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Determination of Bioactive Chemical Composition of *Callosobruchus maculatus* and Investigation of its Anti-Fungal Activity

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Available Online: 10th August, 2016

ABSTRACT

Methanolic extract of bioactive compounds of *Callosobruchus maculatus* was assayed for *in vitro* anti-fungal activity against *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, *Saccharomyces cerevisiae*, *Penicillium expansum*, and *Trichoderma viride*. GC-MS analysis of *Callosobruchus maculatus* revealed the existence of the 4-Cyclooctene-1-carboxaldehyde, Benzenemethanol, 2-(2-aminopropoxy)-3-methyl, 1-(1,4-cyclohexadienyl)-2-methylaminopropane, 2-Undecanone, 6,10-dimethyl-, Tricyclo[10.2.2.2(5,8)octadeca-5,7,12,14,15,17-hexaene, 1-Dimethyl(pentafluorophenyl)silyloxycyclopentane, 3-Methylene-bicyclo[3.2.1]oct-6-en-8-ol, Benzenesulfonamide, N-(bicyclo[2.2.1]hept-5-en-2-ylmethyl), (-)-Norephedrine, 4-Bromo-7-methylenebicyclo[4.2.0]oct-2-ene, Benzene, 1-propenyl-, 1-Hexanol, 2-ethyl-, 1,2-Benzisothiazol-3-amine tbdms, 5-Methyl-6-phenyltetrahydro-1,3-oxazine-2-thione, Propanamide, N-(3-methoxyphenyl)-2,2-dimethyl, 3,6-Octadecadiynoic acid, methyl ester, 1-Chloroundecane, 10-(Tetrahydro-puran-2-yloxy)-tricyclo[4.2.1.1(2,5)]dec-7-en, Pent-1-en-3-one, 1-(2-furyl)-5-dimethylamino, Methyl salicylate, 2-Amino-nicotinic acid methyl ester, 2,5-Dimethylhexane-2,5-dihydroperoxide, 1,4-Oxathiane, 4-oxide, 3-Cyclohexene-1-propanal, Cyclobutane(1,6)spiro(2,3-diazabicyclo[3.1.0]hex-2-ene)-4-, Trans-8-Hydroxy-bicyclo(4,3,0)non-3-ene, Tricyclo[3.3.1.1(3,7)]decane, 1-[(hydrazinocarbonyl)amino]-, (7R)-cis-bicyclo[4.3.0]-3-nonen-7-ol, Ethaneperoxoic acid, 1-cyano-1,4-diphenylpentyl ester, 3-Amino-3-(4-isopropoxy-3-methoxy-phenyl)-propionic acid, Aziridinone, 1,3-bis(tricyclo[3.3.1.1(3,7)]dec-1-yl), Hydrazinecarboxamide, 2-(2,6-cyclooctadien-1-ylidene, Cis-pinen-3-ol, Salicylaldehyde, thiocarbazon, Adamantane, 1-isocyano, 2,7-Methanonaphthalen-3-amine, 1,2,3,4,4a,7,8,8a-octahyd, Pteridine-8-oxide, 6-aldoximino-2-amino-4(3H)-oxo-, 2-Dodecenal, 5H-Cyclohepta-1,4-dioxin, 2,3,4a,6,7,9a-hexahydro-, cis-, Hexadecanoic acid, methyl ester, Cyclohexanebutanoic acid, 2-methyl-3-oxo-, methyl ester, Octadecanoic acid, Butyl 9-tetradecenoate, 7-[3-Chloro-2-hydroxypropyl]guanine, 4-Heneicosanone, 1-cyclopentyl-, 4-Methoxycarbonylmethylundec-3-enedioic acid, dimethyl ester, 5-(4,5-Dihydro-3H-pyrrol-2-ylmethylene)-4,4-dimethylpyrrolidin, Phthalic acid, octyl oct-3-yl ester. The results of anti-fungal activity produced by *Callosobruchus maculatus* showed that the volatile compounds were highly effective to suppress the growth of *Aspergillus niger*. *Callosobruchus maculatus* produce many important secondary metabolites with high biological activities.

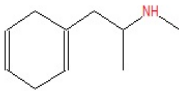
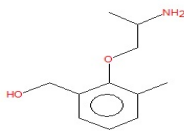
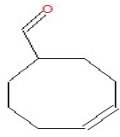

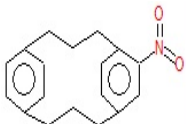
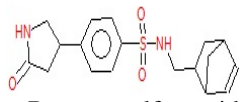
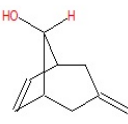
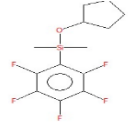
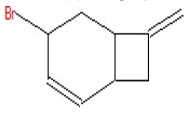
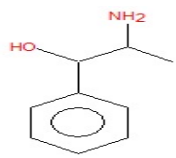
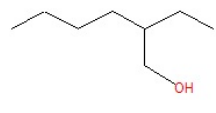
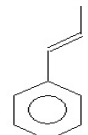
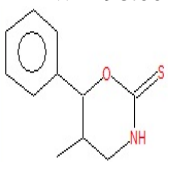
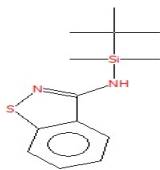
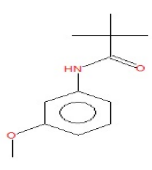
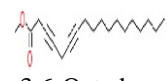
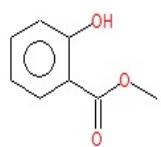
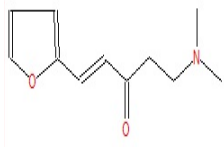
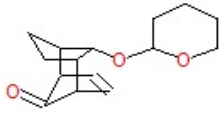

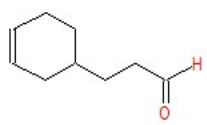
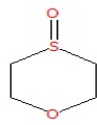
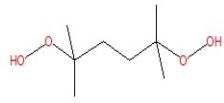
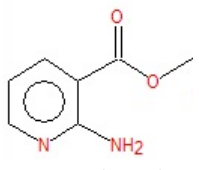
Keywords: GC/MS, Bioactive compounds, *Callosobruchus maculatus*, Anti-fungal.

INTRODUCTION

Callosobruchus maculatus is a species of beetle known commonly as the cowpea weevil or cowpea seed beetle¹. The beetle most likely originated in West Africa and moved around the globe with the trade of legumes and other crops^{1,2}. As only a small number of individuals were likely present in legumes carried by people to distant places, the populations that have invaded various parts of the globe have likely gone through multiple bottlenecks. A female adult can lay over a hundred eggs, and most of them will hatch. She lays an egg on the surface of a bean, and when the larva emerges about 4 to 8 days later, it burrows into the bean³. The bean beetle, *Callosobruchus maculatus*, oviposit their eggs on the cowpea bean. The varieties of cowpea that are most common for the beetle to lay their eggs on are black eyed peas, mung beans, and

adzuki beans⁴. As only a small number of individuals were likely present in legumes carried by people to distant places, the populations that have invaded various parts of the globe have likely gone through multiple bottlenecks. If more than one host is available, the beetle will choose its host depending on the variety and size of the bean as well as the texture of the seed coat⁵. The time it takes the larvae to develop varies across hosts, with longer development times on less suitable hosts⁴. It has been found that beetles that choose to oviposit their eggs on the black eyed pea have a shorter development time, suggesting that the black eyed pea is a more suitable host⁶. Larval crowding can occur when up to 8 or 10 larvae feed and grow within one bean. The emerged adult beetles mate assortatively, meaning they mate with others that developed on the sam

Table 1: Bioactive chemical compounds identified in methanolic extract of *Callosobruchus maculatus*

			
1-(1,4-cyclohexadienyl)-2-methylaminopropane Rt=3.293 Mw=151.1361	Benzenemethanol, 2-(2-aminopropoxy)-3-methyl- Rt=3.259 Mw=195.125929	4-Cyclooctene-1-carboxaldehyde Rt=3.133 Mw=138.1044655	2-Undecanone, 6,10-dimethyl- Rt=3.362 Mw=198.198365
			
Tricyclo[10.2.2.2(5,8)octadeca-5,7,12,14,15,17-hexaene Rt=3.396 MW=281.141579	Benzenesulfonamide, N-(bicyclo[2.2.1]hept-5-en-2-ylmethyl)- RT=3.545 MW=346.135113	3-Methylenebicyclo[3.2.1]oct-6-en-8-ol RT=3.499 MW=136.088815	1 Dimethyl (pentafluorophenyl) silyloxycyclopentane RT=3.430 MW=310.081232
			
4-Bromo-7-methylenebicyclo[4.2.0]oct-2-ene RT=3.722 MW=198.004412	(-)-Norephedrine RT=3.653 MW=151.099714	1-Hexanol, 2-ethyl- RT=3.997 MW=130.135765	Benzene, 1-propenyl- RT=3.785 MW=118.0782504
			
5-Methyl-6-phenyltetrahydro-1,3-oxazine-2-thione RT=4.260 MW=207.071785	1,2-Benzisothiazol-3-amine tbdms RT=4.146 MW=264.111647	Propanamide, N-(3-methoxyphenyl)-2,2-dimethyl- RT=4.552 MW=207.125929	3,6-Octadecadiynoic acid, methyl ester RT=4.626 MW=290.22458
			
Methyl salicylate RT=6.245 MW=152.047344	Pent-1-en-3-one, 1-(2-furyl)-5-dimethylamino- RT=4.872 MW=193.110279	10-(Tetrahydro-puran-2-yloxy)-tricyclo[4.2.1.1(2,5)]dec-7-en RT=4.775 MW=248.141245	1-Chloroundecane RT=4.706 MW=190.1488285
			
3-Cyclohexene-1-propanal RT=8.900 MW=138.1044655	1,4-Oxathiane, 4-oxide RT=8.569 MW=120.0245004	2,5-Dimethylhexane-2,5-dihydroperoxide RT=8.145 MW=178.120509	2-Amino-nicotinic acid methyl ester RT=7.785 MW=152.058578

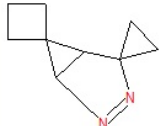
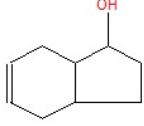
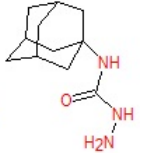
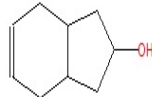
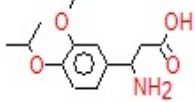
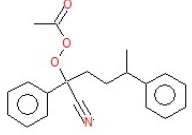
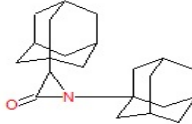
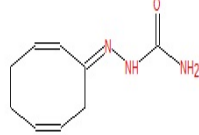
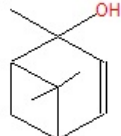
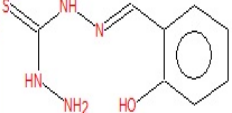
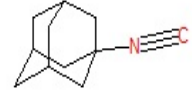
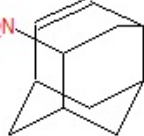
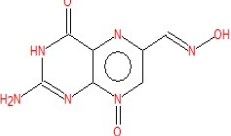

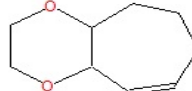
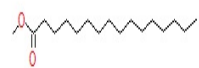
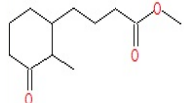
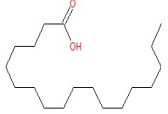
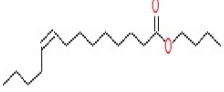
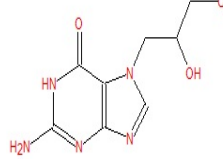

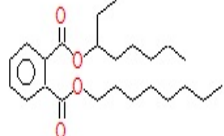
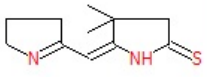
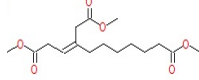
			
Cyclobutane (1,6) spiro (2,3) diazabicyclo [3.1.0] hex-2-ene-4-ol RT=9.152 MW=148.100048	(7R)-cis-bicyclo[4.3.0]-3-nonen-7-ol RT=9.982 MW=138.1044655	Tricyclo [3.3.1.1(3,7)] decane, 1-[(hydrazinocarbonyl)amino] RT=9.713 MW=209.152812	Trans-8-Hydroxy-bicyclo (4,3,0) non-3-ene Rt=9.261 MW=138.1044655
			
3-Amino-3-(4-isopropoxy-3-methoxyphenyl)-propionic Acid RT=10.297 MW=253.131408	Ethaneperoxoic acid, 1-cyano-1,4-diphenylpentyl ester RT=10.136 MW=323.152143	Aziridinone, 1,3 bis (tricyclo [3.3.1.1(3,7)] dec-1-yl)- RT=10.428 MW=325.240564	Hydrazinecarboxamide, 2-(2,6-cyclooctadien-1-ylidene) RT=11.504 MW=179.105862
			
Cis-pinen-3-ol RT=11.916 MW=152.120115	Salicylaldehyde, thiocarbazone RT=12.179 MW=210.057532	Adamantane, 1-isocyano- RT=12.122 MW=161.120449	2,7-Methanonaphthalen-3-amine, 1,2,3,4,4a,7,8,8a-octahyd RT=13.758 MW=163.1361
			
Pteridine-8-oxide, 6-aldoximino-2-amino-4(3H)-oxo- RT=13.896 MW=222.050138	Dodecenal RT=13.198 MW=182.167066	5H-Cyclohepta-1,4-dioxin, 2,3,4a,6,7,9a-hexahydro-, cis- RT=14.382 MW=14.382	Hexadecanoic acid, methyl ester RT=14.577 MW=270.25588
			
Cyclohexanebutanoic acid, 2-methyl-3-oxo-, methyl ester RT=14.806 MW=212.141245	Octadecanoic acid RT=16.831 MW=284.27153	Butyl 9-tetradecenoate RT=17.501 MW=282.25588	7-[3-Chloro-2-hydroxypropyl] guanine RT=17.649 MW=243.052302
			
4-Heneicosanone, 1-cyclopentyl- RT=17.958 MW=378.386166	Phthalic acid, octyl oct-3-yl ester RT=20.018 MW=390.27701	5-(4,5-Dihydro-3H-pyrrol-2-ylmethylene)-4,4-dimethylpyrrolidin RT=18.376 MW=208.103419	4 Methoxycarbonylmethylundec-3-enedioic acid, dimethyl ester RT=18.187 MW=314.17294

Table 2: Antifungal activity of *Callosobruchus maculatus* metabolite products.

Fungi	Metabolite products <i>Callosobruchus maculatus</i> /Antibiotics			
	Metabolite products of <i>Callosobruchus maculatus</i>	Amphotericin B	Fluconazol	Miconazole nitrate
<i>Aspergillus niger</i>	4.00±0.20 ^a	1.00±0.07	1.08±0.04	0.15±0.03
<i>Aspergillus flavus</i>	4.02±0.20	1.04±0.09	1.79±0.20	1.04±0.04
<i>Aspergillus terreus</i>	3.98±0.19	1.99±0.08	1.00±0.07	1.95±0.08
<i>Penicillium expansum</i>	1.67±0.08	2.00±0.20	3.77±0.28	0.86±0.03
<i>Aspergillus fumigatus</i>	4.00±0.20	0.86±0.05	2.07±0.25	1.11±0.06
<i>Trichoderma horzianum</i>	2.00±0.10	1.08±0.04	1.99±0.20	1.08±0.04
<i>Saccharomyces cerevisiae</i>	2.02±0.10	1.33±0.09	1.93±0.20	2.13±0.16
<i>Candida albicans</i>	4.01±0.21	1.00±0.07	0.79±0.05	1.98±0.10
<i>Trichoderma viride</i>	2.51±0.07	1.99±0.20	1.45±0.10	1.47±0.08

^a The values (average of triplicate) are diameter of zone of inhibition at 100 mg/mL crude extract and 30 µg/mL of (Amphotericin B; Fluconazol and Miconazole nitrate).

host bean. The adult requires neither food nor water, but if offered water, sugared water, or yeast, it may consume it. A female given nutrients may lay more eggs⁶. The beetle tolerates a range of humidity and temperature, making it adaptable in climates worldwide. Its developmental time varies with factors such as humidity, temperature, legume type, crowding, and inbreeding levels in the population⁷. Temperature and humidity in legume storage areas are relatively constant and the food density is high⁸. Antibiotic resistance has been a major problem in impeding the efficiency of commonly used antibiotics exhibited by a plethora of microbes as a result of the excessive use of the drugs in medical treatments and animal feed⁹⁻¹². Due to the unpredictable degree of resistance, antibiotic research has now turned its direction to the manipulation of antimicrobial peptides (AMPs). In insects, AMPs are constitutively expressed or highly inducible in response to bacterial infection by various epithelia of midgut and salivary glands, fat bodies and hemocytes in which they are secreted into the hemolymph. In holometabolous insects with complete metamorphosis, AMPs are produced by the fat bodies^{5,13,14} whereas the hemocytes act as the vital synthesizer for AMP in heterometabolous insects with incomplete metamorphosis¹⁵.

MATERIALS AND METHODS

Laboratory culture of *C. maculatus* was obtained from infested seeds from college of science for woman university of Babylon. The stock culture of the insects maintained on *Vigna sinensis*, seeds to obtain newly emerged beetles of same generation, 100 insects were released in plastic containers having 500 of cowpea seed covered by muslin cloth, containers kept in acclimatized chambers at 28 ±2 C° and 65 ±5% humidity, after ten days' adults were removed. The insects emerged after four weeks were used in entire investigation¹⁶. The extraction was performed by adding 100 ml methanol to the whole body insect powder. Methanol was used as solvent control. The studied yeast and fungi, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, *Saccharomyces cerevisiae*, *Penicillium expansum*, and *Trichoderma viride* were isolated and maintained in potato dextrose agar slants. Spores were

grown in a liquid culture of potato dextrose broth (PDB) and incubated at 25°C in a shaker for 16 days at 130 rpm. The tests were carried out in triplicate¹⁷⁻²⁰. The antifungal activity was evaluated by measuring the inhibition-zone diameter observed after 48 h of incubation.

Gas chromatography – mass spectrum analysis

GC-MS is a powerful technique used for many applications which has very high sensitivity and specificity²¹⁻²⁴. The GC-MS analysis of the insect extract was made in a (Agilent 789 A) instrument under computer control at 70 eV^{25,26}. About 1µL of the methanol extract was injected into the GC-MS using a micro syringe and the scanning was done for 45 minutes. The time from when the injection was made (Initial time) to when elution occurred referred to as the Retention time (RT). Helium gas was used as a carrier as well as an eluent. The flow rate of helium was set to 1ml per minute. Compounds were identified by comparing their spectra to those of the Wiley and NIST/EPA/NIH mass spectral libraries²⁷⁻²⁹.

Statistical analysis

Results of the study were based on analysis of variance (ANOVA) using Statistica Software. A significance level of 0.05 was used for all statistical tests.

RESULTS AND DISCUSSION

Identification of biochemical compounds

Gas chromatography and mass spectroscopy analysis of compounds was carried out in methanolic extract of *Callosobruchus maculatus*, shown in Table 1. The GC-MS chromatogram of the peaks of the compounds detected was shown in Figure 1. Chromatogram GC-MS analysis of the methanol extract of *Callosobruchus maculatus* showed the presence of forty-eight major peaks and the components corresponding to the peaks were determined as follows. The First set up peak were determined to be 4-Cyclooctene-1-carboxaldehyde, Benzenemethanol, 2-(2-aminopropoxy)-3-methyl, 1-(1,4-cyclohexadienyl)-2-methylaminopropane, 2-Undecanone, 6,10-dimethyl-, Tricyclo [10.2.2.2 (5,8) octadeca-5,7,12,14,15,17-hexaene, 1-Dimethyl (pentafluoropenyl) silyloxycyclopentane, 3-Methylene-bicyclo [3.2.1] oct-6-en-8-ol, Benzenesulfonamide, N-(bicyclo [2.2.1] hept-5-en-2-ylmethyl), (-)-Norephedrine, 4-Bromo-7-

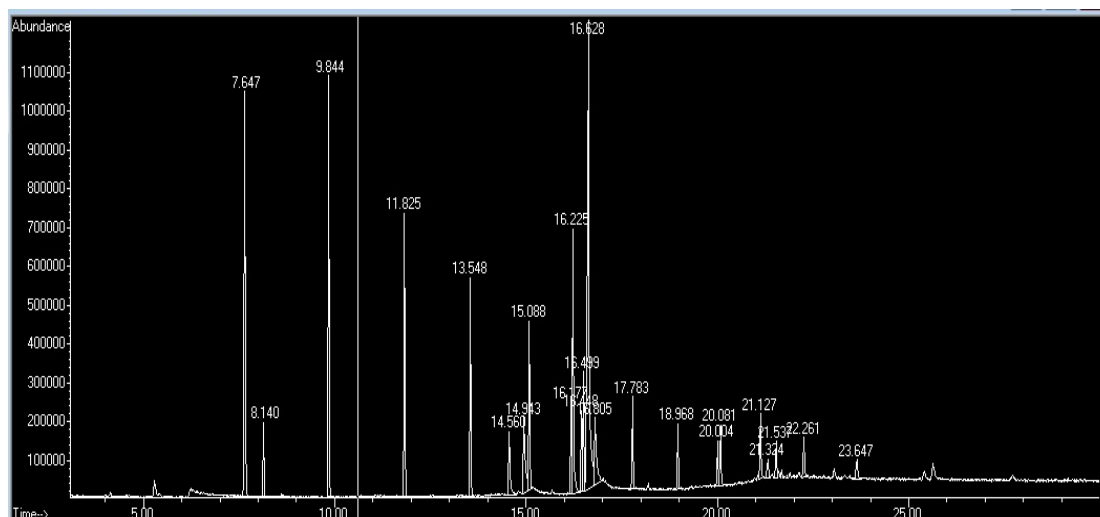


Figure 1: GC-MS chromatogram of methanolic extract of *Callosobruchus maculatus*.

methylenebicyclo [4.2.0] oct-2-ene, Benzene, 1-propenyl, 1-Hexanol, 2-ethyl-, 1,2-Benzisothiazol-3-amine tbdms, 5-Methyl-6-phenyltetrahydro-1,3-oxazine-2-thione, Propanamide, N-(3-methoxyphenyl)-2,2-dimethyl, 3,6-Octadecadienoic acid, methyl ester, 1-Chloroundecane, 10-(Tetrahydro-puran-2-yloxy)-tricyclo [4.2.1.1(2,5)] dec-7-en, Pent-1-en-3-one, 1-(2-furyl)-5-dimethylamino, Methyl salicylate, 2-Amino-nicotinic acid methyl ester, 2,5-Dimethylhexane-2,5-dihydroperoxide, 1,4-Oxathiane, 4-oxide, 3-Cyclohexene-1-propanal, Cyclobutane (1,6) spiro(2,3-diazabicyclo [3.1.0] hex-2-ene)-4-, Trans-8-Hydroxy-bicyclo (4,3,0) non-3-ene, Tricyclo [3.3.1.1(3,7)] decane, 1-[(hydrazinocarbonyl)amino]-, (7R)-cis-bicyclo[4.3.0]-3-nonen-7-ol, Ethaneperoxoic acid, 1-cyano-1,4-diphenylpentyl ester, 3-Amino-3-(4-isopropoxy-3-methoxy-phenyl)-propionic acid, Aziridinone, 1,3-bis(tricyclo[3.3.1.1(3,7)]dec-1-yl), Hydrazinecarboxamide, 2-(2,6-cyclooctadien-1-ylidene, Cis-pinen-3-ol, Salicylaldehyde, thiocarbazono, Adamantane, 1-isocyano, 2,7-Methanonaphthalen-3-amine, 1,2,3,4,4a,7,8,8a-octahyd, Pteridine-8-oxide, 6-aldoximino-2-amino-4(3H)-oxo-, 2-Dodecenal, 5H-Cyclohepta-1,4-dioxin,2,3,4a,6,7,9a-hexahydro-,cis-, Hexadecanoic acid, methyl ester, Cyclohexanebutanoic acid, 2-methyl-3-oxo-, methyl ester, Octadecanoic acid, Butyl 9-tetradecenoate, 7-[3-Chloro-2-hydroxypropyl]guanine, 4-Heneicosanone, 1-cyclopentyl-, 4-Methoxycarbonylmethylundec-3-enedioic acid, dimethyl ester, 5-(4,5-Dihydro-3H-pyrrol-2-ylmethylene)-4,4-dimethylpyrrolidin, Phthalic acid, octyl oct-3-yl ester. Solvent extract was found to exhibit significant inhibitory effects on the fungi ($p < 0.05$). The results of anti-fungal activity produced by *Callosobruchus maculatus* showed that the volatile compounds were the largest zone inhibition in *Aspergillus niger* (4.00 ± 0.20 mm), *Aspergillus terreus* (3.98 ± 0.19 mm), *Aspergillus flavus* (4.02 ± 0.20 mm), *Aspergillus fumigatus* (4.00 ± 0.20 mm), *Candida albicans* (4.01 ± 0.21 mm), *Saccharomyces cerevisiae* (2.02 ± 0.10 mm), *Penicillium expansum* (1.67 ± 0.08 mm), *Trichoderma viride* (2.51 ± 0.07 mm) and *Trichoderma horzianum* (2.00 ± 0.10 mm). *Callosobruchus*

maculatus produce many important secondary metabolites with high biological activities. Based on the significance of employing bioactive compounds in pharmacy to produce drugs for the treatment of many diseases, the purification of compounds produced by *Callosobruchus maculatus* can be useful. Maximum zone formation against *Aspergillus niger* (4.00 ± 0.20 mm) mm, Table 2. AMPs are short bioactive molecules either cationic or non-cationic in nature with broad-spectrum antimicrobial effects against many pathogens^{2,30-36}. AMPs are short bioactive molecules either cationic or non-cationic in nature with broad-spectrum antimicrobial effects against many pathogens^{2,37}. They generally act in destabilizing the cell membrane permeability or interacting with the specific targets in cells which cause signaling pathway disruption³⁸. Interestingly, insect whole body is also an alternative source for the discovery of a new class of small AMPs which may have a role in processes other than immunity. The AMP extraction from the whole body is governed by the following facts: (i) AMP production is not restricted to fat bodies and hemocytes, (ii) unknown physiological location of the molecules and (iii) small-sized body of insects. Initial peptide recovery from insect whole body requires the sample to be first subjected to homogenization³⁹. The beetle is known for attacking the cowpea (*Vigna unguiculata*), but it readily attacks other beans and peas such as the mung bean (*Vigna radiata*) and adzuki bean (*Vigna angularis*). The adult is more likely to seek the legume in which it developed as a larva, but if it is not available or less common, the beetle will utilize another type³⁹.

CONCLUSION

Forty-eight bioactive chemical constituents have been identified from methanolic extract of the *Callosobruchus maculatus* by GC-MS technique. *In vitro* antifungal evaluation of secondary metabolite products of *Callosobruchus maculatus* forms a primary platform for further pharmacological investigation for the development of new potential antifungal compounds.

ACKNOWLEDGEMENTS

I would like to express my deep appreciation to head of department Prof. Dr. Ali Al-Marzooqi and department of Medical Science in college of nursing for providing all necessary facilities to conduct this study.

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