight containers to prevent contamination and moisture absorption.

Fig. 2. PID controlled system producing BPL from biomass produced by mealworms.

The pyrolysis experiments were carried out using a custom-built laboratory-scale pyrolysis system equipped with the proportional-integral-derivative (PID) temperature controller for precise thermal management. The reactor chamber, fabricated from stainless steel, was designed to operate under oxygen-free conditions, ensuring the process occurred under a strictly inert nitrogen atmosphere. Nitrogen gas with a purity of 99 % was continuously supplied at a controlled flow rate of 60 mL min^{-1} to maintain the inert environment and minimize oxidation of the volatile compounds. The entire pyrolysis run was conducted at a constant temperature of 295±5 °C, maintained for 7 h to ensure complete thermal conversion of the biomass material.

The reactor was equipped with a dual-stage condensation system consisting of two water--cooled condensers arranged in series. This configuration enabled the selective separation of heavy and light condensable vapors. During pyrolysis, the vapours generated from thermal decomposition were directed through these condensers, where cooling led to the formation of two distinct liquid fractions based on their boiling points. The heavy fractions were collected in the first condensation unit, while the lighter fractions were captured in the second condenser and stored in separate glass collection flasks.

After the pyrolysis process was completed, the collected liquid fractions were carefully transferred to a separation funnel to undergo phase separation and purification. The funnel was allowed to stand undisturbed for several hours to ensure complete settling of the immiscible layers. Following the separation, the upper and lower liquid phases – representing light and heavy pyrolysis oils respectively – were individually isolated and filtered to remove any particulate residues. These purified fractions were then stored in amber glass bottles at 4 °C prior to subsequent chemical characterization and analysis. Consistent preparation methods, standardized operating conditions and controlled environments were maintained throughout the study.

Identification of phenolic compounds by LC–MS/MS

A Thermo Scientific brand LC–MS/MS device was used to measure the amounts of phenolic components in the biowaste pyrolysis liquid (BPL) studied in this research. After the samples were dissolved in methanol to be 10 mg mL^{-1}, they were filtered through a 0.45 µm syringe filter and transferred to the vial. The chromatographic separation was carried out on an ODS HYPERSIL model (250 mm×4.6 mm, 5 µm) analytical column. The analysis test conditions used in the study are given in Table I.

TABLE I. LC–MS/MS analysis conditions of biowaste pyrolysis liquid (BPL)

	Time, min	A (0.1 % formic acid in water)	B (%, methyl alcohol)
Solvent program	0	100	0
	1	100	0
	22	5	95
	25	5	95
	30	0	100
Solvent flow rate	0.7 mL min^{-1}		
Column oven temperature	30 °C		
Column properties	ODS HYPERSIL 250 mm×4.6 mm, 5 µm column		
Injection volume	20 µL		
Analysis time	34 min		

Mass spectrometric determination was performed using a Shimadzu LCMS-8040 model tandem mass spectrometer equipped with an electrospray ionization (ESI) source operating in both negative and positive ionization modes. LC-ESI–MS/MS data were processed by LabSolutions software (Shimadzu). Multiple reaction monitoring (MRM) mode was used for quantification of phytochemicals. The MRM method was optimized to selectively detect and quantify phytochemical compounds based on scanning of ion transitions from the specified precursor phytochemical to the fragment. Collision energies (*CE*) were optimized to produce the optimal phytochemical fragmentation and maximum transmission of desired product ions.[15] MS analysis, drying gas flow, and MS/MS operating conditions are expressed in Table II.

TABLE II. LC–MS/MS instrument operating conditions

Capillary temperature	300 °C
Vaporizer temperature	350 °C
Sheath gas pressure	30 Arb
Aux gas pressure	13 Arb
Spray voltage (positive polarity)	4000 V
Spray voltage (negative polarity)	2500 V
Discharge current	4.0 µA

Determination of antibacterial activity

The antibacterial activity of the samples was investigated against two gram-negative (*E. coli* ATCC-25922 and *P. aeruginosa* ATCC-27853) and two gram-positive (*E. faecalis* ATCC--23212 and *S. aureus* ATCC-25923) bacterial strains. The antimicrobial activities of the samples were assessed using the agar well diffusion method with some modifications, as described by Karpuz *et al.*[16] For this purpose, Petri dishes prepared with nutrient agar (NA) medium were inoculated with overnight cultures of the bacterial strains using the spread plate technique. To allow diffusion into the agar layer, the inoculated Petri dishes were kept in a laminar air flow cabinet (Thermo Fisher scientific laminar air flow, USA) for approximately 1 h. After this period, wells with a diameter of 6 mm were made in the agar plates using a cork borer. Each well was filled with 50 µL of sample using a micropipette. Ampicillin (working concentration 50 µg mL^{-1}) was used as a positive control. The prepared Petri dishes were then incubated at 37 °C for 24 h in an incubator (Memmert, Germany). At the end of the incubation period, the diameter of the inhibition zones formed around the wells was measured to determine the antibacterial activity.

Statistical analysis

All experimental measurements were performed in triplicate and the results were reported as mean ± standard deviation (*STD*). The data obtained were analysed by one-way analysis of variance (One-way ANOVA) using IBM SPSS Statistics 21 software. Statistically significant differences were accepted at the $p < 0.05$ level.

RESULTS AND DISCUSSION

LC–MS/MS analysis results of phenolic compounds

Phenolic compounds found in plants play a significant role as potent antioxidants capable of neutralizing free radicals in biological systems. These compounds provide various health benefits by reducing cellular damage. Among phenolic compounds, flavonoids are particularly noteworthy due to their broad distribution and diverse biological activities. As key members of the polyphenol family, flavonoids comprise more than 4,000 compound types present in different plant organs, including roots, flowers, leaves and fruits. The biological properties of these compounds form an essential basis for understanding the health effects of plant-based foods.

To accurately identify phenolic and flavonoid compounds, advanced analytical techniques are employed, and in this context, mass spectrometry (MS) is frequently preferred, particularly for analyses requiring high sensitivity and accur-

acy.[8] Furthermore, the LC–MS/MS technique offers higher selectivity and sensitivity compared to other liquid chromatography methods, making it widely used for quantitative analyses. This method provides effective results in the detailed analysis of flavonoids and other phenolic compounds.[17]

In line with this information, LC–MS/MS analysis was employed in the present study to identify the phytochemicals present in the BPL. Among the 32 standard compounds used by LC–MS/MS, seven phytochemical compounds were detected. The distribution of the quantitative findings of the phenolic compounds in the bio-waste is presented in Table III. Additionally, the analytical performance of the LC–MS/MS method is shown in Table IV. The main peak chromatogram rep-

TABLE III. Distribution of quantitative findings of phenolic compounds of BPL; the mean of three replicate samples ($n = 3$) ± *STD* ($p < 0.05$); NF – not found

Compound	Content, μg phenolic compound/g pyrolysis liquid
Gallic acid	NF
Protocatechuic acid	NF
Protocatechuic aldehyde	NF
Sesamol	NF
Gentisic acid	24.75±1.21
Catechin	NF
Chlorogenic acid	NF
Epicatechin	NF
Caffeic acid	4.38±0.13
Vanillin	NF
Syringic acid	NF
p_Coumaric acid	NF
Taxifolin	NF
Ferulic acid	18.47±1.34
Salicylic acid	36.26±1.56
4-Hydroxybenzoic acid	37.35±1.98
Hesperidin	NF
Rosmarinic acid	NF
Oleuropein	NF
Luteolin-7-glucoside	NF
Rutin	NF
Resveratrol	NF
Ellagic acid	NF
Cinnamic acid	358.05±11.32
Naringenin	37.11±2.12
Quarcetin	NF
Luteolin	NF
Apigenin	NF
Pinocembrin	NF
Chrysin	NF
Galangin	NF
Flavone	NF

resenting the composition of the bio-waste obtained by the LC–MS/MS method is provided in Fig. 3. The quantities of the flavonoid compounds and phenolic acid types, identified in the extracts are reported as μg analyte/g extract.

TABLE IV. Analytical performance of LC–MS/MS method; linear range: 0.125–10 ppm

Phenolic compound	Equation	R^2
Gallic acid	$Y = -1772.12 + 66065.7X$	0.9949
Protocatechuic acid	$Y = 27640.8 + 35692.1X$	0.9926
Protocatechuic aldehyde	$Y = 506291 + 900489X$	0.9963
Sesamol	$Y = 190429 + 294285X$	0.9965
Gentisic acid	$Y = 81035.3X$	0.9979
Catechin	$Y = -46484.8 + 91012.1X$	0.9987
Chlorogenic acid	$Y = 21653.3X$	0.9937
Epicatechin	$Y = -137604 + 217920X$	0.9951
Caffeic acid	$Y = 166126 + 470479X$	0.9981
Vanillin	$Y = 18407.3 + 4851.4X$	0.9937
Syringic acid	$Y = 150673–55037.7X$	0.9149
p_Coumaric acid	$Y = 79931.9 + 73318.2X$	0.9927
Taxifolin	$Y = 8374.89 + 42570.1X$	0.9979
Ferulic acid	$Y = 96528.3 + 177434X$	0.9941
Salicylic acid	$Y = 485398 + 972318X$	0.9960
4-Hydroxybenzoic acid	$Y = 537544 + 881649X$	0.9951
Hesperidin	$Y = 1.43184e + 006X$	0.9977
Rosmarinic acid	$Y = -127182 + 253431X$	0.9980
Oleuropein	$Y = 375327 + 1.76005e + 006X$	0.9969
Luteolin-7-Glycoside	$Y = 135610X$	0.9985
Routine	$Y = 2.20056e + 006 + 1.00456e + 007X$	0.9977
Resveratrol	$Y = 97778.9 + 63601.2X$	0.9988
Ellagic Acid	$Y = 745549 + 1.07598e + 006X$	0.9977
Cinnamic Acid	$Y = 536496X$	0.9957
Naringenin	$Y = 2.35935e + 006X$	0.9986
Quercetin	$Y = 111352 + 2.43427e + 006X$	0.9941
Luteolin	$Y = 1.48908e + 007X$	0.9981
Apigenin	$Y = 831953X$	0.9987
Pinocembrin	$Y = 789059X$	0.9979
Krisin	$Y = 1.33937e + 007X$	0.9972
Galangin	$Y = 1.66971e + 006X$	0.9929
Flavone	$Y = 1.57469e + 007 + 3.51252e + 007X$	0.9954

As seen in Table III, cinnamic acid was the phenolic compound detected at the highest concentration (358.05 μg/g), making it the predominant phenolic compound in the extract. Salicylic acid (36.27 μg/g), 4-hydroxybenzoic acid (37.35 μg/g), and naringenin (37.11 μg/g) were also found in considerable amounts and are important in terms of biological activity (anti-inflammatory, antioxidant effects). The hydroxycinnamic acid derivatives such as caffeic acid and ferulic acid were also present, contributing to the antioxidant potential of the extract. Most

flavonoid derivatives (*e.g.*, quercetin, apigenin, rutin) were not detected, suggesting that the phenolic composition of the extract is predominantly centred around phenolic acids. The LC–MS/MS analysis revealed that phenolic acid derivatives were the major constituents in the analysed sample. Notably, cinnamic acid, salicylic acid, and 4-hydroxybenzoic acid were detected at high concentrations. In contrast, most flavonoid compounds were below the detection limit or absent in the sample. These findings suggest that the antioxidant capacity of the extract may largely be attributed to its phenolic acid content. Table IV shows the analytical performance of the LC–MS/MS method for the samples.

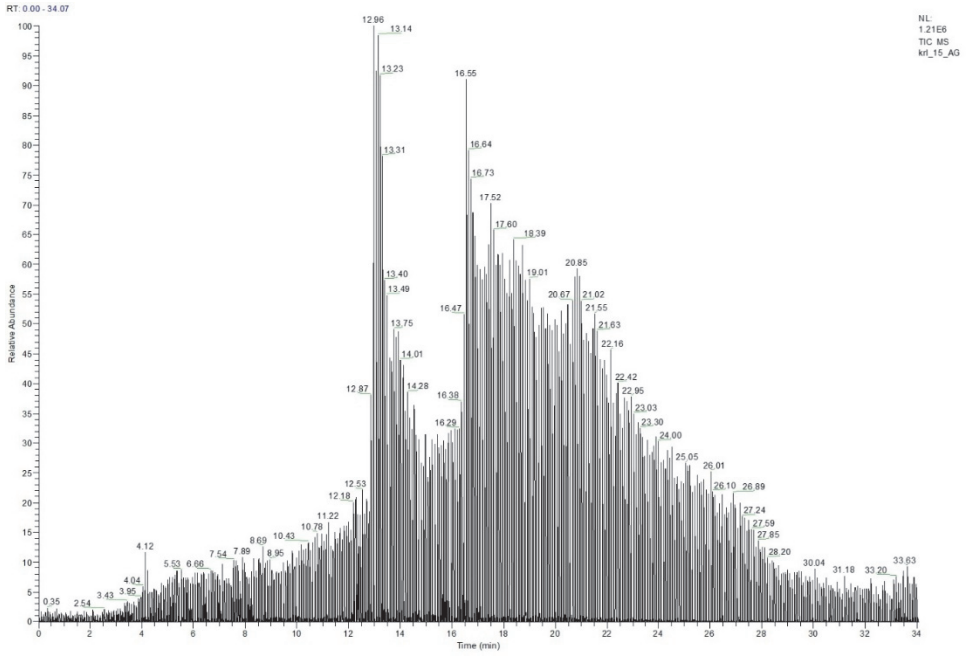

Fig. 3. LC–MS/MS total ion chromatogram (TIC) of biowaste pyrolysis liquid (BPL).

Antibacterial analysis results

The antibacterial properties of BPL were investigated using the agar well diffusion method. Antibacterial activity was evaluated against two gram-negative (*E. coli* and *P. aeruginosa*) and two gram-positive (*E. faecalis* and *S. aureus*) bacterial strains. These strains were selected due to their significant threat to human health and the urgent need for new antibiotics to combat them. Gram-positive and gram-negative bacteria represent major global public health concerns.[14] Ampicillin antibiotic was used as a positive control to determine antibacterial efficacy. Images of the Petri dishes obtained from the experiment are presented in Fig. 4.

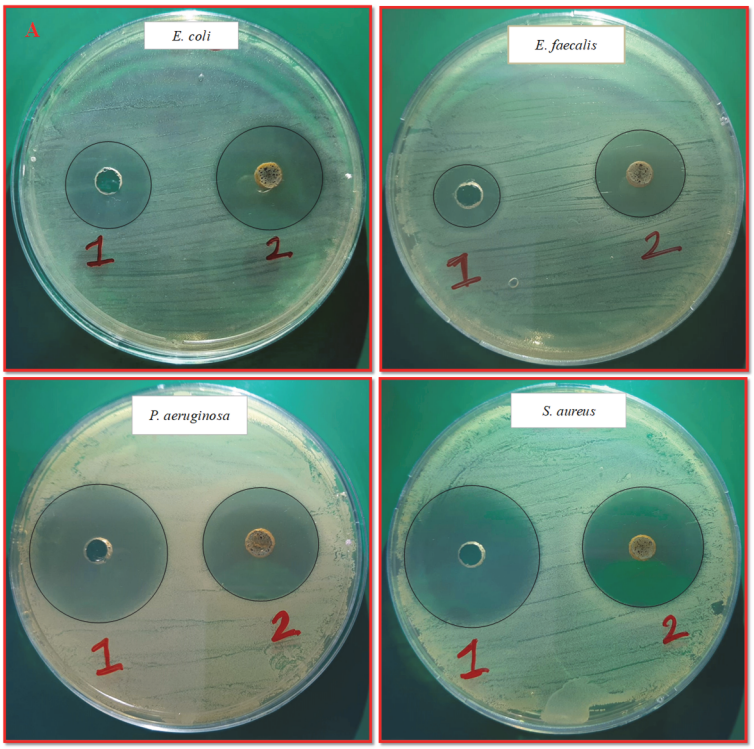

Fig. 4. Petri plates showing the antibacterial activity; 1: ampicillin (50 μg mL^{-1}) and 2: BPL sample (20 mg L^{-1}).

It is well known that the antibacterial activity of NPs is dependent on their concentration and size.[18] Therefore, preliminary experiments were first conducted in this study to determine the minimum BPL concentration. Based on this information, the inhibitory zones were assessed to evaluate the effects of the BPL sample on bacterial growth inhibition. The BPL sample exhibited good antibacterial activity against all bacterial strains tested in this study. The highest antibacterial activity of the BPL sample was observed against *S. aureus*, with an inhibition zone of 29.5±0.3 mm, followed by *P. aeruginosa* (23.5±0.2 mm) and *E. coli* (21.7±0.1 mm). The ampicillin antibiotic used in the study also demonstrated antibacterial activity against all bacterial strains (Fig. 5). Overall, the BPL sample showed good antibacterial efficacy when compared with the antibiotic ampicillin.

CONCLUSION

The present study successfully demonstrated that biowaste pyrolysis liquids (BPL) obtained from *T. molitor* adults and larvae exhibit promising antibacterial properties against both gram-positive and gram-negative bacterial strains. The agar well diffusion results showed clear inhibitory zones, with the strongest effects obs-

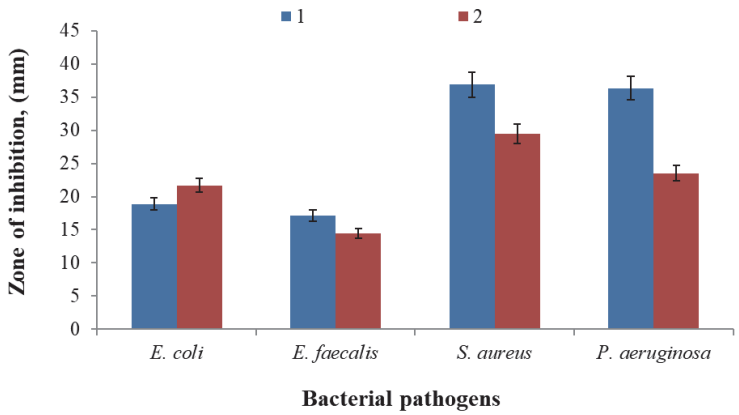

Fig. 5. Antibacterial activity of BPL sample against tested bacterial strains based on inhibition zone diameters; 1: ampicillin (50 μg mL^{-1}) and 2: BPL samples (20 mg L^{-1}). The values were the mean of three replicate samples ($n = 3$) ± *STD*. Statistical analysis was performed using one-way ANOVA ($p < 0.05$) in IBM SPSS Statistics 21.

erved against *S. aureus* and *P. aeruginosa*, suggesting the broad-spectrum antibacterial potential of BPL. These findings are particularly noteworthy given the growing threat of antibiotic-resistant bacteria, highlighting the importance of exploring the alternative antimicrobial agents derived from natural and sustainable sources.

The LC–MS/MS phytochemical analysis revealed that the major contributors to the observed biological activities were phenolic acids, especially cinnamic acid, which was present at remarkably high levels. This compound, along with salicylic acid and 4-hydroxybenzoic acid, is known for its antioxidant, anti-inflammatory, and antimicrobial activities, supporting the observed antibacterial effects of the BPL samples. Interestingly, most flavonoid derivatives were absent or below detection limits, suggesting that the antimicrobial activity is largely attributable to phenolic acids rather than flavonoids.

The thermal degradation process during pyrolysis appears to selectively enrich certain bioactive compounds while eliminating others, offering a unique method for the obtaining of antibacterial agents from insect biowastes.[19] This aligns with the current interest in circular bioeconomy approaches that aim to upcycle waste materials into high-value products with therapeutic potential.[20,21]

While the study provides some valuable insights, further research is needed to understand the mechanism of antibacterial action, optimize the pyrolysis conditions for maximum yield of active compounds and assess the safety and cytotoxicity of these extracts in clinical settings. Moreover, investigating the synergistic effects of BPL with conventional antibiotics, may open new avenues for combating multidrug-resistant pathogens.

In conclusion, the findings support the potential of insect-derived pyrolysis liquids as an innovative and sustainable source of antibacterial agents. With further

refinement and validation, BPL extracts could contribute to the development of novel antimicrobial formulations, addressing a critical need in global health.

ИЗВОД

ОДРЕЂИВАЊЕ АНТИБАКТЕРИЈСКЕ АКТИВНОСТИ И ХЕМИЈСКОГ САСТАВА ПИРОЛИТИЧКЕ ТЕЧНОСТИ НАСТАЛЕ ИЗ НУТРИТИВНОГ ОТПАДА ОДРАСЛОГ ЦРВА И ЛАРВЕ *Tenebrio molitor* L. (Coleoptera: Tenebrionidae)

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У овој студији су испитиване антибактеријске особине и фитохемијски састав пиролитичке течности добијене из биоотпада одраслог црва и ларве *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). Биоотпад је подвргнут пиролизи на 295±5 °C током 7 h у атмосфери азота, користећи лабораторијски систем са двоструким кондензатором у циљу раздвајања тешких и лаких фракција. Пиролитичка течност биоотпада (BPL) је пречишћена и анализиран је хемијски састав применом LC–MS/MS. Идентификовано је седам главних фенолних једињења, од којих је цинаминска киселина била најприсутнија (358,05 μg/g), а затим салицилна киселина, 4-хидроксибензојева киселина и нарингенин. Већина флавоноида је била одсутна или испод детекционог лимита, указујући да су фенолне киселине доминантне у екстракту. Антибактеријска активност BPL је тестирана спрам два соја Грам-негативних (*E. coli* ATCC-25922, *P. aeruginosa* ATCC-27853) и два соја Грам-позитивних бактерија (*E. faecalis* ATCC-23212, *S. aureus* ATCC-25923), користећи метод дифузије у агару. Резултати су показали значајан антибактеријски ефекат спрам *S. aureus* (29,5±0,3 mm) и *P. aeruginosa* (23,5±0,2 mm), као и *E. coli* (21,7±0,1 mm). Добијени подаци указују на антибактеријски потенцијал пиролитичких течности пореклом од инсеката, пре свега због присуства фенолних киселина. Ова студија истиче важност испитивања биоотпада у циљу развоја биоактивних једињења која се могу применити у антибактеријској терапији.

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