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Thème

CONTRIBUTION TO THE STUDY OF THE ANTIMICROBIAL ACTIVITY OF EXTRACT FROM BLACK SOLDIER FLY LARVAE (*HERMETIA ILLUCENS*) AGAINST HARMFUL BACTERIA

Soutenu publiquement, le 12 / 06 / 2023, devant le jury composé de :

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DEDICATIONS

We humbly extend our deepest appreciation and dedicate this research to the remarkable individuals and collectives whose unwavering support and unwavering commitment have profoundly shaped our academic and professional journey.

First and foremost, we express our profound gratitude to our beloved families, whose strong support, understanding, and wholehearted encouragement have been the foundation of our endeavors. Their steadfast belief in our capabilities and their selfless sacrifices have propelled us forward on our path of knowledge and achievement.

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Abdelaziz & *Anfel*

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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ABSTRACT

The rise of antibiotic resistance has necessitated the search for alternative antimicrobial agents. This study investigates the antimicrobial effects of black soldier fly larvae (*Hermetia illucens*) extract against harmful bacteria. Crushing (C) and drying (D) treatment methods were employed, followed by maceration and evaporation under reduced pressure. After testing eight solvents in a preliminary test, three solvents were selected based on polarity, yield, and antimicrobial activity. The antimicrobial activity of the extract was tested against *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The preliminary test revealed that all extracts showed significant inhibition against the tested Gram-positive bacteria with no observed inhibitory effect against the tested Gram-negative bacteria. Further examination revealed that the extract concentration is an important factor in the antimicrobial effects of these extracts. The treatment of Gram-negative bacteria exaggerates a higher extract concentration than the Gram-positive bacteria. The results showed that methanol extract is optimal in targeting both tested Gram-positive and Gram-negative at a minimum concentration of 20 mg/mL, hexane extract is optimal against the tested Gram-positive bacteria at a concentration of 10 mg/mL, and methylene chloride extract has comparatively lower activity at a minimum concentration of 20 mg/mL. These findings suggest that black soldier fly larvae extract could be used as a natural antimicrobial agent, with significant implications for the development of new alternatives for traditional antibiotics.

Keywords: *Hermetia illucens*, larvae extract, antimicrobial activity, harmful bacteria, AMPs.

RESUME

L'augmentation de la résistance aux antibiotiques a rendu nécessaire la recherche d'agents antimicrobiens alternatifs. Cette étude examine les effets antimicrobiens de l'extrait de larves de la mouche soldat noire (*Hermetia illucens*) contre les bactéries nuisibles. Des méthodes de broyage (C) et de séchage (D) ont été utilisées, suivies d'une macération et d'une évaporation sous pression réduite. Après avoir testé huit solvants dans un essai préliminaire, trois solvants d'extraction ont été sélectionnés en fonction de polarité, rendement et activité antimicrobienne. L'activité antimicrobienne a été testée contre *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* et *Escherichia coli*. Le test préliminaire a révélé que tous les extraits présentaient une inhibition significative contre les bactéries Gram-positives testée ; sans effet inhibiteur observé contre les souches Gram-négatives testée. Un examen plus approfondi a révélé que la concentration de l'extrait est un facteur important dans les effets antimicrobiens de ces extraits. Le traitement des bactéries Gram-négatives exige une concentration d'extrait plus élevée que celui des bactéries Gram-positives. Les résultats ont montré que l'extrait de méthanol est optimal pour cibler les bactéries Gram-positives et Gram-négatives testées à une concentration minimale de 20 mg/mL, l'extrait d'hexane est optimal contre les bactéries Gram-positives testées à une concentration de 10 mg/mL, et l'extrait de chlorure de méthylène a une activité comparativement plus faible à une concentration minimale de 20 mg/mL. Ces résultats indiquent que l'extrait de larve de mouche soldat noire pourrait être utilisé comme agent antimicrobien naturel, avec des implications significatives pour le développement de nouvelles alternatives aux antibiotiques traditionnels.

Mots clés : *Hermetia illucens*, extrait de larves, activité antimicrobienne, bactéries pathogènes, PAMs.

ملخص

إن ارتفاع نسب مقاومة المكروبات للمضادات الحيوية قد استدعى البحث عن عوامل بديلة مضادة للميكروبات. تهدف هذه الدراسة إلى استكشاف التأثيرات المضادة للميكروبات لمستخلصات يرقات ذبابة الجندي الأسود (*Hermetia illucens*). تم استخدام طرق المعالجة بالسحق (C) والتجفيف (D) في هذه الدراسة، تلتها عملية النقع والتبخير تحت الضغط المنخفض. بعد اختبار ثمانية مذيبات للاستخلاص كاختبار أولي، تم اختيار ثلاث مذيبات بناء على القطبية، الانتاج و كذا النشاط المضاد للميكروبات. تم دراسة النشاط المضاد للمكروبات ضد *Listeria monocytogenes*، *Staphylococcus aureus*، *Pseudomonas aeruginosa* و *Escherichia coli*. كشف الاختبار الأولي أن جميع المستخلصات أظهرت قدرة مهمة على تثبيط نمو البكتيريا الموجبة الغرام المدروسة مع عدم ملاحظة أي تأثير ضد البكتيريا السالبة الغرام. كما كشف لنا مجموع التجارب أن تركيز المستخلص عامل مهم يتحكم في التأثير على نمو الميكروبات. كما أن التأثير على تثبيط البكتيريا السالبة للغرام يتطلب تركيزًا أعلى من المستخلص مقارنةً بالبكتيريا الموجبة للغرام. أظهرت النتائج أن مستخلص الميثانول هو الأمثل في استهداف كل البكتيريا المدروسة موجبة وسالبة الجرام عند تركيز لا يقل عن 20 مجم / مل، و أن مستخلص الهكسان هو الأمثل ضد البكتيريا موجبة الجرام المختبرة بتركيز 10 مجم / مل، كما أن مستخلص كلوريد الميثيلين يحتوي على نشاط أقل نسبيًا عند تركيز لا يقل عن 20 مجم / مل. تشير النتائج إلى أنه يمكن استخدام مستخلص يرقات ذبابة الجندي الأسود كعامل طبيعي مضاد للميكروبات، ويمثل هذا مبادرة مهمة في سبيل تطوير بدائل جديدة للمضادات الحيوية التقليدية

الكلمات المفتاحية: *Hermetia illucens*، مستخلص اليرقات، النشاط المضاد للميكروبات، البكتيريا الضارة، AMPs.

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LIST OF ABBREVIATIONS

AAD : Antibiotic-associated diarrhea

AMPs : Antimicrobial Peptides

ATCC : American Type Culture Collection

BSF : Black Soldier Fly

BSFL: Black Soldier Fly Larvae

CDC : Centers for Disease Control and Prevention

CFU : Colony-Forming Unit

CRE : Carbapenem-resistant *Enterobacteriaceae*

DMSO : Dimethyl Sulfoxide

FAs : Fatty Acids

HCL : Hydrochloric Acid

MCFAs : Medium-chain Fatty Acids

MDR : Multi Drug Resistant

mg : Milligram

MH : Mueller Hinton

mL : Milliliter

mm : Millimeter

MRSA : Methicillin-resistant *Staphylococcus aureus*

NaCl : Sodium Chloride

SFAs : Saturated Fatty Acids

WHO : World Health Organization

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INTRODUCTION

Introduction

The emergence of antibiotic-resistant bacteria in the 21st century has posed a major global healthcare challenge. These bacteria are resistant to the most commonly used antibiotics (Alanis, 2005). Making them difficult to treat and increasing the risk of serious infections and death. Pathogenic bacteria, either facultative or obligate, are important agents that can cause severe intoxication or infectious diseases (Fuxa & Tanada, 1991). Some of these bacteria also have the potential to cause significant environmental damages, such as corrosion in infrastructure, which can lead to considerable economic losses (Geweely, 2011). In food industry, bacterial biofilms give rise to a huge problem by increasing the resistance of bacteria against harsh physicochemical conditions (Esbelin *et al*., 2018). Similarly, waterborne pathogens pose a major concern for water quality as they are associated with various diseases (Pandey *et al*., 2014).

Currently, various processes are applied to combat microorganisms. One of the most frequently operated approaches is the use of antibiotics. These compounds are either natural or synthetic substances that exhibit the capacity to kill or restrain the growth of bacteria (Grandclaudon *et al*., 2020). Since their discovery, antibiotics have been employed in the treatment of various bacterial infections that affect humans, animals (Arsene *et al*., 2021), and plants, (McManus *et al*., 2002). Chemical agents like disinfectants are widely used in various sectors, including food processing, agriculture, healthcare, domestic households, cosmetics and pharmaceutics (Kim *et al*., 2018; Mc Carlie *et al*., 2020).

These methods can be efficient in combating bacteria. However, they can have adverse effects on both humans and environment. Antibiotic administration can cause intestinal issues such as antibiotic-associated diarrhea (AAD) by provoking an imbalance in the commensal gut microbiota (McFarland, 1998). Moreover, antibiotics have secondary effects, including nausea, vomiting, fever, leukopenia, thrombocytopenia, drug-induced hepatitis, and nephrotoxicity (Cunha, 2001). Also, several studies indicated the toxicity of numerous disinfectants (Plewa *et al.*, 2017), where the extended and the high-level exposure to these chemicals can cause several health problems, including respiratory diseases and skin irritation (Yemiş & Yenil, 2020). The use of disinfectants can also contribute to the evolution of resistance (Madigan *et al.*, 1997). In the environment, antibiotics as an ecological factor have the potential to affect microbial communities (Ding & He, 2010). They can alter the phylogenetic structure and disturb the ecological function in the micro-ecosystem (Ding & He, 2010).

Nowadays, the most significant concern is antibiotic resistance, where pathogenic bacteria can survive exposure to a drug that normally kills them or inhibits their growth (CDC, 2022). The extensive use and misuse of antibiotics leads to the increase of antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA; Köck *et al*., 2010). They can also contribute to a ubiquitous presence of trace concentrations of antibiotic compounds in various environments (Roose-Amsaleg & Laverman, 2016), where they are considered as dangerous pollutants (Vignesh *et al*., 2011). The use of antibiotics in agriculture and aquaculture can also lead to the spread of antibiotic resistance and increase the expansion of multidrug-resistant bacteria (Proia *et al*., 2013). The rapid expansion of bacterial resistance threatens the remarkable health benefits that have been obtained with antibiotics (Rossolini *et al*., 2014). Many infections have become nearly impossible to treat with the current antimicrobial agents (Davies, 2006). The Centers for Disease Control and Prevention (CDC) reported that in 2019 alone, resistant pathogens were responsible for causing 2.8 million infections and 32,000 deaths (CDC, 2019). The expenses of both diagnosis and treatment of bacterial infections have become remarkably high as a result of antibiotic resistance (Alanis, 2005).

Lately, the emergence of multidrug-resistant (MDR) bacteria has shown a huge increase, both in their absolute numbers as well as their distribution and frequency amongst bacterial communities (Roca *et al*., 2015). This has been worsened by a concurrent reduction in the detection and development of new antibacterial agents over the past decade. (Perros, 2015). There is a 90% rejection rate for the new antibiotics' approval, with the discovery of only a few novel classes from the 1980s to the early 2000s (Luepke *et al*., 2017). The current situation in antibiotic research is paradoxical, with a decrease in the discovery of new antibiotics but an increasing need for their discovery (Stanton, 2013). Despite the growing need for new antibiotics, many companies have become less concerned about antibiotic development due to economic, scientific, and regulatory reasons (Luepke *et al*., 2017). Nowadays, only a few pharmaceutical companies work on antibiotic discovery programs. (Harbarth *et al*., 2015). As a result, during the last two decades, a major gap has emerged in the discovery of antibiotic alternatives (Harbarth *et al*., 2015). All of these pressing issues require urgent moves to discover new alternative options to antibiotics and chemical agents.

Some of the possible alternatives to overused antibiotics are antimicrobial compounds obtained from natural sources (Marusich *et al*., 2020). In addition to being a lower risk for not targeting the good bacteria, the effectiveness of alternative treatments can potentially be similar to antibiotics (CDC, 2022). Alternative treatments are still under research, some of the

promising alternatives are bacteriophages, plant extracts, and antimicrobial peptides (AMPs) (Wittebole *et al.*, 2014). Bacteriophages are a type of virus that specifically targets bacteria, making them a promising antibacterial agent (Wittebole *et al.*, 2014). Plants are largely used in ancient medicine (Savoia, 2012), the plant extracts have interesting antimicrobial activity (Vondruskova *et al.*, 2010). Furthermore, AMPs belong to a class of peptides that exhibit important antimicrobial activity and are known for their wide spectrum of targets (Cheng *et al.*, 2014).

It has been revealed that several insects have antimicrobial properties and substances that can prevent microbial infections. These substances can be generated on the surface or in their digestive system (Hazlett & Wu, 2011). Many insects like the honeybee, house fly, centipede, provide various compounds and peptides that can be strong pharmaceuticals (Pemberton, 1999) the larvae of *Lucilia sericata* has the ability to inhibit the growth of Gram-negative and Gram-positive bacteria, including resistant strains like methicillin-resistant *Staphylococcus aureus* (MRSA) (Bexfield *et al.*, 2008).

The black soldier fly (*Hermetia illucens*) is an insect that belongs to the *Diptera* family, particularly the *Stratiomyidae* order, and is originally found in regions with temperate tropical climates (Singh & Kumari, 2019). Black soldier fly larvae (BSFL) have been shown to possess a remarkable ability to recycle a diverse array of waste materials, including but not limited to fruit and vegetable waste, abattoir waste, human feces, and food waste (Lalander *et al.*, 2019). farming the black soldier fly (BSF) gives an opportunity to produce nutrient-rich animal feed, organic fertilizer, and biobased using various organic wastes. The use of BSF larvae or pupae in the poultry, fish, and swine diets presents a potential alternative to other conventional feed ingredients like fish meal and soybean meal (Surendra *et al.*, 2020). BSFL don't accumulate pesticides or mycotoxins, and they have a high saturated fat content, making them safer and more economically viable than other insects (Purschke *et al.*, 2017). Furthermore, the presence of bioactive compounds in BSFL offers additional benefits to animal diets (Surendra *et al.*, 2020).

The black soldier fly (BSF) has the appearance of a wasp with unique color patterns of white and black throughout its body (Tomberlin *et al.*, 2002). The BSF is a eurygamous insect (mating on flight), adults can survive on water alone for about two weeks (Caruso *et al.*, 2013). Eggs can live for approximately four days, while the larval stage lasts for about 15 days (Tomberlin *et al.*, 2002). The prepupae stage follows, during which the larvae empty their

digestive tracts and scatter in search of a dry place to pupate away from nutrition sources (Sheppard *et al*., 1994). Pupae need approximately 15 days to develop (Surendra *et al*., 2020).

The fact that BSF can thrive in rough environments means that it possesses an innate immunity that can produce a range of substances, including peptides (Choi *et al.*, 2018). This includes the production of a variety of antimicrobial peptides (Harlystiarini *et al.*, 2019). BSF is a highly optional source for AMPs research due to its accessibility and board range of immune system responses (Sultana *et al*., 2021). The risk of growing bacterial resistance against AMPs obtained from BSF may be low. Thus, AMPs derived from this insect may represent inspiring alternatives to antibiotics (Xia *et al.,* 2021). The lipid composition of BSFL can vary depending on the larvae processing method, resulting in different fatty acid profiles (Caligiani *et al.*, 2019). BSFL exhibit a remarkable balance between saturated and unsaturated fatty acids (FAs), which confers them with significant antiviral, antiprotozoal, and antibacterial properties (Makkar *et al.*, 2014). Additionally, BSF has rapid growth with low fed costs, which makes it an appropriate source for the mass production of natural driven alternative (Lee *et al*., 2020). Previous studies investigating the antibacterial activity of BSFL extract, such as those conducted by Park *et al.* (2014), Choi *et al.* (2018), Alvarez *et al.* (2019), and Lee *et al.* (2020) have principally focused on immunizing BSFL by introducing bacteria into the body of the larvae. However, some other studies, specifically those of Choi *et al.* (2012), Harlystiarini *et al.* (2019), Auza *et al*. (2020), and Qosimah *et al.* (2023) where they have demonstrated the antibacterial activity of BSFL extract against pathogenic bacteria in non-immunized BSFL.

The black soldier fly larvae are natural decomposers and can be found in challenging environments that are associated with pathogenic microorganisms like fungi and bacteria (Park *et al.*, 2014). It is known that such environments may have an influence on the evolution of the larvae's innate immune system (Harlystiarini *et al.*, 2019). Based on the existing evidence of the antibacterial activity of BSFL extract in non-immunized larvae, and considering the practicality of reproducing industrial conditions, we have adopted the non-immunization method in our study. The thing that can allows us to conduct researches that align more closely with real-world practices.

The study conducted by Harlystiarini *et al.* (2019) demonstrated that using methanolic extraction the antibacterial activity of BSFL extract against ram-negative bacteria *Salmonella sp.* and *E. coli* at a concentration of 320 mg/mL. Additionally, Choi *et al.* (2012) revealed the antibacterial activity of the same extract against Gram-negative bacteria, particularly *Neisseria gonorrhoeae*, *Shigella sonnei*, and *Klebsiella pneumoniae* at a concentration of 20 mg/mL.

However, their study found no activity against Gram-positive bacteria. In contrast, Qosimah *et al.* (2023) reported the presence of an activity against the Gram-positive bacteria, mainly *Staphylococcus aureus* at a concentration of 320 mg/mL, while no effect against the Gram-positive bacterium *Aeromonas* was observed. Additionally, the study conducted by Auza *et al*. (2020) demonstrated the activity of BSFL extract against *Salmonella typhimurium*, *E. coli* and *P. aeruginosa* at the concentration of 325 mg/mL.

Given the considerable challenges that bacterial problems present to both human health and the environment, and the growing concerns surrounding the safety and efficacy of traditional approaches, especially considering the emerging issue of antibiotic resistance, it is clear that a new approach is needed. Moreover, we have noticed that many of the recent studies have been limited in the choice of solvents, which may reduce the opportunity of discovering the best antibacterial activity of BSFL. In this context, and basing on the previous studies, our study aims to assess the effectiveness of a new alternative for combating harmful bacterial effects by offering a viable solution to conventional methods. It is possible that BSFL extract has a strong potential antimicrobial activity against different bacteria strains and can be considered a promising alternative to traditional antibacterial approaches, especially with the remarkable biology of BSFL. This could lead us to expect more important antibacterial activity regarding the inhibition spectrum and administrated concentration. Our study serves to evaluate the antimicrobial properties of BSFL extract against various bacterial strains, including *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli*, with two different methods of treatment. It also includes controlling various solvents and their mixtures to determine the optimal solvent for yield and antimicrobial activity. This approach increases the chance of discovering potent antibacterial activity. Moreover, it provides an insight into the nature of bioactive molecules present in black soldier larvae.

Our manuscript is divided into four chapters, following the IMRaD methodology. In the introduction, we present an overview of the threat posed by bacterial infections as well as the detrimental impact of current methods employed to combat them, particularly in light of the growing issue of antibiotic resistance. We emphasize the need for alternatives, and we propose the utilization of black soldier fly larvae (BSFL) extract as a promising and natural alternative to conventional approaches. The materials and methods section provides a detailed description of the techniques and laboratory equipment utilized in our experiment. Then, we demonstrate our findings in the results section and provide their analysis and interpretation in the

discussions section. Finishing our manuscript with a conclusion and perspectives for further studies.

METHODOLOGY

1. Study area

The experiments were conducted at the Laboratory of Research and Biotechnology, Faculty of Natural and Life Sciences, university of Ghardaïa, during the period of March to May 2023.

2. Experiment design

Our protocol involves processing live larvae samples using two treatment methods (Figure 1,a), then thawing them till reaching smooth consistency (Figure 1,b). After that, the samples were subjected to maceration (Figure 1,c), filtration (Figure 1,d), and evaporation under reduced pressure (Figure 1,e). These steps allow us to extract bioactive compounds that are then stored at 4 °C (Figure 1,f) for later use. These extracted compounds are then tested against a variety of bacteria using the agar disc and well diffusion assays (Figure 1,g). The inhibition zone is then measured to assess the efficacity of this extract (Figure 1,h). The figure bellow is an illustration that summarizes the protocol followed in this study.

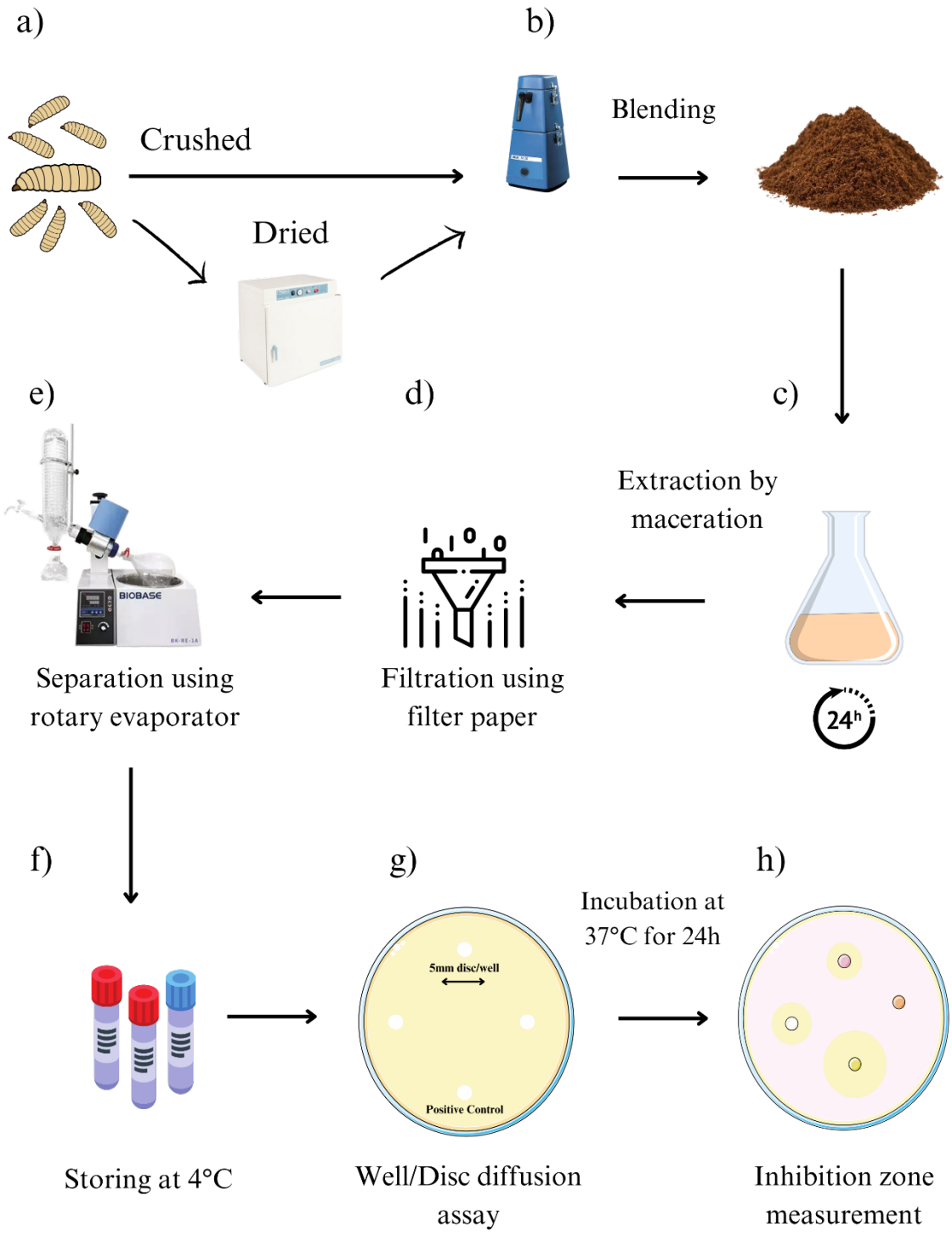

Figure 1. Illustration that presents the protocol adopted in the study.
a: sample preparation; **b**: blending; **c**: maceration; **d**: filtration; **e**: evaporation; **f**: storing; **g**: well/disc diffusion; **h**: measurement

3. Sample preparation

The insects used in this study were 15-day-old black soldier fly larvae (Choi *et al.*, 2012) (Figure 2; *Hermetia illucens*) obtained from a local breeding farm (IziYummy) in Beni Isguen, Ghardaïa, Algeria. The larvae were fed on a regular schedule of every three days, and the feeding ratio was 5 kilograms of food per kilogram of larvae. The larvae's diet was composed of 65% potato scraps, 30% bran, and 5% dried bread. Insect material is kept in a plastic container with a perforated lid.

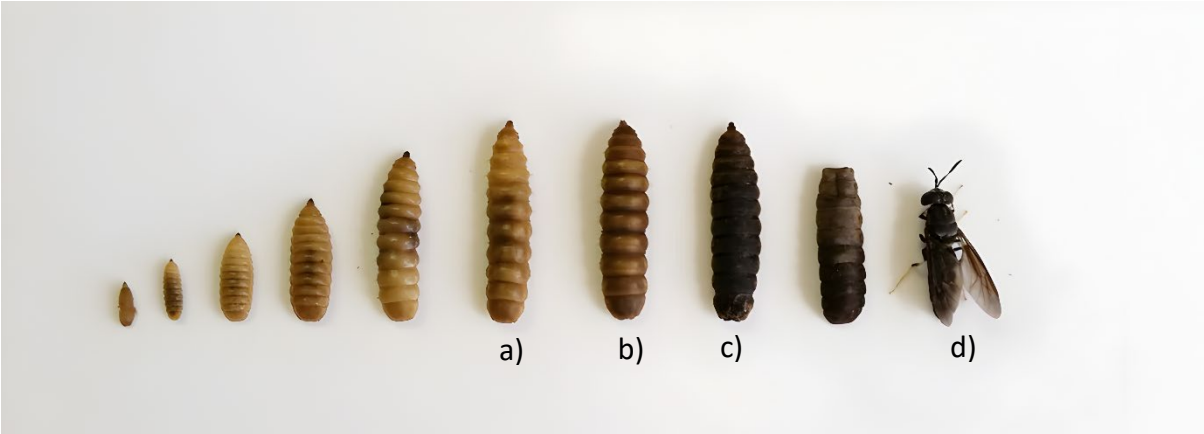

Figure 2. Black soldier fly life cycle (Obie's Worms Black Soldier Fly Life Cycle, n.d.).
a: larvae; **b**: pre pupae; **c**: pupae; **d**: adult

The larvae were subjected to a thorough washing with water and sterilization using a bleach dilution. Following this, the larvae were rinsed with water again to ensure cleanliness (Figure 3). In order to determine the most effective treatment method, two distinct techniques were considered. The first method was referred to as the crushing method (C), and it involved thawing the larvae and processing them directly in a blender (IKA® M20 Universal Mill). The second method, known as the drying method (D), entailed the larvae being dried in an oven (Raypa®) at a temperature of 65°C for 24 hours (Auza *et al.*, 2020), after which they were ground in the blender until a smooth texture was achieved (Figure 4).

Figure 3. BSF larvae cleaning. **a**: rinsing; **b**: sterilizing; **c**: sterilizing

Figure 4. BSF larvae drying and thawing. **a**: fresh larvae; **b**:dried larvae; **c**: crushed larvae

4. Evaluation of solvents

To determine the most effective solvents for extraction, a preliminary test was carried out with a range of solvents and solvent mixtures with varying polarities and properties. The objective was to gain insight into the nature of the active molecule. For each treatment method (C and D), eight solvents were employed (Table I), including methanol as per Harlystiarini *et al*. (2019) protocol, a mixture of H_2O, methanol, and HCl (90; 9; 1%) according to Heakal *et al*. (2021), a combination of methanol, H_2O, and acetic acid (90; 9; 1%) following Vogel *et al*. (2018) methodology, acetone following Almeida *et al*. (2020) protocol, as well as hexane, butanol, ethyl acetate, and methylene chloride.

Table I. Used solvents and solvent mixtures.

Solvent Label	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
Organic Solvents	Methanol	H2O; methanol; HCl (90;9;1%)	Methanol, H2O, acetic acid (90;9;1%)	Acetone	Hexane	Butanol	Ethyl acetate	Methylene chloride

5. Extraction method

The extraction process was performed using a modified version of the protocol described by Choi *et al*. (2012). Two grams of crushed larvae (C) and two grams of dried larvae (D) were added to 20 mL of each solvent at a volume ratio of 1:10 (Figure 5). The mixture was then placed on a shaker at 160 rpm and 25 °C for 24 hours. Subsequently, each sample was filtered using filter paper, and the solvents were removed by evaporation under reduced pressure using

a rotary evaporator at 40 °C. The resulting extracts were stored at -4 °C in a refrigerator until further use.

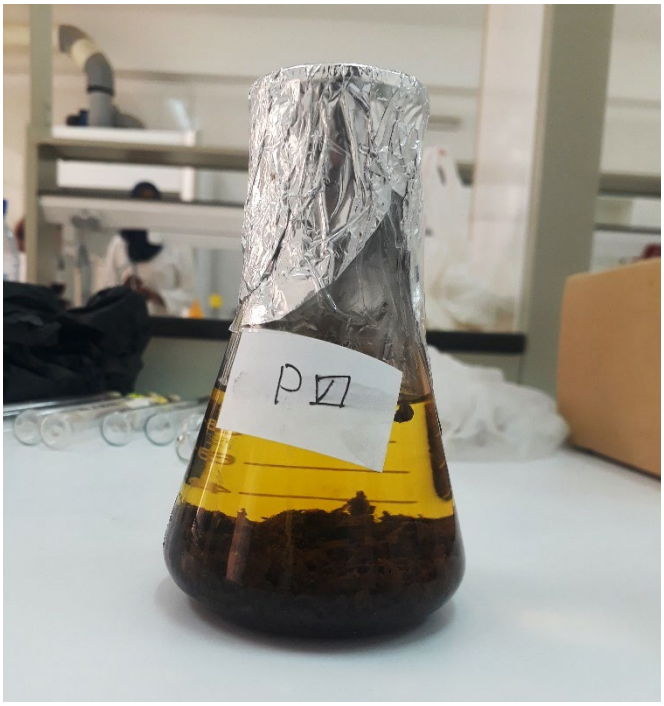

Figure 5. Extraction by maceration.

The weight of the final extract of each solvent was measured in a balance. The yield (Y) was calculated using the following equation:

Equation 1. BSFL extract yield.

$$Y = weight\ of\ extract \div initial\ weight\ of\ crushed\ larvae$$

6. Bacterial strains

Four common pathogen bacterial strains (two Gram positives and two Gram negatives) were tested in order to evaluate the effectivity of the extract, namely *Pseudomonas aeruginosa* (ATCC 9027), *Listeria monocytogenes* (ATCC 13932), *Staphylococcus aureus* (MRSA 639c), and *Escherichia coli* (ATCC 8739). The four strains were sub cultured on nutrient agar (Annex) and incubated at 37 °C for 18 hours.

7. Agar disc diffusion assay

Five-millimeter Whatman discs (No 3) were made and sterilized under UV light for 30 minutes on each side. Then, the extracts were dissolved in dimethyl sulfoxide (DMSO) before utilization. Subsequently, 10 µL of each extract was added onto sterile discs, and 10 µL of

DMSO was added to additional discs as a negative control. The discs were then allowed to dry for 30 minutes in sterile conditions. Gentamicin discs were used as positive controls.

To reach a 0.5 McFarland standard, the subcultured bacteria colonies were added to physiological water (NaCl 9%) and the turbidity of the suspensions was adjusted to an absorbance of 0.08 to 0.12 at 625nm using a spectrophotometer (SECOMAM UviLine® 9400C), which is approximately equal to (1-2) $\times 10^8$ CFU/mL.

Using a sterile swab, the inoculums were streaked onto Muller Hinton agar (MH; Annex) medium, after which the discs were placed on the surface of the agar. The petri dishes were then incubated at 37 °C for 24 hours, and the zones of inhibition around each disc were measured and recorded.

8. Agar well diffusion assay

The bacterial inoculum was prepared as described before, and using a sterile swab, the inoculum is spread uniformly on the surface of the MH agar medium. In this assay, five-millimeter wells were made in the agar medium using a sterile cork borer. The BSF larvae extracts were added to the wells using a micropipette, along with positive and negative control discs. The inoculated plates were then incubated under the same conditions as the disc diffusion assay, and the zones of inhibition around each well were measured and recorded.

9. Inhibition zone measurement:

The inhibition zones were measured using a ruler. Both horizontal and vertical axis were measured, and their average was then taken as a final result for each zone, respectively.

10. Solvent selection

Based on the results obtained from the preliminary test, a total of three solvents were selected for the final test. The solvents were divided into three groups based on their polarity, namely polar, nonpolar, and intermediate polarity. The selection of solvents and treatment method was based on their antimicrobial activity and yield of extraction for each solvent. The solvents chosen for the final test include methanol (DI), hexane (CV), and methylene chloride (CVIII).

To ensure consistency, ten grams of treated larvae were added to 100 mL of the respective selected solvent in a 1:10 volume ratio. The mixture was then placed on a shaker at 25 °C and

160 rpm for 24 hours. The same extraction process and storage conditions as previously described were followed (sub-section 5).

RESULTS

1. Solvent preliminary test

1.1. Extract weight

Various organic solvents were evaluated for their suitability in extracting bioactive compounds from black soldier fly larvae. Following the extraction by maceration, all solvents underwent low-pressure evaporation using a rotary evaporator, and the resulting extract weights were measured and represented in Figure 6. The extracts were labeled CI to CVIII and DI to DVIII, corresponding to the two treatment methods (C & D) employed and the eight organic solvents utilized in the study. The results demonstrated that solvents II, III, and VII yielded a significantly higher weight compared to the rest of the solvents. Similarly, the samples subjected to the drying treatment (D) generally yielded a higher extract weight.

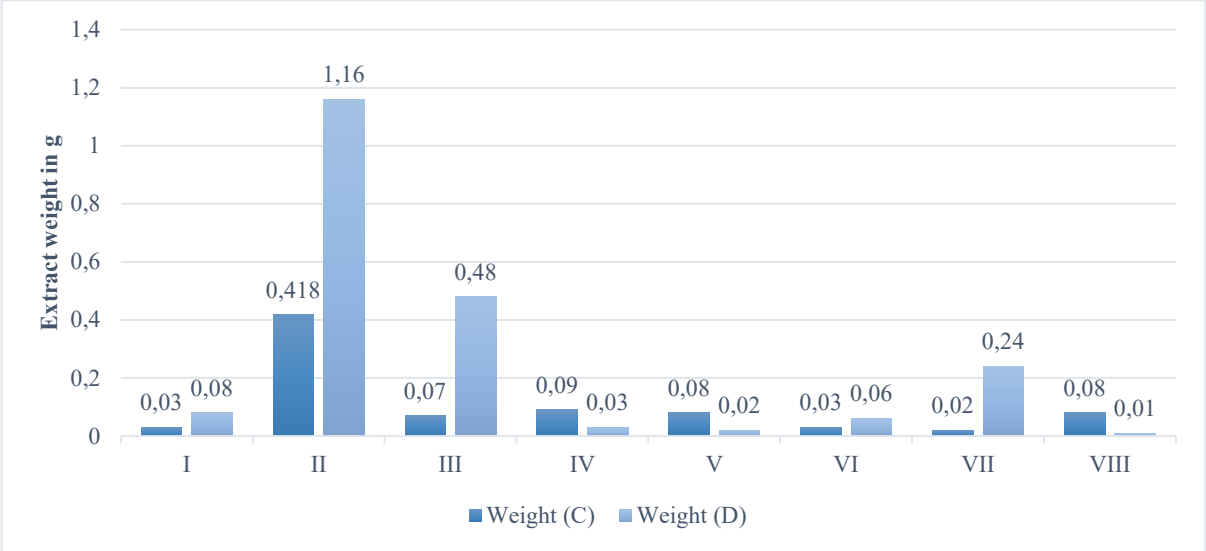

Figure 6. Extracts weight chart

1.2. Extract yield

The yield of the extracts varies between 0.07% and 5.80%. The mixture of solvents showed the highest yield percentage among all the extracts, with 5,80% in the mixture of H_2O, methanol, HCl (90;9;1%), and 2.4% in the mixture of methanol, H_2O, acetic acid (90;9;1%). As for the treatment method, the yield differs from a solvent to another. However, most solvents exhibited a higher yield with the drying method. The resulting yields achieved from these diverse extraction solvents are presented on Table II below.

Table II. The different solvents and volumes used along with the weights of larvae and its extracts yield.

	Organic Solvents							
	Methanol	H_2O methanol HCl	Methanol H_2O acetic acid	Acetone	Hexane	Butanol	Ethyl acetate	Methylene chloride
Solvent Label	I	II	III	IV	V	VI	VII	VIII
Larvae (g)	2	2	2	2	2	2	2	2
Volume (mL)	20	18; 1.8; 0.2	18; 1.8; 0.2	20	20	20	20	20
Yield (C)	0.15%	2.09%	0.35%	0.45%	0.40%	0.15%	0.10%	0.40%
Yield (D)	0.40%	5.80%	2.4%	0.15%	0.10%	0.30%	1.20%	0.07%

1.3. Antibacterial activity of extracts

The antimicrobial activity of the extracts obtained from black soldier fly larvae was assessed against a variety of bacterial strains. The agar disc diffusion method was employed to determine the inhibitory effect of the extracts on bacterial growth Concentrations of 20 to 120 mg/mL were used for the extracts. The results indicated varying degrees of antimicrobial activity among the different extracts.

The antimicrobial activity of all tested extracts was found to be significantly effective against L. monocytogenes, with zone sizes ranging from 8 to 15 mm (Figure 7). The zones of inhibition observed for both sets of extracts (C & D) were slightly similar, indicating comparable effectiveness against the tested bacteria. The methylene chloride extract produced an inhibition zone of 14-15 mm, which is the widest inhibition zone against the tested bacteria. Followed by a 9-13 mm inhibition zone exhibited by methanol, butanol, hexane, and ethyl acetate extracts. finally, the extract mixtures labeled II and III produced the smallest inhibition zone of 8 and 10 mm respectively.

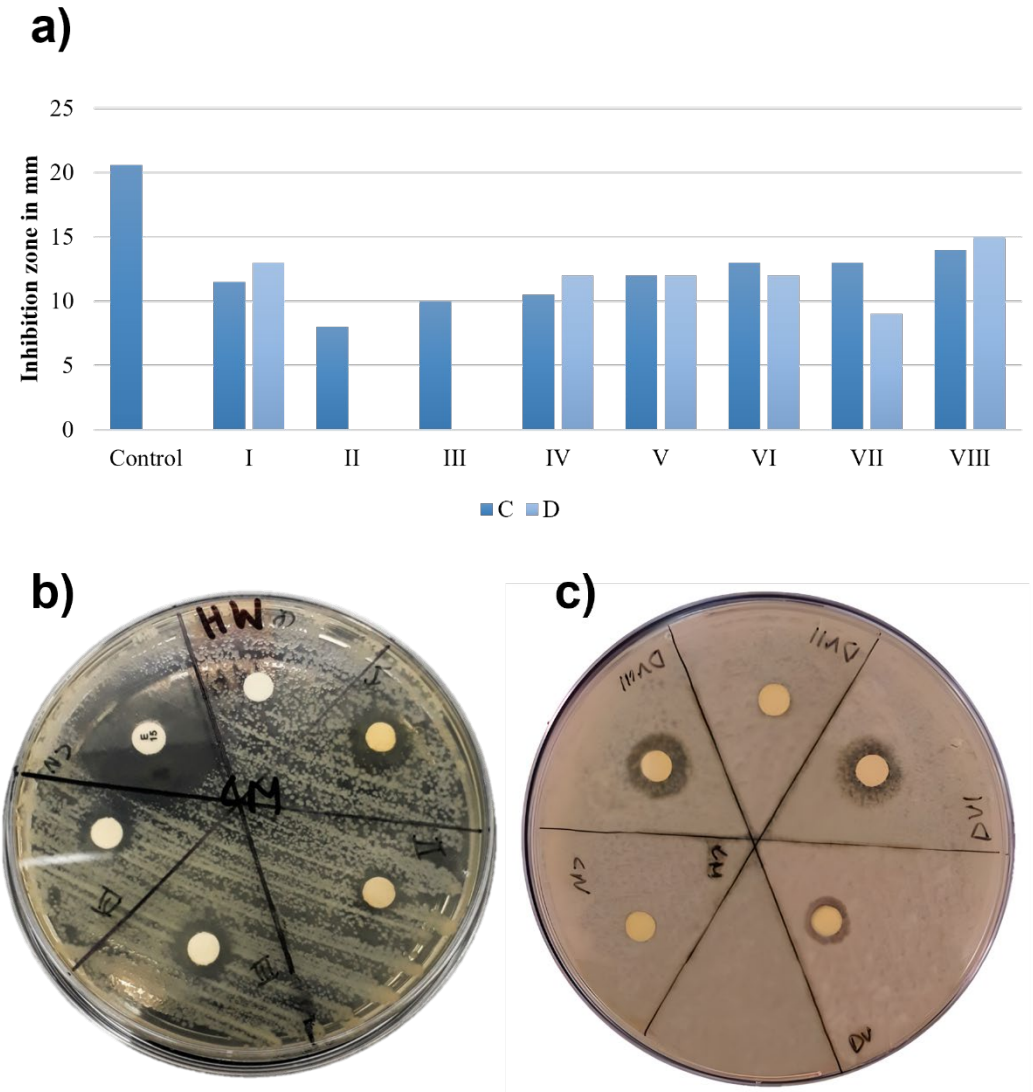

Figure 7. Antibacterial effects and inhibition zone of different extracts obtained from BSF larvae against *L. monocytogenes*. **a**: inhibition zone chart; **b**: inhibition zone of extracts CI-CIV; **c**: inhibition zone of extracts DV-DVIII

Our results revealed a significant antimicrobial activity of all tested extracts against *S. aureus*, as indicated by varying inhibition zones ranging from 8 to 13 mm (Figure 8). All the extracts exhibited significant antibacterial activity against *S. aureus*, displaying a range of inhibition zones varying from 8 to 13 mm. Both treatment methods (C & D) demonstrated similar activity in the same extract. Notably, the methanol extract exhibited the highest activity with a significant inhibition zone of 13 mm. Following closely behind was the mixture of methanol, H2O, and acetic acid (90:9:1%), which showed an inhibition zone of 12 mm. After that, the methylene chloride extract showed an activity with an inhibition zone of 11 mm. The remaining solvents demonstrated activity equal to or above 9 mm, with the exception of the mixture of H2O, methanol, and HCl (90:9:1%), which exhibited the lowest activity at 8 mm.

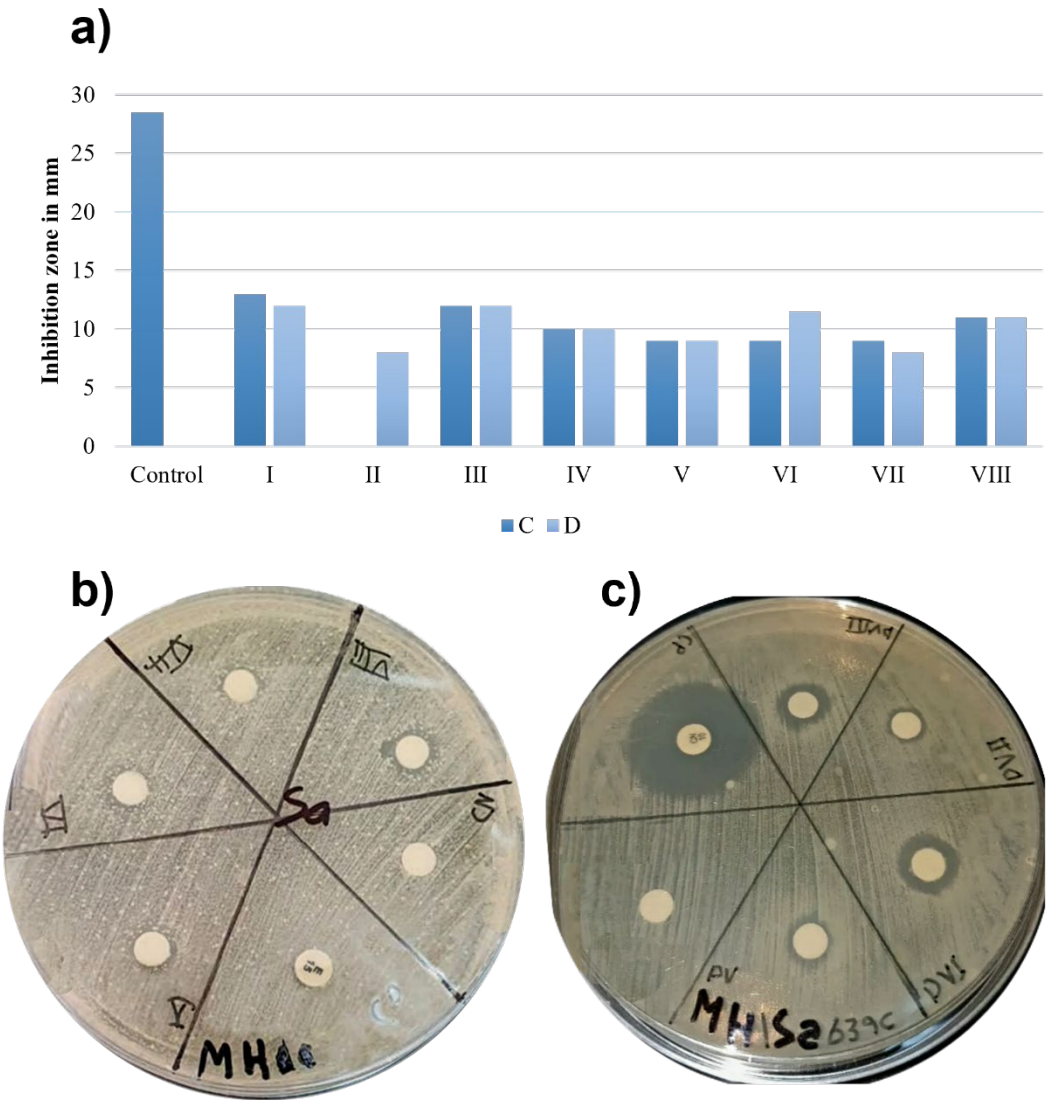

Figure 8. Antibacterial effects and inhibition zone of different extracts obtained from BSF larvae against *S. aureus*. **a**: inhibition zone chart; **b**: inhibition zone of extracts CV-CVIII; **c**: inhibition zone of extracts DV-DVIII

Our results showed that no visible antimicrobial activity was observed against the Gram-negative strains, *E. coli* and *P. aeruginosa* (Figure 9 and 10).

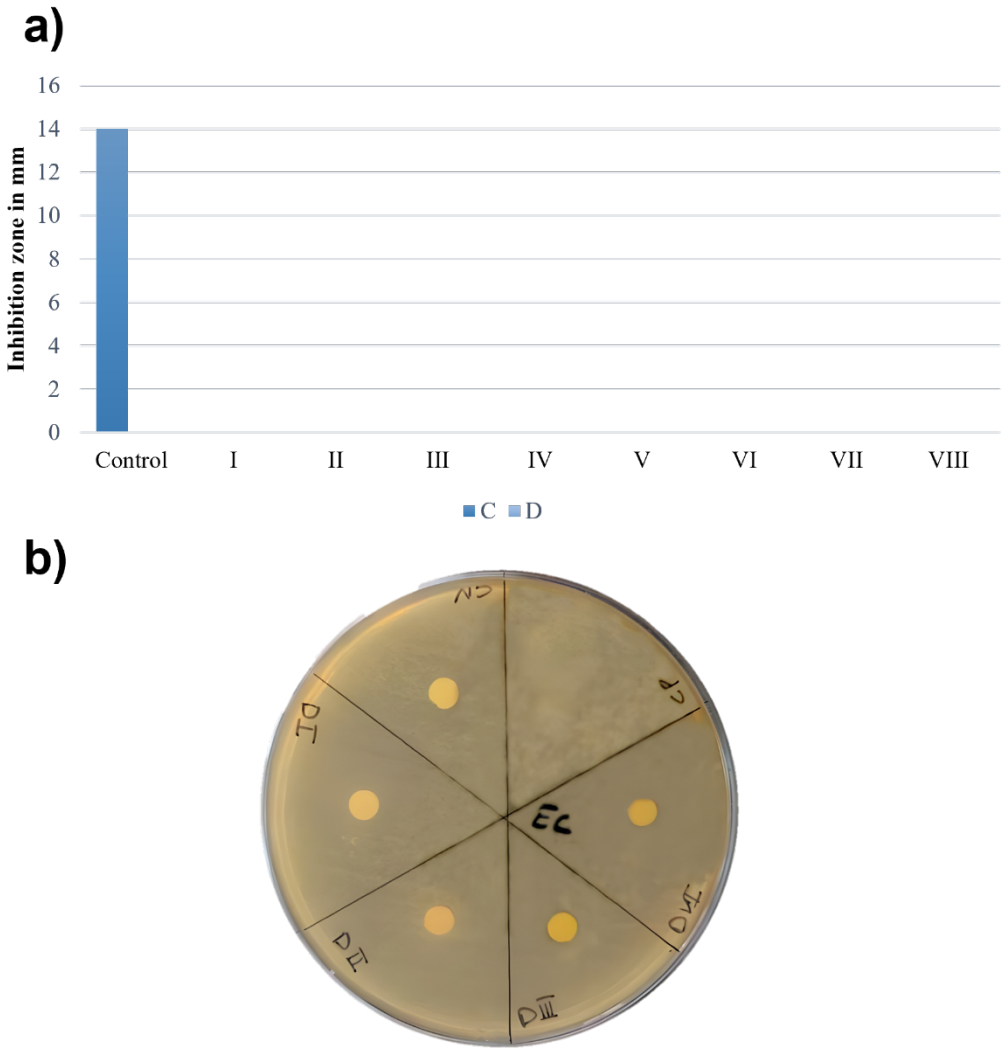

Figure 9. Antibacterial effects and inhibition zone of different extracts obtained from BSF larvae against *E. coli*. **a**: inhibition zone chart; **b**: inhibition zone of extracts DI-DIV

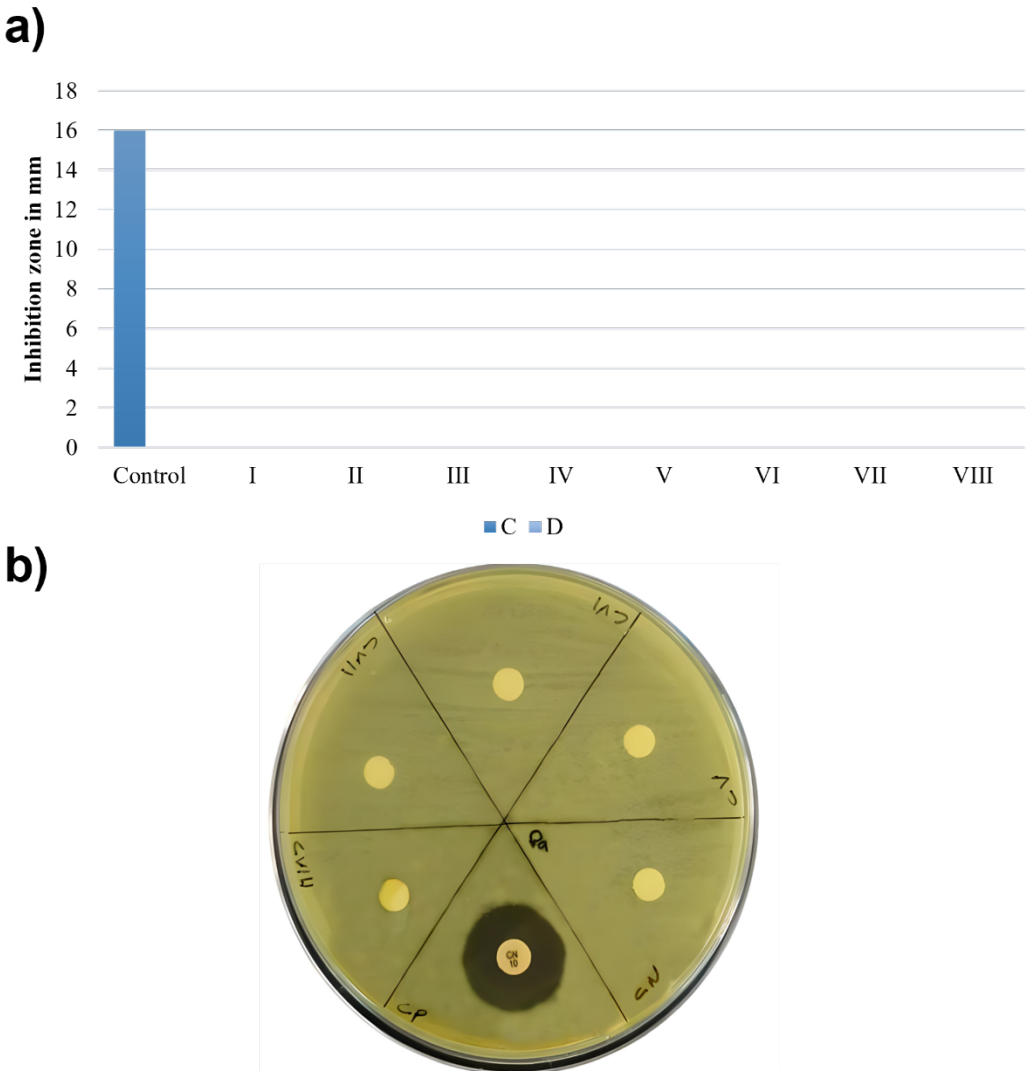

Figure 10. Antibacterial effects and inhibition zone of different extracts obtained from BSF larvae against *P. aeruginosa*. **a**: inhibition zone chart; **b**: inhibition zone of extracts CV-CVIII

2. Antibacterial activity of black soldier fly larvae extracts

The antimicrobial activity of the extracts was evaluated at different concentrations, including 10, 20, 40, 80, and 160 mg/mL, against a range of bacterial strains. The purpose was to assess the effectiveness of the extracts as antimicrobial agents at varying concentrations. The results revealed varying degrees of inhibition against the tested bacterial strains (Figure 11). Higher concentrations generally exhibited greater inhibitory effects, as indicated by larger zones of inhibition. The specific concentrations at which significant antimicrobial activity was observed varied depending on the bacterial strain and the extract, notably 20 mg/mL. These findings suggest that the antimicrobial effectiveness of the extracts is concentration-dependent and can vary among different bacterial species.

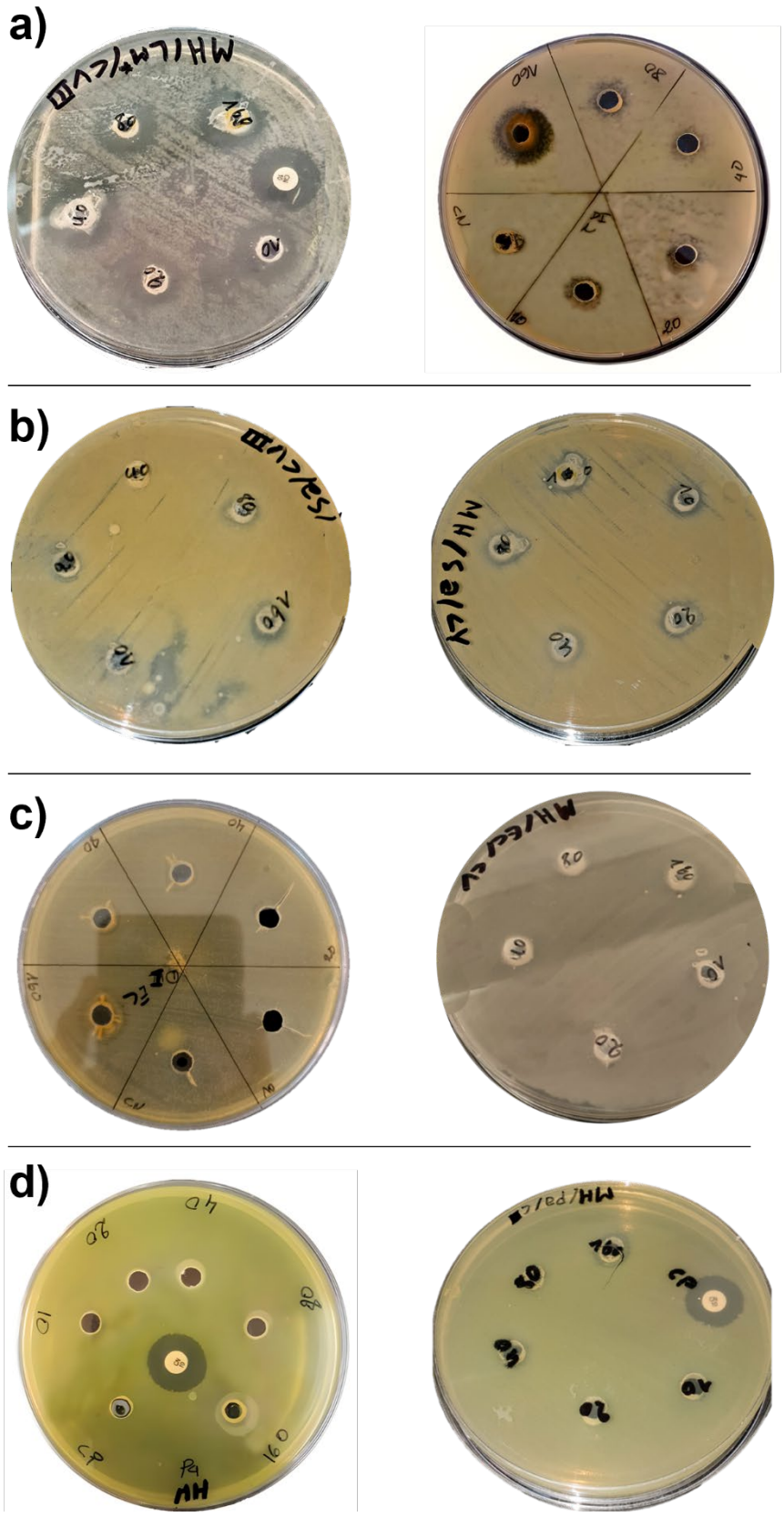

Figure 11. Inhibition zone of different extracts against harmful bacteria.
a: *L. monocytogenes* ; **b**: *S. aureus*; **c**: *E. coli* ; **d**: *P. aeruginosa.* The bacteria were treated with various concentrations (10-160 mg/mL).

The methanol extract demonstrated a significant inhibitory effect against all bacterial strains tested. Notably, a zone of inhibition with a diameter of 20 mm was observed against *Staphylococcus aureus* at a concentration of 160 mg/mL. Additionally, at a concentration of 20 mg/mL, the extract proved efficient against both *S. aureus* and *E. coli*, with inhibition zone diameters of 10 mm and 6 mm, respectively.

Table III. Diagram of inhibition zone and antibacterial activity of methanol extract (DI).

Microorganism		Concentration (mg/mL)				
		10	20	40	80	160
Gram-positive Bacteria	*L. monocytogenes*	-	+	+	+	+
	S. aureus	-	-	-	+	+
Gram-negative bacteria	*P. aeruginosa*	-	-	+	+	+
	E. coli	-	-	-	+	+

>6 to 10 mm (+), 10 to 14 mm (++), 14 to 18 mm (+++), 18 to 22 mm (++++), and no activity (-).

The hexane extract showed a remarkable inhibitory activity against all the tested bacterial strains at a concentration of 160 mg/mL, where the extract displayed a substantial inhibitory effect, resulting in a significant zone of inhibition with a diameter of 20 mm against *L. monocytogenes*. Furthermore, even at a lower concentration of 10 mg/mL, the hexane extract demonstrated considerable activity against *L. monocytogenes* and *S. aureus*, exhibiting zone sizes of 15 mm and 11 mm, respectively. However, its activity against *P. aeruginosa* and *E. coli* was relatively lower, with zone diameters of 9 mm and 6 mm, respectively, at a concentration of 160 mg/mL.

Table IV. Diagram of inhibition zone and antibacterial activity of hexane extract (CV).

Microorganism		Concentration (mg/mL)				
		10	20	40	80	160
Gram-positive Bacteria	*L. monocytogenes*	-	-	+	++	+++
	S. aureus	-	+	++	+++	++++
Gram-negative bacteria	*P. aeruginosa*	-	-	-	++	+++
	E. coli	-	+	++	++	++

>6 to 10 mm (+), 10 to 14 mm (++), 14 to 18 mm (+++), 18 to 22 mm (++++), and no activity (-).

The methylene chloride extract exhibited antimicrobial activity against all tested bacterial strains, at a concentration of 80 mg/mL. Notably, the extract showed particular efficacy against *P. aeruginosa*, displaying activity even at a lower concentration of 40 mg/mL and resulting in an inhibition zone of 8 mm. Additionally, *L. monocytogenes* showed susceptibility to this extract at a concentration of 20 mg/mL, yielding an inhibition zone of 6 mm.

Table V. Diagram of inhibition zone and antibacterial activity of methylene chloride extract (CVIII).

Microorganism		**Concentration (mg/mL)**				
		10	20	40	80	160
Gram-positive Bacteria	*L. monocytogenes*	++	+++	+++	++++	++++
	S. aureus	++	++	++	++	++
Gram-negative bacteria	*P. aeruginosa*	-	-	-	+	+
	E. coli	-	-	-	-	+

>6 to 10 mm (+), 10 to 14 mm (++), 14 to 18 mm (+++), 18 to 22 mm (++++), and no activity (-).

DISCUSSION

DISCUSSION

This study provides an overview of the antimicrobial activity of the black soldier fly (*Hermetia illucens*) larvae (BSFL) extract as an alternative for the antibiotics and the antibacterial chemical agents that are currently used. The BSFL were treated with two methods, including crushing (C) and drying (D), followed by an extraction using maceration and evaporation techniques. The yields of the extracts varied depending on the treatment approach and solvent used. The selection of the treatment approach and the choice of solvents can significantly impact the resulting extract yield.

The agar disc diffusion method was employed only to determine whether the solvents possessed any antibacterial activity considering the limited quantity of extracts available for testing.

All of the extracts utilized in the preliminary test demonstrated a significant antibacterial activity against Gram-positive bacteria, specifically in *L. monocytogenes* and *S. aureus*. However, these extracts did not show any activity against both *E. coli* and *P. aeruginosa*, proving BSFL extract more effective against Gram-positive bacteria compared to Gram-negative bacteria. This finding aligns with the research conducted by Mamman *et al*. (2005), which shows that Gram-negative strains demonstrated significantly higher resistance compared to Gram-positive strains.

Having an activity in all the extracts of all the solvents that extract molecules with different polarities indicates that the BSFL extract may contain multiple bioactive compounds. It is known that the humoral immunity is associated with the synthesis and secretion of AMPs within the lipid fraction, which are subsequently released into the hemolymph. (Hoffmann & Reichhart, 2002; Tsakas & Marmaras, 2010). BSFL contain an important amount of fatty acids, which confers them with significant antiviral, antiprotozoal, and antibacterial properties (Makkar *et al.*, 2014).

In order to investigate the activity of BSFL extract as antimicrobial agents, the antimicrobial activity of the extracts was evaluated at different concentrations, five concentrations were tested, including 10, 20, 40, 80, and 160 mg/mL, against the previous bacterial strains. We have selected three different solvents with varying polarities in order to test a wide range of bioactive compound polarities in the BSFL extract. The selection was based on polarity as well as antimicrobial activity and yield.

Methanol was selected as the polar solvent, while hexane was chosen as the nonpolar solvent. Additionally, methylene chloride was opted for as the solvent with intermediate polarity. Based on both antibacterial activity and yield, the appropriate treatment method (crushing or drying) was chosen for each solvent. We noticed that the wells are more suitable for containing the recommended volume in order to benefit from the full potential generated by the extract. On the other hand, the discs demonstrated an inability to absorb the entire volume needed to exhibit a significant antibacterial activity.

The methanol extract exhibited a significant zone of inhibition against all tested bacterial strains at a concentration of 80 mg/mL, with a zone of inhibition reaching the diameter of 20 mm against *Staphylococcus aureus* at a concentration of 160 mg/mL. Furthermore, the concentration of 20 mg/ml displayed an activity against both *S. aureus* (10 mm) and *E. coli* (6 mm). The finding of Mohtar *et al*. (2014) demonstrated a significant effect of optimizing the extraction procedure using methanolic extraction of AMP from whole body extracts of insects. As previously mentioned, AMPs represent an important part of humoral immunity of the insect, defensins are a type of AMPs that are found in insects. Generally, the mechanism of action of these AMPs is to create channels within the bacterial cytoplasmic membrane. Xia *et al.* (2021) demonstrated that defensins are the most dominant AMP in BSFL. Specifically, defensins have a high affinity to cardiolipin, a predominant phospholipid in bacteria. The interaction between defensins and phospholipids can lead to the induction of microheterogeneity in the lipid membrane, this process is likely related to the formation of channels which are responsible for the biological activity of defensin (Yi *et al.*, 2014), including the inhibition or even the stop of the enzyme catalytic activity (Jia *et al.*, 2019), and the damage of the cell wall of bacteria which can result the bacterial death (Qosimah *et al.*, 2023). The study conducted by Choi *et al.* (2012) reported that the methanol extract exhibited a higher susceptibility to Gram-negative bacteria compared to Gram-positive bacteria. Generally, Gram-positive bacteria are characterized by a cell wall composed of only a thick single-unit peptidoglycan layer, while the cell wall of Gram-negative bacteria consists a thin peptidoglycan layer accompanied with an outer membrane containing lipopolysaccharide (LPS), lipoprotein, and phospholipids. The raison that possibly makes defensins more effective in Gram-negative bacteria than Gram-positive bacteria for having a high attachment to cardiolipin (Yi *et al.*, 2014). Park & Yoe (2017) reported that cecropin is an AMP driven from *H. illucens* that have an antibacterial activity against *E. coli*.

In contrast to the findings of Choi *et al.* (2012), the research published by Qosimah *et al.* (2023) reported that the methanol extract obtained from black soldier fly larvae (BSFL) had a more pronounced effect on Gram-positive bacteria such as *S. aureus* compared to Gram-negative bacteria like *Aeromonas.* This can be related to the presence of other antimicrobial agents within the innate immune system of BSFL. One such agent is fatty acids, as indicated by the study conducted by Auza *et al*. (2020).

In our study, the methanol extract showed an activity against all tested bacterial strains, both Gram-negative and Gram-positive. This may indicate the presence of compounds with a broad spectrum of activity or multiple antibacterial compounds (that may include both AMPs and fatty acids) which supports the observations of Van de Plas *et al.* (2008) who noted that the methanol extract possesses unique properties that trigger diverse antibacterial effects against the tested bacterial strains. It is also possible that a combination of compounds is working synergistically against both Gram-positive and Gram-negative bacteria.

Staphylococcus aureus and *L. monocytogenes* were significantly sensible to the hexane extract, indicating that it has a considerable potential against these bacteria. The extract displayed a 20 mm diameter zone of inhibition towards *L. monocytogenes* at a concentration of 160 mg/mL. Furthermore, this extract showed substantial action against *L. monocytogenes* and *S. aureus* even at a lower concentration of 10 mg/mL, with zone diameters of 15 mm and 11 mm, respectively. This shows the extract's effectiveness against these Gram-positive bacteria. However, it is worth mentioning that the hexane extract presented comparatively lower activity against both *P. aeruginosa* (9 mm) and *E. coli* (6 mm), suggesting limited efficacy against Gram-negative bacteria. The selection of hexane as a solvent was based on its polarity, which favors the extraction of polar molecules. This orientation towards polar molecules leads us to consider fatty acids as potential active components, as BSFL are known to be a rich source of such compounds (Harlystiarini *et al.*, 2019). The lipid composition of BSFL can vary depending on the larvae processing method, resulting in different fatty acid profiles (Caligiani *et al.*, 2019). Saturated fatty acids (SFAs), including lauric acid, stearic acid, palmitic acid, and capric acid, have been reported to exhibit broad-spectrum antimicrobial properties (Casillas-Vargas *et al.*, 2021). The primary target of medium-chain fatty acids (MCFAs). Their action causes membrane damage, facilitating the entry of antimicrobial compounds into the cytoplasm, thereby accelerating bacterial death. Additionally, the damaged cell membranes allow hydrogen ions from the extracellular fluid to enter the cells, acting as potent bacterial-killing agents (Kim & Rhee, 2016). Fatty acids can also prevent bacterial

adhesion and biofilm formation while maintaining an acidic pH on the body's surface, which contributes to their antimicrobial effects (Jumina *et al.*, 2019). Disruption of bacterial membranes or inhibition of fatty acid synthesis are reported mechanisms through which fatty acids inhibit bacterial growth (Thormar & Hilmarsson, 2011).

BSFL have been found to have a high content of lauric acid in their lipid profile (Marusich *et al.*, 2020). In the study performed by Harlystiarini *et al.* (2019), the BSFL has a lauric acid content of 49.18%. Lauric acid is known to exhibit antimicrobial activity by disrupting cell membranes (Committee, 2015), inactivating enzymes, and denaturing cell proteins (Pezzi *et al.*, 2017). The wide-ranging antimicrobial activity of fatty acids in general, and specifically lauric acid, may be the main contributing factor to the significant antibacterial activity observed against *L. monocytogenes* and *S. aureus*.

The low antibacterial activity against Gram-negative bacteria can be attributed to the findings of Casillas-Vargas *et al.* (2021), which suggest that lauric acid has more potential as an antibacterial agent against Gram-positive bacteria such as *Staphylococcus* and *Streptococcus* compared to Gram-negative bacteria like *E. coli*. Lauric acid alone exhibits antimicrobial effects against Gram-positive bacteria and yeasts, but not against Gram-negative bacteria (Van Huis *et al.*, 2013; Van Huis & Oonincx, 2017).

Furthermore, Choi & Jiang (2014) reported that hexanedioic acid isolated from *H. illucens* larvae effectively inhibits the growth and proliferation of both Gram-positive and Gram-negative bacteria in a concentration-dependent manner. We propose that hexanedioic acid, being effective against Gram-negative bacteria, may be the primary agent responsible for its effect on *P. aeruginosa* and *E. coli*. However, the low yield of hexanedioic acid compared to other fatty acids (only 2.0% extracted by methanol, according to Choi & Jiang, 2014) could explain its lower antibacterial activity compared to Gram-positive bacterial strains.

The methylene chloride extract displayed an activity against all tested bacterial strains both Gram-positive and Gram negative, starting from a concentration of 80 mg/ml. Furthermore, specifically against *P. aeruginosa*, the extract demonstrated activity even at a lower concentration of 40 mg/ml, resulting in an inhibition zone of 8 mm. Similarly, it also exhibited an activity against *L. monocytogenes* at a concentration of 20 mg/ml, with an inhibition zone of 6 mm. Moreau *et al.* (2003) demonstrated the significant extraction capability of methylene chloride for nonpolar compounds. Additionally, according to Ben Brahim *et al.* (2022), methylene chloride has been successfully utilized for extracting AMPs

from certain plant tissues. It is possible that the activity of methylene chloride extract caused by both AMPs and fatty acids.

It was demonstrated that extracts of BSFL fed a cellulose and protein-rich diet exhibits an antimicrobial activity against Gram-negative bacteria, while larvae fed chitin, cellulose, and plant oil performs higher activity against Gram-positive bacteria (Vogel *et al*., 2018) additionally Vogel *et al* (2018) demonstrated that the production of AMPs by BSF is also diet-dependent. The change of diet may explain the different results of the studies.

Each selected solvent exhibited distinct activity levels. Methanol emerged as the most potent solvent, displaying significant activity against all bacterial strains. Meanwhile, hexane extract proved to be the optimal performance in combating Gram-negative bacteria. Although the methylene chloride extract exhibited comparatively lower activity compared to the other two extracts, it still demonstrated effectiveness against all bacterial strains. Notably, all observed activities were found to be concentration-dependent.

All the selected solvents demonstrated notable antibacterial activity against the tested bacterial strains. The antibacterial efficacy varied based on the specific characteristics of each solvent, which directly influenced the extraction of distinct bioactive molecules. These bioactive molecules exhibited varying reactivity against Gram-positive and Gram-negative bacteria. Particularly, recent studies indicated that the AMPs and fatty acids, such as lauric acid, were identified as the primary antibacterial agents extracted from black soldier fly larvae.

It is worth mentioning that the agar well diffusion method exhibited superior efficacy compared to the disc diffusion method, suggesting that the volume absorbed plays an important role in the manifestation of antibacterial activity.

Among all the extracts, the methanol extract exhibited the highest effectiveness, successfully targeting both Gram-positive and Gram-negative bacterial strains at relatively low concentrations. In the case of Gram-negative bacteria, the hexane extract displayed remarkable antibacterial activity, achieving notable efficacy even at a low concentration of 10 mg/mL. Conversely, the methyl chloride extract demonstrated antibacterial activity against all tested bacterial strains, albeit with a relatively lower potency compared to the methanol extract.

This study makes a notable contribution by uncovering new insights into the antimicrobial activity of black soldier fly larvae (BSFL) extract. Based on the available information, this study can be considered as a first report of BSFL extract activity against *L. monocytogenes*.

It should also be noted that our study demonstrates that a low concentration of 10 mg/mL is reliable in generating significant antimicrobial activity against *L. monocytogenes* and *S. aureus*, with inhibition zones measuring 14 mm and 10 mm in diameter, respectively. This finding is particularly noteworthy when compared to previous investigations conducted by Choi *et al.* (2012), Harlystiarini *et al.* (2019), Auza *et al.* (2020), and Qosimah *et al.* (2023), which reported antibacterial activity at concentrations of 20 mg/mL, 160 mg/mL and 325 mg/mL, respectively. The demonstrated efficacy of the BSFL extract at such a reduced concentration underscores its potential as a potent antimicrobial agent warranting further investigation and development.

CONCLUSION

CONCLUSION

This study provided highlights on the effectiveness of the black solider fly larvae extract (*Hermetia illucens*) as a natural driven antibacterial alternative to the traditional antibacterial approaches, where the efficacity of BSFL extract was tested against harmful bacteria. This research contributes to the suggestions of new antibacterial candidates to replace the conventional antibacterial agents in the light of antibiotic. The primary objective of this research is to assess the efficacy of BSFL extract as a potential alternative to the standard antibacterial methods, offering a potential solution to the issue of antibiotic resistance.

Two weeks old BSFL were treated with two different methods, then extracted using eight different solvents. The BSFL extracts were tested against Gram-positive and Gram-negative bacterial strains, namely *L. monocytogenes* (ATCC 13932), *S. aureus* (MRSA 639c), *P. aeruginosa* (ATCC 9027), and *E. coli* (ATCC 8739), This experiment aid in identifying the optimal solvent concerning yield and antimicrobial activity, it also provides insight into the nature of bioactive molecules.

The preliminary test showed an activity of all extracts against the tested Gram-positive bacteria. However, they didn't show any inhibition zone in the Gram-negative bacteria. Three of the previous solvents with various polarities were selected for a further test where the extracts were evaluated with multiple concentrations, including from 10 to 160 mg/mL.

Methanol extract exhibited a significant antibacterial activity against all tested bacteria with important zones of inhibition at the concentration 80 mg/mL and 20 mg/mL against tow bacterial strains. Same concentrations were effective in the Methylene chloride extract but with a lower zone of inhibition. While hexane extract exhibited a significant antibacterial activity against the tested Gram-negative bacteria at a remarkably low concentration of 10 mg/mL, whereas in Gram-positive bacteria the activity was at the concentration of 160 mg/mL. These findings suggest that the activity exhibited by BSFL extracts can be linked to the presence of bioactive compounds, notably AMPs and fatty acids, particularly defensin and lauric acid, respectively.

This study demonstrated that the BSFL extract is effective against *P. aeruginosa* (ATCC 9027), *L. monocytogenes* (ATCC 13932), *S. aureus* (MRSA 639c), and *E. coli* (ATCC 8739) with a concentration-dependent manner. Where the tested Gram-positive bacteria are more sensible to the extract than Gram-negative bacteria. In addition, the study showed that methanol extract surpasses the other extracts in targeting all the tested bacterial strains in this research

both Gram-positive and Gram-negative bacteria, while hexane extract showed an optimal performance against the tested Gram-positive bacteria.

It's worth mentioning that this study makes a notable contribution by unveiling new insights into the antimicrobial activity of black soldier fly larvae extract against *L. monocytogenes*. To the best of our knowledge, this study represents the first report of the antibacterial activity of BSFL larvae extract at an exceptionally low concentration of 10 mg/mL against *L. monocytogenes* and *S. aureus* with an inhibition zone with the dimeter of 14 mm and 10 mm respectively.

Our findings about antibacterial activity of the different extracts obtained from BSFL encourage us to carry out further, more in-depth research in the future in order to explore the extensive capabilities of this insect. This mainly involves:

- Examine the antimicrobial activity of BSFL extract against a broader range of microorganisms, including bacteria, fungi and yeast, to evaluate its efficacy across different types of pathogens.
- Conduct *in vivo* analysis to accurately evaluate the efficiency of BSF larvae under conditions closer to real-life application.
- Evaluate the efficacity of the antimicrobial activity of BSF extract during different stages of its life cycle.
- Identify and characterize the bioactive compounds that compose the BSFL extracts.
- Explore different treatment methods to gain insight into the method that provides the best yield.

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ANNEXES

ANNEXES

Culture media composition

Condalab

Nutrient Agar ISO

Cat. 1060

For the cultivation of non-fastodious microorganisms in water, feces and from clinical samples.

Practical information

Aplications	Categories
Growth	Mesophilic aerobic
Non selective enumeration	Mesophilic aerobic

Industry: Water / Clinical / Food

Regulations: ISO 10273 / ISO 11133 / ISO 19250 / BAM / ISO 6579

CE IVD

Principles and uses

Nutrient Agar is a general purpose medium, not selective but suitable for the cultivation of a wide variety nonfastidious microorganisms. It can be used as a colony count medium in sanitation, medical and industrial bacteriology.

There are many uses for Nutrient Agar in the bacteriological analysis of drinking water, wastewater, milk and other foods. The American Public Health Association (APHA) suggested the formula of Nutrient Agar as a standard culture medium used in water testing.

It is also used in the multiplication of microorganisms to produce vaccines and antigens in general, in the tests of sensitivity and resistance, and as a base to prepare an enriched medium by adding ascitic fluid, etc. It is used to grow microorganisms and for subsequent biochemical tests.

The Gelatin peptone and Beef extract provide nitrogen, vitamins, minerals and amino acids essential for growth. Bacteriological agar is the solidifying agent.

ISO 6579, ISO 19250 and ISO 10273 recommend this medium to obtain presuntive Salmonella and Yersinia isolated colonies respectively. Good growth will appear as translucent colonies.

Formula in g/L

Bacteriological agar	15	Peptone	5
Meat extract	3		

Preparation

Suspend 23 grams of the medium in one liter of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. Sterilize in autoclave at 121 °C for 15 minutes. Cool to 45 ºC and dispense into appropriate containers.

Instructions for use

» For clinical diagnosis, the type of sample is any clinical sample, especially feces.

- Inoculate on the surface with a handle or swab (the plates).
- Incubate plates and tubes with a tight cap at 35±2 °C for 18-24 hours.
- Reading and interpretation of the results.

» For other uses not covered by the CE marking:

Detection of Salmonella spp. and Yersinia enterocolitica according to ISO 6579, ISO 19250 and ISO 10273:
- Select one typical or suspect colony from each selective medium, if it one turns out to be negative select at least other four.

- Streak the selected colonies onto the surface of the Nutrient Agar.
- In the case of epidemiological studies, it is recommended to identify at least five colonies.
- Should there be less than five typical or suspicious colonies on a plate, all the typical or suspicious colonies will be used for confirmation.
- Incubate at 36±2 ºC for 24±3 hours.
- For isolating of Yersinia enterocolitica incubate at 30 ºC for 18-24 hours.

Quality control

Solubility	Appareance	Color of the dehydrated medium	Color of the prepared medium	Final pH (25ºC)
w/o rests	Fine powder	Beige	Amber slightly opalescent	6,8 ± 0,2

Microbiological test

According to ISO 11133:
Incubation contitions: Productivity qualitative: E.coli (37±1 ºC / 24±2 h), Salmonella typhimurium (34-38 ºC / 24±3 h), Yersinia enterocolitica (30±1 ºC / 24±2 h).
Inoculation conditions: (10^3-10^4 CFU).

Microorganisms	Specification
Salmonella typhimurium ATCC 14028	Good growth (2)
Escherichia coli ATCC 25922	Good growth (2)
Escherichia coli ATCC 8739	Good growth (2)
Yersinia enterocolitica CECT 9144	Good growth (2)

Storage

Temp. Min.:2 ºC
Temp. Max.:25 ºC

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Norma UNE-EN-ISO 6579. Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of Salmonella spp.
ISO 10273 Microbiology of Food and animal feeding stuffs- Horizontal method for the detection of presumptive pathogenic Yersinia enterocolitica.
ISO 19250 water quality-detection of Salmonella spp.

Condalab

Mueller Hinton Agar

Cat. 1058

For sensitivity tests on antibiotics and sulfamides, and for the primary isolation of Neisseria and other pathogens from clinical samples

Practical information

Aplications	Categories
Antimicrobial susceptibility tests	General use

Industry: Clinical

CE IVD

Principles and uses

Mueller Hinton Agar, together with the Mueller Hinton Broth (Cat. 1214), is used to test the antimicrobial susceptibility of rapidly growing aerobic organisms from clinical samples and has become the standard medium for the Bauer Kirby method in accordance to the standards of the Clinical and Laboratory Standards Institute (CLSI) and European Committee and Antimicrobial Susceptibility Testing (EUCAST).

The main objective of in vitro antimicrobial susceptibility testing is to provide a guide for the therapeutic management of infectious diseases through the sensitivity or resistance of facultative aerobic and anaerobic pathogenic bacteria to different antimicrobial compounds.

Because it is impossible to predict the susceptibility of a bacterium responsible for a specific infection to antimicrobials, the antibiotic susceptibility tests carried out in the microbiological laboratory become an essential instrument for the therapeutic management of patients.

In the medium, beef infusion and acid casein peptone (H) provide nitrogen, vitamins, minerals and amino acids essential for growth. The starch absorbs any toxic metabolite produced by microbial growth and the bacteriological agar is the solidifying agent.

Formula in g/L

Acid casein peptone (H)	17,5	Bacteriological agar	17
Beef infusion	2	Starch	1,5

Preparation

Suspend 38 grams of the medium in one liter of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. Dispense into appropriate containers and sterilize in autoclave at 121 ºC for 15 minutes. Cool to 45 or 50 ºC and add defibrinated blood if desired. The blood mixture should be chocolated by heating to 80 ºC for 10 minutes if Neisseria development is desired. DO NOT OVERHEAT. To remelt the cold medium, heat as briefly as possible.

Instructions for use

For clinical diagnosis, the type of sample is bacteria isolated from urine:
- Inoculate according to the Bauer-Kirby method.
- Incubate in aerobic conditions at 35±2 ºC for 24 -48 hours.
- Reading and interpretation of the results.

For sensitivity tests on antibiotics according to EUCAST:
- Dispense medium into sterile Petri dishes to give a level depth of 4±0,5 mm (approximately 25 mL in a 90 mm circular plate, 31 mL in a 100 mm circular plate, 71 mL in a 150 mm circular plate, 40 mL in a 100 mm square plate).
- Adjust the density of the organism suspension to McFarland 0,5 by adding saline or more bacteria. A denser inoculum will result in reduced zones of

inhibition and a decreased inoculum will have the opposite effect.
- The suspension should optimally be used within 15 min and always within 60 min of preparation.
- Dip a sterile cotton swab into the suspension.
- To avoid over-inoculation of gram negative bacteria, remove excess fluid by pressing and turning the swab against the inside of the tube.
- For gram positive bacteria, do not press or turn the swab against the inside of the tube.
- Apply disks within 15 min of inoculation.
- Incubate at a temperature of 35±2 ºC for 24 hours.
- Zone edges should be read at the point of complete inhibition as judged by the naked eye with the plate held about 30 cm from the eye.
- Read MH plates from the back against a dark background illuminated with reflected light.
- In case of distinct colonies within zones, check for purity and repeat the test if necessary.
- For Proteus spp., ignore swarming and read inhibition of growth.
- In case of double zones, read the inner zone.

For cultivating of Neisseria specimens:
- Incubate in plates at a temperature of 35±2 ºC in a CO_2 atmosphere for 18-24 hours.

Quality control

Solubility	Appareance	Color of the dehydrated medium	Color of the prepared medium	Final pH (25ºC)
Slightly opalescent	Fine powder	Cream	w/o blood: Amber opalescent. with blood: Red	7,4±0,2

Microbiological test

Disk diffusion sensitivity testing. Incubation conditions: (35±2 ºC / 24 h).

Diammeter halo in mm.

Microorganisms	Gentamycin 10 µg	Ampicillin 10 µg	Tetracycline 30 µg	Polymyxin B 300 µg	SXT: Trimethoprim (1,25µg)+Sulfamethoxazole (23,75 µg)
Escherichia coli ATCC 25922 CLSI	19-26	15-22	18-25	13-19	23-29
Escherichia coli ATCC 25922 EUCAST	19-26	15-22		13-19	23-29
Staphylococcus aureus ATCC 25923 CLSI	19-27	27-35	24-30		24-32
Staphylococcus aureus ATCC 25923 EUCAST					
Pseudomonas aeruginosa ATCC 27853 CLSI	17-23			14-18	
Pseudomonas aeruginosa ATCC 27853 EUCAST	17-23				
Enterococcus faecalis ATCC 29212 CLSI					
Enterococcus faecalis ATCC 29212 EUCAST					26-34
Staphylococcus aureus ATCC 29213 CLSI					
Staphylococcus aureus ATCC 29213 EUCAST	19-25		23-31		26-32

Storage

Temp. Min.:2 ºC
Temp. Max.:25 ºC

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