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In vitro screening the antibacterial activity of four whole body eusocial insects extracted by polar solvents

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Abstract
Nowadays, insect body derivative products were used as one of alternatives to solve the bacterial resistance to current antibiotics. Bacteria growth inhibition with dry body extracts of the following social hymenopteran insects: honey bee *Apis mellifera* and three ant species; *Monomorium pharoanis*, *camponotus xerxes* and *Crematogastor auberti* against the bacteria: Gram - positive *Staphylococcus aureus* and Gram - negatives; *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The antibiotics Ceftriaxone (CN), Gentamycin (CRO) and Tetracycline (TE) as standard drugs and negative control by DMSO treatment. Antibacterial activity was measured by disc diffusion test. *A. mellifera* body extract with the solvents (methanol, hexane) was found less effective than dichloromethane solvent. Also, bacteria susceptibility were varied between Gram stains bacteria and their species. At 5.0 mg/ml concentration of MeOH, extract of *M. pharoanis* and *A. mellifera* were inhibited activity of *staph. aureus* and *K. pneumoniae* 30.33 and 13.67 mm respectively. *K. pneumoniae* was more sensitive to *M. pharoanis* and *C. xerxes* extracts with 20.0 and 15.67 mm. *Pseudo. aeruginosa* was found resistant to all the standard drugs and *K. pnoumoniae* no affected by CRO antibiotic. Methanol body extract of the ants; *M. pharoanis*, *C. xerxes* and *C. auberti* and dichloromethane *A. mellifera* extracts are promise alternatives in refination and identification of newly antibacterial compounds.

Keywords: insect body extract, antibacterial, *Apis mellifera*, *Monomorium pharoanis*, *Camponotus xerxes*, *Crematogastor auberti*

Introduction

Insect body and its components have been used for treatment may diseases in traditional medicine [1]. Nowadays, it was found that insects have active immunity defense system represented by antimicrobial ingredients which targeted wide spectrum of pathogens [2] While be historically, antibiotics have been categorized in two main groups on bases of Gram - positive and Gram - negative bacteria species [3]. Antibacterial resistance threatens the effective treatment of increasing bacterial infections on the world level [4]. The crude extract of eastern subterranean termite, *Reticuliterma flavepes* inhibits a broad spectrum of multidrug resistant (MDR) and MDR human pathogens [5]. The excretion and secretion of the fresh maggots has antibacterial and antiviral activity [6]. Cockroach, *Periplaneta americana* habitat in damp places with human association as household pest, its gut and exoskeleton extracts had selected as antibacterial which inhibited *Escherichia coli* and *Staphylococcus aureus* without effect on *Pseudomonas aeruginosa* [7].

Many insects can induce antimicrobial peptides which synthesize in hemolymph by reflex to pathogens infection as defensive innate immunity, with more than 150 antimicrobial peptides were identified and purified from different insect taxa [8]. American cockroach hemolymph has antimicrobial effect on susceptible and resistant strains of nosocomial bacteria; *E. coli* and *S. aureus* [9]. By whole body extraction method by polar solvents [10], were screened antimicrobial peptides of three ant species in genus *Monomorium* and tested their antibacterial activity as alternative antibiotic source. Ant metapleural glands have significant immune defense peptide profile [11]. Ants and honey bees are eusocial hymenopteran insects, they live in dense population with a high disease transmission probability, therefore, they must develop defensive abilities against infectious pathogens by means antimicrobial secretions [12]. Sting secretion of the valentine ant, *Crematogastor scutellaris* (Oliver) have a strong antimicrobial activity against pathogenic bacteria and fungi [13]. Other social insect, *Apis mellifera* was under pathogenic stress, so, it developed individual innate immunity system [14]. Two antimicrobial peptides was synthesized by honey bees; definsin I produced by salivary glands and represented the social immunity, hemolymph and fat bodies synthesized definsin II whom expression was individual immunity [15].

In view the previous studies on bacteria growth inhibition by insect extracts, the present study aims to inspect for antibacterial activity in eusocial insect bodies with chosen solvents.

Materials and Methods

Materials

Insects

The experimental insects were collected from Al-kaim town, Al Anbar Province, Iraq (34 ° 23′28 N 40 ° 59′16 ″ E) Honey bees, *Apis mellifera* were collected around the honey bee hives by handle net. Worker ants; *Monomorium pharoanis*, *Camponotus xerxes* and *Crematogastor auberti* by hand were reared at early march near the colonies within the swarming season.

Bacteria for Bioassay

The pathogenic human bacteria: one Gram– positive bacterium *Staphylococcus aureus* and four Gram- negatives; *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were brought from Microbiology laboratory / Research Unit / Biology Department / College of Education for pure sciences / Mosul University – Iraq (36 ° 22′35 N 43 ° 08′32 ″ E).

Solvents

The polar solvents with gradual polarity Index; Methanol (5.1), dichloromethane (3.1), ethanol (0.654) and hexane (0.1) were used in extract preparation.

Culture Media

Muller – Hinton agar media were purchased from NEOGEN Culture Media (foodstafety.neogen.com**)**

Methods

Preparation of insect body extract

After collecting the insect species specimens, they were dried in oven at 35C° for 24 hrs. Then, the whole body insects were grounded up to be powdered, the insect materials were macerated in the selected solvents for three days and twice filtrated by Wattmann filter papers no.1 under low pressure. The filtrations were left over night for solvents evaporation. The crude extract were kept at 4C° till beginning of the experiments. Stock solution and desired concentration were prepared with DMSO solvent.

Bacteria recovery

The bacterial species were inoculated on the plates and cultured in the incubator at 37 C° for 24 hrs, while stock bacteria plates were kept at 4 C°.

Antibacterial susceptibility test

In vitro, antibacterial activity of the insect body extracts and positive control (antibiotics) were estimated by growth inhibition zones after Kirb – Bauer disc diffusion susceptibility test protocol [16]. Paper discs (5mm) were punched out of whatmann filter papers, then sterilized in oven at 160 C° for one hr. and left to cool, the discs were dipped in the applied concentration for half hr. and air dried. Control and extract treated discs were fixed on agar media surface. Petri dish plates were incubated for 24 hrs. at 37 C°, the diameters of the inhibited zones excluding the disc diameter were calculated by caliper [17]. The experiments were conducted in three replications, the data were analyzed by ANOVA, Duncan Multiple Range Test at P 0.05 [18]. Means of the Inhibition zones were ranked according to the extract antibacterial activity based on [19] method; ≥ 8 mm good, 6 – 7 mm moderate, 4 – 5 mm weak and 2 – 3 mm very weak.

Results

Table 1 shows the inhibition zones of one Gram – positive and four Gram – negative bacteria, as response to antimicrobial activity of 5.0 mg/ml *Apis mellifera* extract. The extract was prepared by the solvents; Dichloromethane, methanol and hexane. The extracts with those solvents were appeared antibacterial activity against Gram positive *Staphylococcus aureus* (15.0, 13.67 and 13.33 mm) in comparison to Gram – negative bacteria especially *Pseudomonas aeruginosa* (4.0, 4.67 and 7.33 mm) and *Klebsiella pneumoniae* (8.67, 4.33 and 7.0 mm) respectively. The last two Gram – negatives *Escherichia coli* and *Proteus mirabilis* were more susceptible than the two others to *A. mellifera* extract.

Table 1: Antibacterial activity of *Apis mellifera* extract (5.0 mg/ml) against Gram – negative and Gram - positive tested bacteria.

Extraction solvent	Inhibition Zone (mm)				
	Gram - positive	Gram - negatives			
	Staph. aureus	*E. coli*	*P. mirabilis*	*Pseudo. aeruginosa*	*K. pneumonia*
Dichloromethane	15.0±1.0a	12.33±0.56b	12.67±1.53a	4.0±1.0d	8.67±1.53c
Methanol	13.67±1.5a	13.33±0.56b	10.67±1.5b	4.67±1.15c	4.33±1.53c
Hexane	13.33±0.5a	11.33±1.67a	11.67±1.5a	7.33±1.5b	7.0±1.0b

- Means with horizontal different letters significant at p ≤ 0.05.

On the other hand, fig 1 evokes variable inhibition effect of *A. mellifera* extract with the different applied solvents. The extract with dichloromethane have more effect on the bacteria; *Staph. aureus*, *P. mirabilis* and *K. pneumonia*e extracts.

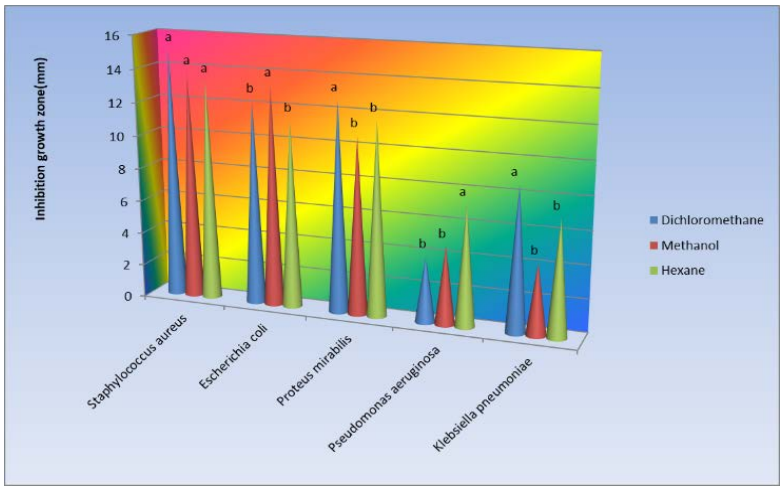

Fig1: Antibacterial activity of *Apis mellifera* extract on tested bacteria prepared with different solvents.

The ant, *Monomorium pharoanis* extract have distinguishable antibacterial activity represented by inhibition zones after extraction with methanol and hexane solvents. The inhibition growth zone depends on the solvent of extraction; therefore, methanol extract were caused 30.33, 25.33, 27.67, 8.0 and 20.0 mm inhibition zones for growth of *Staph. aureus*, *E. coli*, *P. mirabilis*, *Pseudo. aeruginosa* and *K. pneumoniae* respectively. Besides as the previous bacterial sequence for hexane extract were 12.67, 10.33, 8.67, 4.33 and 7.0 mm respectively (table 2).

Table 2: Inhibition zones of bacteria growth treated with 5.0 mg/ml *Monomorium pharoanis* extract.

Extraction solvent	Inhibition Zone (mm)				
	Gram – positive	Gram - negatives			
	Staph. aureus	*E. coli*	*P. mirabilis*	*Pseudo. aeruginosa*	*K. pneumoniae*
Methanol	30.33±1.53a	25.33±1.53b	27.67±2.09b	8.0±1.0d	20.0±2.08c
Hexane	12.67±0.58a	10.33±0.58ab	8.67±1.53bc	4.33±1.53d	7.0±1.73c

- Means ± standard deviation (SD) in horizontal column with different letters are significant at $P \leq 0.05$.

Also, fig 2 exhibits *M. pharoanis* in methanol extract more antibacterial effective than hexane in comparison between that two solvents; (30.3, 2.7 mm), (27. 3, 10.3 mm), (27.7, 8.7 mm), (8. 0, 4.3 mm) and (20.0, 7.0 mm) against the bacteria *Staph. aureus*, *E. coli*, *P. mirabilis*, *Pseudo. Aeruginosa* and *K. pneumoniae* respectively.

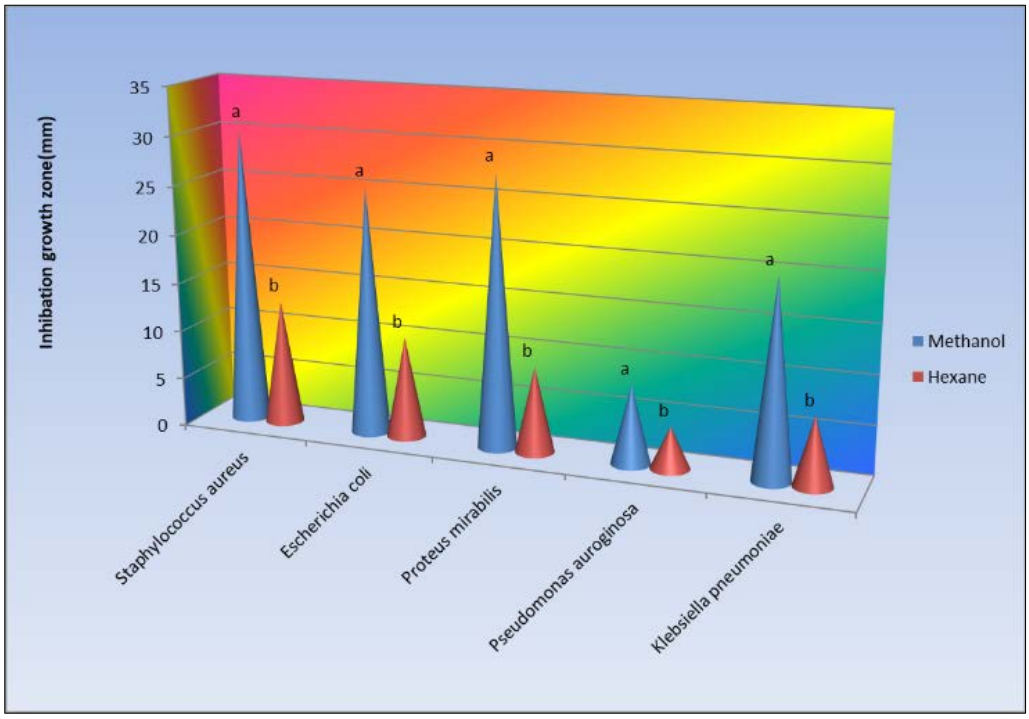

Fig 2: Variation in bacteria inhibition treated by ant *Monomorium pharoanis* extract prepared with two different solvents.

Ethanolic extract of harvest ant, *Camponotus xerxes* and valentine ant *Crematogastor auberti* were caused inhibition zones for used bacteria at 5.0 mg/ml concentration. For *C. xerxes*, the inhibition zones were ranged between 23.3 mm to 11.3 mm and *C. auberti* from 14.3 to 6.0 mm in case *Staph. aureus* and *Pseudo. aeruginosa* treatments (table 3).

Table 3: Variation of bacteria growth inhibition by ethanolic extract of the ants, *Camponotus xerxes* and *Crematogastor auberti* (5.0 mg/ml).

Ant extract	Inhibition Zone (mm)				
	Gram - positive	Gram - negatives			
	Staph. aureus	*E. coli*	*P. mirabilis*	*Pseudo. aeruginosa*	*K. pneumoniae*
Camponotus xerxes	23.33±1.15a	21.67±1.53a	18.33±1.53b	11.33±1.53d	15.67±0.53c
Crematoga-stor auberti	14.33±1.15a	11.67±1.53b	10.33±1.53b	6.0±1.0 c	7.33±0.58c

- Means with different letters in horizontal column are significant at $p \leq 0.05$.

Fig 3 evokes antibacterial activity dominance of *C. xerxes* in relation to *C. auberti*, the most differences between them were (23.3, 14.3 mm), (21.7, 11.7 mm) and (18.3, 10.3 mm) for *Staph. aureus*, *E. coli* and *P. mirabilis* respectively. Also, in spite of low effect, but clear differences between the two ant extracts for *Pseudo. aeruginosa* and *K. pneumoniae*.

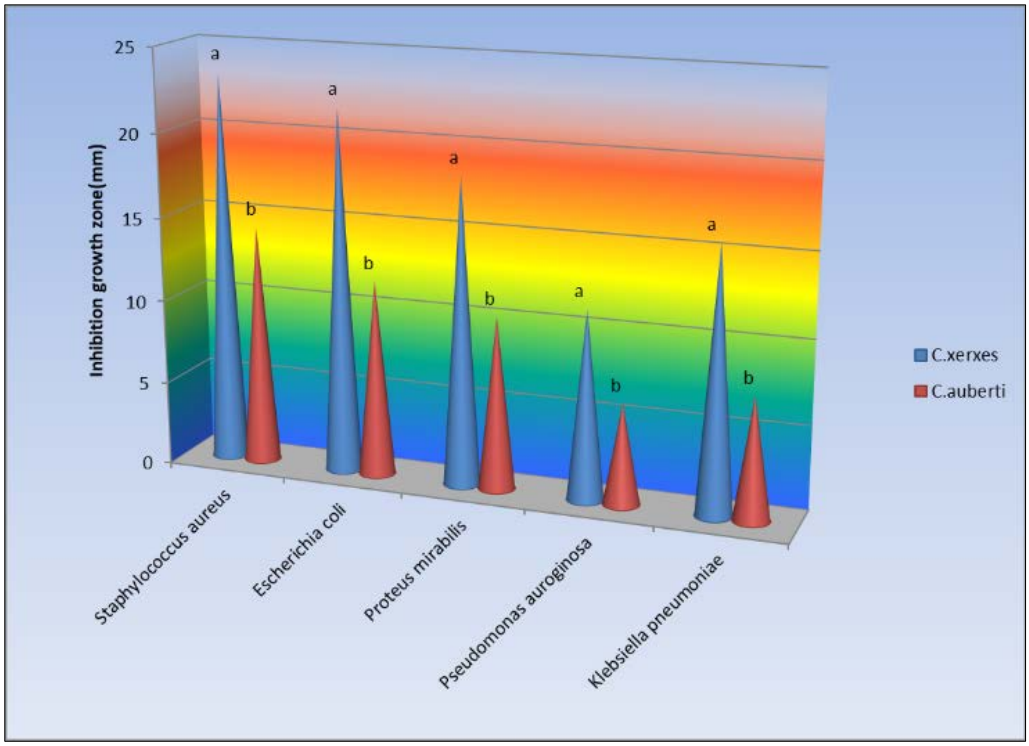

Fig 3: Antibacterial action of the ants *Camponotus xerxes* and *Cremotogastor auberti*.

The Inhibition zones by the antibiotics (Standard drugs) were revealed by fig 4. All the three antibiotics have no activity against *Pseudo. aeruginosa* besides *K. pneumoniae* treatment with Gentamycin. However, susceptibility of the other three applied bacterial species were varied according to the antibiotic usage and bacterium species. Thereafter, growth zones for; Ceftriaxone (CRO) ranged between 25.3 to 6.3 mm for *Staph. aureus* and *P. mirabilis*, Gentamycin (CN) from 35.0 to 11.7 mm in *P. mirabilis* and *Staph. aureus* treatment and Tetracycline (TE) caused inhibition zones for treated bacteria with range 14.7 – 5.3 mm at *Staph. aureus* and *P. mirabilis* treatment.

Discussion

The objective of this study is to evaluate the inhibition growth activity of selected eusocial insects. Hypothetically, social insects are good source for antibacterial activity to protect themselves and their broads in high density habitat with easily pathogens transmission. The growing problem of multidrug - resistance in bacteria populations were encouraged research inspection for new antibiotics and made a milestone in human battle with antibiotic resistance bacteria [20, 21]. The polar solvents; methanol, dichloromethane, ethanol and hexane were chosen for insect body extraction. Because the solvent chosen is one of the effective factors in antibacterial activity of the insect body extracts [19]. Therefore, the polar solvents; methanol, dichloromethane ethanol and hexane were choose for insect body extraction. Ants are considered a potential source for antimicrobial peptides [22], their metapleural glands are the main source of antimicrobial activity in many ant species [23].

Our results on bacteria growth inhibition of eusocial insects: honey bee and three ant local species; *Monomorium pharoanis*, *Camponotus xerxes* and *Crematogaster auberti* have satisfied antibacterial activity at the experimental treatments with 5.0 mg/ml. Methanol extract of honey bee and ant *M. pharoanis* have more efficiency than dichloromethane and hexane extracts against the tested Gram positive and Gram negatives bacteria due to high solubility the active components in methanol solvent. On the other hand, significant variation in antibacterial activity of ethanolic between *C. xerxes* and *C. auberti* extracts. The experimental extracts with different solvents, *Staph. aureus* had mostly the widest growth inhibition zone (tables 1, 2, 3), this bacterium is abundant in the wounds [24]. In comparison for growth inhibition zone diameter between Gram negative marker bacteria, *Escherichia coli* and *Proteus mirabilis* were more susceptible to the applied extracts than *Pseudomonas aeruginosa* and *Klebsiella pneumoniae.*

Ants are considered a potential source for antimicrobial peptides [22]. Ethanolic extract of *C. auberti* have good inhibition zones against the tested bacteria, while *C. xerxes* with moderate to good inhibition activity. These findings context with [25] results for *Crempatogaster* sp., and *M. crinicepls* among three *Monomorium* ssp. extracts which have strong antibacterial activity [10].

Significantly, most the applied honey bee and ants extracts possessed good antimicrobial activity in comparison with tested synthetic antibiotics; Ceftriaxone (CRO), Gentamycin (CN) and Tetracycline (TE) at 10.0 mcg/ml concentration. Fig 4 indicates to that *Pseudo. aeruginosa* and *K. pnoumoniae* resistance to CN and CRO antibiotics by developing act compounds in the ant extracts.

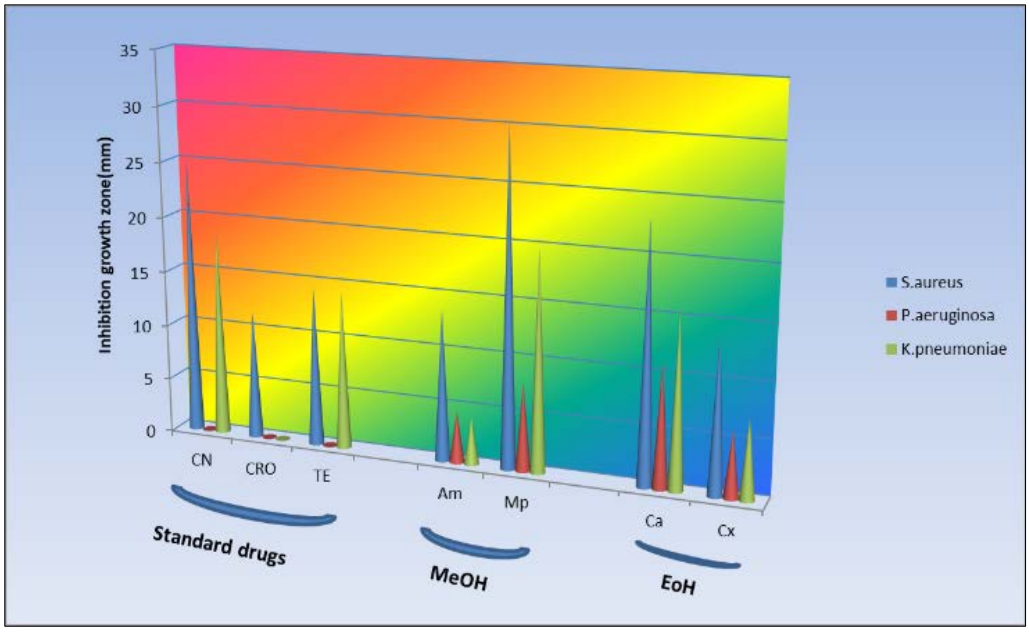

Fig 4: Inhibition growth zones of methanol extract of *Apis mellifera* (Am) and *Monomorium pharoanis* (Mp), besides ethanolic extract of *Cremotogastor auberti* (Ca) and *camponotus xerxes* (Cx) 5.0 mg/ml in comparison with action of the antibiotics; Cefriaxone (CRO), Gentamycin (CN) and Tetracycline (TE) 10.0 mcg.

Conclusion

In this experimental study, insect body extract of social and in environmental stress insects were appeared antibacterial activity against wide spectrum of Gram negative bacteria and Gram positive *S. aureus*. The indicator of this antibacterial activity was represented by growth inhibition zones with disc diffusion test. Methanol and ethanol extracts of the ants: *Monomorium pharoanis*, *camponotus xerxes* and *Crematogastor auberti* have better effect than standard drugs (CN, CRO and TE antibiotics) at 5.0 mg / ml concentration.

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