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Separation of bioactive compounds from Haemolymph of scarab beetle *Scarabaeus sacer* (Coleoptera: Scarabaeidae) by GC-MS and determination of its antimicrobial activity

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Abstract

This study aimed to investigate the bioactive compounds in the haemolymph of scarab beetle *Scarabaeus sacer* by using the Gas chromatography–mass spectrometry (GS-MS) analysis. The identification of the bioactive compounds is based on peak area, retention time, molecular formula and molecular weight. There are 129 compounds are detected in the haemolymph of scarab beetle and 43 of them were reported to have a bioactivity. The most analyzed bioactive compounds are alcohols, steroids, fatty acids and terpenoids. The current study also test the antimicrobial activity of scarab beetle haemolymph against gram-negative bacteria (*Escherichia coli, Enterobacter cloacae*, gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis*), and fungi (*Aspergillus fumigatus* and *Candida albicans*). The haemolymph has highest antibacterial activity against gram negative bacteria *Enterobacter cloacae, Escherichia coli* respectively and against gram-positive bacteria *Bacillus subtilis, Staphylococcus aureus* respectively. No antifungal activity has been detected.

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Keyword
Haemolymph, GS-MS, gram negative bacteria, gram positive bacteria, antimicrobial activity.

Introduction

Coleoptera is one of the largest order of insects with about 370,000 insect species described worldwide. The family Scarabaeidae encompasses over 30.000 species of beetles worldwide; they are often called scarabs. Dung beetles are a major insect group (Coleoptera: Scarabaeidae) distributed globally except Antarctica with a high number of diversity comprising approximately 6,200 species and nearly 267 genera (Tarasov and Génier, 2015). These species are coprophagous in nature which live freely in soil and mostly feed on both wet and dry dung materials of herbivorous mammals. The undigested excreta of mammals are utilized as food and nesting material throughout their life cycle, hence, they possess many ecologically beneficial functions. The dung beetles play a vital role in nutrient recycling by decaying organic matter and developing soil aeration (Manning et al., 2016) thereby, reducing the greenhouse gas fluxes (Slade et al., 2016). It also improves plant growth and grain production (Koyama et al., 2003). *Scarabeus sacer* is considered a species

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of genus *Scarabaeus*, occurs in coastal dunes and marshes around the Mediterranean Basin. It can be found across north Africa, Southern Europe and parts of Asia (Afghanistan, Corsica, Cyprus, France, Iran, Israel, Italy, Morocco, Sardinia, Sicily, Sudan and Syria). *Scarabaeus sacer* is a species of dung beetle belonging to the family Scarabaeidae (Long, 1836)

Insects are known for their ability to resist infection. They protect themselves against bacterial infection by secreting a battery of antimicrobial peptides (AMP) into the hemolymph. Hemolymph, also known as the insect blood, is a clear fluid, with or without yellow or greenish pigmentation. It constitutes 16-40% of the body weight of certain insects. The volume and component of hemolymph are vary in different types of insects and their developmental stages. It spends much of its time flowing freely within body cavities where it makes direct contact with all internal tissues and organs. Therefore the circulation would help to transport the AMP to its target site (Kurata, 2006). In insects, AMPs / polypeptides are manufactured mainly in a fat body (similar to mammalian liver) and are released into hemolymph where they play a vital role in innate immune systems and host defense mechanisms, and having a broad spectrum of activity against both gram + ve and gram -ve bacteria and against fungi (Januszanis et al., 2013)

However, misuse of antibiotics intake has caused many problems, such as the appearance of antibiotic-resistant bacteria, weakening of disease resistance in livestock, and ecosystem pollution (Looft et al., 2012). Insects exhibit innate immune systems that produce potent AMPs to protect them from pathogen invasion, and these AMPs are viewed as strong natural antibiotic applicants (Kalsy et al., 2020). The insect innate immune system is categorized into cellular and humoral immunity. Cellular immunity involves the phagocytosis of bacteria, fungi, and protozoa, and nodule formation and encapsulation, while humoral immunity involves the secretions of proteins and peptides produced in fat and blood cells to hemolymph in response to infection (Wu et al., 2018).

AMPs secreted by the humoral immune response are classified according to their structure and amino acid sequence into cecropins, defensins, proline-rich peptides, glycine-rich peptides, and lysozymes and are found in various insect orders including Coleoptera, Diptera, Hymenoptera, and Lepidoptera (Michael Zasloff, 2002). Melittin is familiar AMP contained in bee venom and its antimicrobial activity was observed greatly in methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-positive and Gram-negative bacteria (Pashaei et al., 2019). AMPs are small molecules that vary in size, ranging from 10 to 100 amino acid residues and are produced by all living organisms. The rich diversity of insects makes them rich sources of AMPs. The black soldier fly *Hermetia illucens* L. (Diptera: Stratiomyidae), particularly, able to live in hostile environments rich in microbial colonies, making it one of the most promising sources of AMPs (Moretta et al., 2020).

Nowadays, the using of GC-MS technique is important in analyzing and separation the compounds found in plants extracts (Eva de Rijke et al., 2006)and also the haemolymph of arthropods. This will be a new method for discovering a future drugs to be used in traditional medicine system. In this article, the haemolymph of scarab beetle was analyzed by GC-MS and resulting in many bioactive compounds.

Materials and Methods

Collection of Insects

Scarab beetle was collected from Baltim in the Kafr El Sheikh Governorate, in the north coast of Egypt.

Withdrawing of Hemolymph

Scarab beetle body surface cleaned with 70% alcohol. Then, in order to collect haemolymph, hind pair legs were cut from coxa, and haemolymph fluid was extracted with a capillary tube placed into micro tubes containing EDTA. Haemolymph was centrifuged at 10000 × g for 10 minutes and the supernatant was collected for the antimicrobial testing and stored in 4°C.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

Haemolymph was collected and 30 milligrams were homogenized in 1ml methanol centrifuged at 4500 rpm for 10 minutes, the supernatant was taken to GC-MS. The chemical composition of samples was performed using Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 50 C and then increased by 5°C /min to 230°C for 2 min. increased to the final temperature 290°C by 30°C /min and hold for 2 min. The injector and MS transfer line temperatures were kept at 250, 260°C respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 3 min and diluted samples of 1 µl were injected automatically using Auto-sampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40–1000 in full scan mode. The ion source temperature was set at 200 °C. The components were identified by comparison of their retention times and mass spectra with those of the WILEY 09 and NIST 11 mass spectral databases.

Antimicrobial Activity Assay:

The antimicrobial activity was investigated in the haemolymph against microorganisms. All microbial strains were provided from the culture collection of the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. The antimicrobial profile was tested against two Gram-positive bacterial species (*Bacillus subtilis*, *Staphylococcus aureus*), two Gram-negative bacterial species (*Escherichia coli*, *Enterobacter cloacae*) and two fungi (*Aspergillus fumigatus* and *Candida albicans*) using a modified well diffusion method. Briefly, 100 µl of the test bacteria/or fungi were grown in 10 mL of fresh media until they reached a count of approximately 10^8 cells/ml for bacteria or 10^5 cells/mL for fungi (Ibrahim et al., 2014). One hundred µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained and tested for susceptibility by well diffusion method on Mueller-Hinton and Sabaroud agar (Clinical and Laboratory Standards Institute, 2012.) One hundred µL of each sample (at 10 mg/ml) was added to each well (10 mm diameter holes cut in the agar gel). The plates were incubated for 24-48 h at 37 °C (for bacteria and yeast) and for 48 h at 28 °C (for filamentous fungi). After incubation, the microorganism's growth was observed. The resulting inhibition zone diameters were measured in millimeters and used as a criterion for antimicrobial activity. If an organism is placed on the agar, it will not grow in the area around the well if it is susceptible to the chemical. This area of no growth around the disc is known as a "Zone of inhibition" or "Clear zone". The size of the clear zone is proportional to the inhibitory action of the compound under investigation. Solvent controls (DMSO) were included in every experiment as negative controls. DMSO was used for dissolving the tested compounds and showed no inhibition zones, confirming that it has no influence on growth of the tested

microorganisms. Gentamycin and ketoconazole (Sigma Aldrich, USA) were used as standard antibacterial and antifungal drugs at 30 and 50ug/ml, respectively.

MIC Determination

The tested extract was screened in vitro for their antibacterial and antifungal activities at a different concentration to determine the lowest concentration inhibiting the growth of the organism that recorded as the MIC (Ibrahim et al., 2014).

Results and Discussion

In the present article, the separation of compounds in the haemolymph of scarab beetle by using GC-MS analysis gas separation technique resulting in 43 bioactive compounds as shown in table (1). The identification of the bioactive compounds is based on peak area, retention time, molecular formula and molecular weight.

In this study, the antibacterial effectiveness of the haemolymph against gram -V bacteria (*Escherichia coli*, *Enterobacter cloacae*, against gram +V bacteria (*Staphylococcus aureus*, *Bacillus subtilis*) as well as antifungal activity (*Aspergillus fumigatus*, *Candida albicans*) is investigated. Antibacterial activity against *Bacillus subtilis* was first, followed by *Enterobacter cloacae*, *Escherichia coli* and *Staphylococcus aureus* respectively. No antifungal activity has been investigated yet (Table 2). The minimum inhibitory concentration (MIC) is measured for the tested sample and it was 2500 μg/ml against *E. coli*, 1250 μg/ml against *Enterobacter cloacae*, *Bacillus subtilis* and 10000 μg/ml against *Staphylococcus aureus r*espectively (Table 3).

Table 1. Bioactive compounds in haemolymph of scarab beetle separating by GC-MS

No.	Compound Name	Molecular Formula	Chemical nature	MWT	Area %	RT	Bioactivity
1	Trichloromethane	CHCl3	Trihalomethane	118	3.57	4.06	Anti-virus, anti-cancer, anti-mutagenic, anti-allergic and anti-ulcer **(Ali et al., 2015)**
2	Tert-Hexadecanethiol	C16H34S	Thio-Alcohol	258	0.11	5.16	Enzyme activators **(Rajendran et al., 2017)**
3	Ethanol, 2 octadecyloxy-	C20H42O2	Alcohol	314	0.11	5.16	Antimicrobial activity **(Sudhandra Karthi, 2016)**
4	1-Heptatriacotanol	C37H76O	Alcohol	536	0.48	39.87	Anticancer, antineoplastic and anti-HIV **(Kala and Ammani, 2017)**
5	1,4-Benzenediol, 2-(1,1-dimethylethyl)-5-(2-propenyl)-	C13H18O2	Alcohol	206	2.66	25.14	Anticancer, Antioxidant activity and pesticidies **(Swamy et al., 2017)**
6	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	C19H34O2	Alcohol	294	0.40	39.20	Antimicrobial activity **(Hadi et al., 2016)**
7	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C20H40O	Terpene alcohol	296	6.43	30.04	Antimicrobial **(Ponmathi Sujatha et al., 2017)** Precursor of synthetic forms of vitamin E and vitamin K1 **(Devakumar et al., 2017)**

8	Dotriacontane	C32H66	Saturated hydrocarbons	450	0.11	5.16	Antimicrobial, antifungal, anti-inflammatory, cytotoxic activity **(Harris, 1992)**
9	Isochiapin B	C19H22O6	Terpenoids	346	0.32	10.83	Anti-insect Antitumor agent **(Elsharkawy, 2016)**
10	14-á-H-Pregna	C21H36	Steroids	288	0.32	10.83	defense chemical and Diabetic retinopathy prevention (**Durak and Kalender, 2007**)
11	Cycloheptasiloxane, tetradecamethyl-	C14H42O7Si7	Organo-Silicone compound	518	1.69	15.25	Antimicrobial, Antiseptic, Hair Conditioning Agent, Skin- Conditioning Agent-Emollient and Solvent (**Mary and Giri, 2018**)
12	4H-1-Benzopyran-4 one, 2-(3,4-dimethoxyphenyl)-3, 5-dihydroxy-7-methoxy-	C18H16O7	Ketonic comound	344	0.38	17.48	Antioxidant, antimicrobial, cancer enzyme inhibitors in pharmaceutical, cosmetics, and food industries (**Albergoni et al., 1980**)
13	Cyclooctasiloxane, hexadecamethyl-	C16H48O8Si8	Alkanes	592	2.84	19.68	Antimicrobial (**Al Bratty et al., 2020**)
14	Cyclododecasiloxane, tetracosamethyl	C24H72O12Si12	Alkanes	888	2.84	19.68	Hepatoprotective, antispasmotic, antirheumatic (**Al Bratty et al., 2020**)
15	9,12-Octadecadienoic acid (Z,Z)-, 2,3- bis [(trimethysilyl)oxy]propyl ester	C27H54O4Si2	Linoleic acid ester	498	0.74	21.58	Anti-inflamation (**Rani and Kapoor, 2019**)
16	4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3, 5-dihydroxy-7-meyhoxy-	C18H16O7	Phenolic compound	344	0.28	25.36	Antioxidant, antimicrobial, cancer enzyme inhibitors in pharmaceutical, cosmetics, and food industries (**Huang and Irwin, 2006**)
17	9,12,15-Octadecatrienoic acid ,2,3-bis[(Trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-	C27H52O4Si2	Alpha linoleic acid	496	0.28	25.36	anti-oxidant, anti-diabetic, and anti-inflammatory (**Rajendran et al., 2017**)
18	Eicosamethyl,cyclodecasiloxane	C20H60O10Si10	Organohetero-silane	740	2.59	27.05	Prevent degenerative diseases (**Budayatin et al., 2021**)
19	Silcone oil	N/A	polysiloxane compounds	0	2.59	27.05	Toxic for bed-bug(insecticide) (**Zha et al., 2018**)
20	1H-Purin-6-amine,[(2-flourophenyl)methyl]	C12H10FN5	Amino compounds	243	2.59	27.05	Anti-oxidant (**Budayatin et al., 2021**)
21	Neophytadiene	C20H38	Alkenes	278	6.43	30.04	analgesic, antipyretic, anti-inflammatory,

							antimicrobial, and antioxidant compound (**Venkata raman et al., 2012**)
22	2,6,10-Trimethyl,14-ethylene-14-pentadecene	C20H38	Alkenes	278	6.43	30.04	Antiproliferative activity (**Devakumar et al., 2017**)
23	Phytol Isomer	C20H40O	Diterpene	296	0.29	30.93	antimicrobial, antioxidant, and anticancer activities (**Rani and Kapoor, 2019**)
24	7-Methyl-Z-tetradecen-1-ol acetate	C17H32O2	Acetate ester	268	0.64	31.36	Anticancer, anti-inflammatory, hepatoprotective (**Hameed et al., 2015**)
25	2-Dodecen-1-yl(-)succinic anhydride	C16H26O3	SuccinicAcid anhydride	266	0.13	33.83	Antineoplastic agents, Antioxidants, Antimicrobial (**Jatin and Sonawani, 2016**)
26	Hexadecanoic acid, methyl ester	C17H34O	Palmitic acid ester	270	2.09	34.34	Anti-oxidant, decrease blood cholesterol, anti-inflammatory (**Hema et al., 2015**)
27	Pentadecanoic acid, 14-methyl-, methyl ester	C17H34O	Ester	270	2.09	34.34	Antimicrobial, antifungal (**Beschi et al., 2021**)
28	Dasycarpidan-1-methanol, (Ester)	C20H26N2O2	Ester	326	0.74	21.58	Antimicrobial (**Rani and Kapoor, 2019**)
29	Dibutyl phthalate	C16H22O4	Benzoic acid ester	278	0.98	38.03	Use in cosmotics (**Mary Ann Liebert, 1985**), ectoparasiticide (**Prabhu et al., 2018**)
30	Phthalic acid, butyl undecyl ester	C23H36O4	Ester	376	0.98	38.03	Antimicrobial Antibacterial & Anti-inflammatory (**Al-Gara'Awi et al., 2019**)
31	Phthalic acid, butyl tetradecyl ester	C26H42O4	Ester	418	0.98	38.03	Antimicrobial activity (**Bekele et al., 2016**)
32	11,14-Eicosadienoic acid, methyl ester	C21H38O2	Ester	322	0.40	39.20	Anti-inflammatory, anti-oxidant, anti-arthritic, anti-coronary. (**Chinnasamy P.S et al., 2018**)
33	1,2-Benzenedicarboxylic acid, butyl decyl ester	C22H34O4	Ester	362	5.04	39.35	Antimicrobial activity (**Shoge et al., 2016**)
34	Diisooctyl phthalate	C24H38O4	Phthalic acid derivative (Ester)	390	1.80	50.09	Antimicrobial activity (**Ali Shafaghat, 2012**)
35	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	C21H36O4	Linoleic acid ester	352	0.87	52.61	Analgesic, Antipyretic, Anticonvulsant, Antiseptic (**Srivastava et al., 2015**)
36	9,12,15-Octadecatrienoic acid, 2-phenyl-1,3-dioxan-5-yl ester	C28H40O4	Phenolic ester	440	0.46	58.55	Antimicrobial & anti-inflammatory (**Kadhim et al., 2017**)
37	Cholest-5-en-3-ol, 24-propylidene-, (3á)-	C30H50O	Fatty acid	426	6.09	63.51	Antibacterial activity (**Hussein et al., 2017**)
38	Cis-13-Eicosenoic acid	C20H38O2	Fatty acid	310	0.08	33.78	Anti-inflammatory activity (**Sosa et al., 2016**)

39	Ppropiolic acid, 3-(1-hydroxy-2-isopropyl-5 methylcyclohexyl)-	C13H20O3	Fatty acid	224	1.72	37.54	Anti-angiogenic activity against solid tumor growth **(Hussein et al., 2016)**
40	2-Nonadecanone 2,4-dinitrophenylhydrazine	C25H42N4O4	Nitrogen compound	462	0.36	50.66	Antimicrobial activity **(Muthulakshmi et al., 2012)**
41	Ethyl iso-allocholate	C26H44O5	Steroid derivative	436	0.48	39.87	Antimicrobial,Antioxidant, anti-inflammatory & anti-arthritic antiasthmatic **(Sheela and Uthayakumari, 2013)**
42	1,2-15,16-Diepoxyhexadecane	C16H30O2	Epoxide	254	0.40	39.20	Antitumor, anti-inflammatory (**Hameed et al., 2016**)
43	Milbemycin B, 6,28-anhydro-15-chloro-25-isopropy l-13-dehydro-5-O-demethyl-4-methyl	C33H47ClO7	------	590	0.33	58.77	Anti MRSA **(Vilas and Amit, 2015)**

The GC-Ms analysis on the haemolymph of *Scarabeus sacer* revealed presence of some bioactive compounds such as alcohols, terpenoids, ketones, phenolic compounds, alkanes, alkenes, amino-compounds, Fatty acids and steroids. Alcohols were discovered to have antimicrobial activity (Gołębiowski et al., 2012). Isochiapin B, 3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol and Phytol Isomer are terpenoids present in haemolymph of *S. sacer*. Terpenoid compounds (Phorbol, Isochiapin B, stigmasterol acetate, and b-sitosterol) were detected in essential oil of *Achillea fragmmentissma* that well known for their biological activities as anti-insect and anti-tumor agents (Elsharkawy, 2016). Terpenes are bioactive compounds detected in *Ulva fasciata*, *U. lactuca* and *Corallina mediterranea* seaweeds extract and steroids were detected in the extracts of *U. fasciata*, and *Amphiroa anceps* seaweeds (Mofeed et al., 2021). Most of these compounds exhibit biological activities such as anticancer, antiviral, antioxidant, and anti-inflammatories (Jiang et al., 2017). Phytol isomer is a diterpenes identified in the haemolymph of adult *S. sacer*. phytol is a bioactive compound that has a potent anticancer activity (Sheeja et al., 2016). It also serve as a chemical attractant for parasitoids, according to research on these species*: Lucilia sericata* (Gobiowski et al., 2012c), *Leptinotarsa decemlineata* (Nelson et al., 2003). Alkanes can help distinguish organisms by acting as a chemical signal (Lockey 1988). Alkanes were also marked in the surface lipids of *Liposcelis bostrychophila*, *Cryptolestes ferrugineus* (Howard and Lord 2003) and Laelius utilis (Howard, 1992). Dotriacontane is saturated hydrocarbons present in haemolymph of scarab beetle and reported to has antimicrobial, antifungal, anti-inflamatory and cytotoxic activity (Harris, 1992). Also the hydrocarbons used to distinguish between the male and female of *Sarcophaga* species (Moore et al., 2021). Alkanes were also marked in the surface lipids of *Liposcelis bostrychophila*, *Cryptolestes ferrugineus* (Howard and Lord, 2003) and *Laelius utilis* (Howard R. w, 1992). Larvae of potato beetle contain hydrocarbons of high molecular weight, particularly tetrapentacontane (C54H 110), pentapentacontane (C55H 112) and heptapentacontane (C57H 116) (Ardenne et al., 1965).

Pentadecanoic acid, 9,12,15-octadecatrienoic acid, hexadecanoic acid methyl ester were detected in the haemolymph of adult *S. sacer*. Pentadecanoic acid and 9,12,15-octadecatrienoic acid reported to have anti-inflammatory, antimicrobial, antioxidants, and antiproliferative activity (Rani and Kapoor, 2019). Hexadecanoic acid methyl ester is also known as palmitic acid ester and efficiently used as an antioxidant, pesticide, anti-

androgenic, nematicide, flavoring agent, hypocholesterolemic, and lubricant (Karthikeyan and Sudan, 2017). Also hexadeconic acid was the major fatty acid in *Sargassum granuliferum* seaweed which prevents the biofilm forming bacteria (Bakar et al., 2017).

Esters with even longer C22, C24, C42 and C46 carbon chains were determined in *Aleurotithius timberlakei* (Nelson et al., 1997). In the haemolymph of scarab beetle, fatty acids such as – Cis-13-eicosenoic acid, Cholest-5-en-3-ol, 24-propylidene- (3á), and Ppropiolic acid, 3-(1-hydroxy-2-isopropyl-5 methylcyclohexyl) were detected. In case of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), the presence of C6, C9, C10, C12, C14, C16 and C18 fatty acids were found. Fatty acids C18 and C20 are found also in *Bombyx mori* and *Blatella germanica* (Barlow J. S., 1964). Fatty acids C18:1, C18:2 and C18:3 were estimated in the larvae of *Drosophila melanogaster*, *Musca domestica* and *Galleria mellonella* (Barlow J. S., 1964). Fatty acids C16 – C18 have also been determined in the surface lipids of *Cryptolestes ferrugineus* and *Liposcelis bostrychophila* insects (Howard and Lord, 2003). Cholest-5-en-3-ol, 24- propylidene have been detected in the methyl extract of *Sargassum crassifolium* (Albratty et al., 2021) and (Erwan Plouguerné et al., 2006) seperated Cholest-5-en-3-ol, 24- propylidene from the red alga *Grateloupia turuturu*.

In the adult scarab beetle haemolymph, there is a ketonic compounds such as 4H-1-Benzopyran-4 one, 2-(3, 4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy that act as Antioxidant, antimicrobial, cancer enzyme inhibitors in pharmaceutical, cosmetics, and food industries (Albergoni et al., 1980). Also ketones are separated from *Tessaratoma papillosa* (Zhang et al., 2009). The relationships of these analyzed compounds in insects play vital roles as they can be transmitters of information and signals (Taylor et al., 2012)and also serve as pheromones (Noguez et al., 2013). Ethyl iso-allocholate is a steroid derivative compounds detected in the haemolymph of adult scarab beetle and in the black fruit of *Pistacia lentiscus*, this steroid compound has antimicrobial, anti-inflammatory, anticancer, antiasthma and diuretic activities (Daffodil D Almeida et al., 2012)

Silcone oil, milbemycin B, 6, 28-anhydro-15-chloro-25-isopropyl-13-dehydro-5-O-demethyl-4-met-, and Dibutyl phthalate were detected in the haemolymph of adult scarab beetle. Zha et al., 2018, reported that silicone oil in the bed bug is cytotoxic and has an insecticidal activity that can kill insects by physical mean that affecting on tracheal system causing asphyxiation of insects. Anti MRSA activity was reported for milbemycin B, 6, 28-anhydro-15-chloro-25-isopropyl-13-dehydro-5-O-demethyl-4-met- (Vilas and Amit , 2015) and Dibutyl phthalate act as ectoparasiticide (Prabhu et al., 2018).

Table 2. Antimicrobial activity (as a mean zone of inhibition) of the haemolymph of adult scarab beetle

Sample / Tested microorganisms	Heamolyph	Control
FUNGI		**Ketoconazole**
Aspergillus fumigatus	NA	17
Candida albicans	NA	20
Gram Positive Bacteria:		***Gentamycin***
Staphylococcus aureus	8	24

Bacillus subtilis RCMB 015 (1)	15	26
Gram Negatvie Bacteria:		***Gentamycin***
Escherichia coli	10	30
Enterobacter cloacae	14	27

*NA: No activity.

Table 3. The antimicrobial activity as Minimum Inhibitory concentration (MIC) in μg/ml of the tested microorganisms. The test was done using the diffusion agar technique

Sample / Tested microorganisms	Heamolyph	Control
Gram Positive Bacteria:		***Gentamycin***
Staphylococcus aureus	10000	3.9
Bacillus subtilis	1250	1.95
Gram Negatvie Bacteria:		***Gentamycin***
Escherichia coli	2500	1.95
Enterobacter cloacae	1250	3.9

*NA: No activity.

The present works approved that the haemolymph of *S. sacer* possesses antibacterial activity against gram –negative bacteria (*Escherichia coli*, *Enterobacter cloacae*) and against gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*). No antifungal activity had been investigated against *Aspergillus fumigatus* and *Candida albicans*. There are many works hassling to our work; the methanol extract of oriental hornet *Vespa orientalis* and *Zophobas mori* (Coleoptera:Tenebrionidae) larva show antibacterial activity against *E. coli* and no antifungal activity (HASSAN et al., 2015). Contrary to our results, the whole body extract of housefly maggots show no activity against *E. coli* and exhibit antifungal activity (Meylaers et al., 2004). While (Hou et al., 2007) documented that the extract of the housefly maggots have higher activity against Gram- positive bacteria than Gram negative bacteria and had not antifungal activity yet.

Most of insect extracts show antibacterial activity against Gram-positive and Gram-negative bacteria, the silk worm *Bombyx mori* (Seiichi Hara and Yamakawa, 1995), the European bumble bee, *Bombus pascuorum* (Rees JA et al., 1997) and *Tenebrio molitor* larvae (Lee et al., 1998). On the other hand, some other insects revealed activity only against Gram-positive bacteria as *Aedes aegypti* (Lowenberger et al., 1995), *Chironomus plumosus* (Lauth et al., 1998) and *Anopheles gambiae* (Vizioli et al., 2001).

Synthetic antibiotics and antimicrobials have contributed to public health and stimulated the growth of livestock. Conversely, overuse and abuse of antibiotics and antimicrobial drugs may causes drug-resistant bacteria, which threaten public and livestock health. Several studies reported that insects manufacture antimicrobial peptides (AMPs)

which act as a natural antibiotic (Vetterli et al., 2018). Insects not only perform different roles in the environment, but also host a variety of community of microorganisms. The complicated cellular and humoral mechanisms include the innate immune system of an insect (Kanost et al., 2004). The cellular mechanism is rely on phagocytosis process which is activated by enzymes and invading microorganisms then encapsulated by the hemolymph. Moreover, The humoral response is represented in the production of broad-spectrum antimicrobial peptides (AMPs), reactive oxygen or nitrogen intermediates, and complex enzymatic cascades that help to regulate hemolymph coagulation or melanization (Ahmed MH Ali et al., 2020). The presence of microorganisms invading insects causes the fat body to rapidly synthesize AMPs, which are then released into the hemolymph (Hoffmann and Reichhart, 2002). Previous research shows that each insect species produces a distinct antimicrobial peptide that acts against specific microorganisms (Yi et al., 2014). On the other hand, in order to enhance the insect's defense system against other pathogens, some of the peptides are expressed simultaneously, encouraging synergism (Rahnamaeian et al., 2015). As such, AMPs have a specific modes of action, such as altering the electrochemical gradient at the membrane, producing reactive oxygen/nitrogen species (ROS/RNS) that cause cell death, inhibiting protein synthesis, and permeabilizing the cell membrane (Thevissen et al., 2004). AMPs have pharmacological properties such as low molecular weight, high water solubility, broad-spectrum antimicrobial activity, and low levels of cytotoxicity (Lei et al., 2019). (Turillazzi et al., 2004) reported that the antibacterial activity in salivary secretions of *Polistes dominulus* larvae inhibits growth of Gram positive *Bacillus subtilis* and Gram negative *E. coli*. There are number of studies that have tested ability of the insect extracts against pathogenic bacteria, especially antimicrobial peptides extracted from various insects maggots (Guo et al., 2007), dung beetles (Mohtar et al., 2014), Red Palm Weevil (Chernysh et al., 2015), pupae of the giant silk moths (Sewify et al., 2017).

Contrary to our study on the haemolymph of adult *S. sacer*, the non-induced hemolymph of dung beetle, *Onthophagus taurus* did not show inhibitory activity against any of the bacterial strains and fungus. It does not mean that peptides are absent but it may be present in smaller quantity so that no visible action in in-vitro studies is detected (Patil and Kumar, 2013). But the immune induced hemolymph exhibits activity against all tested bacteria and no activity against fungus. Therefore, the peptide is active against prokaryotes and doesn't affect the fungus which is a eukaryote. Many studies on insect species assert that bacteria injected into the haemocoel stimulate the synthesis of number of peptides and proteins which are active singly or in concert against the invaders and are secreted into the hemolymph (Gillespie JP et al., 1997).

Conclusions

On conclusion, antimicrobial activity of haemolymph of adult *S. sacer* may be due to presence of the previous bioactive compounds which separated by the GC-MS technique. Future studies are necessary to purify the compounds with antimicrobial activity and investigate their antitumor effect against different cell lines.

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