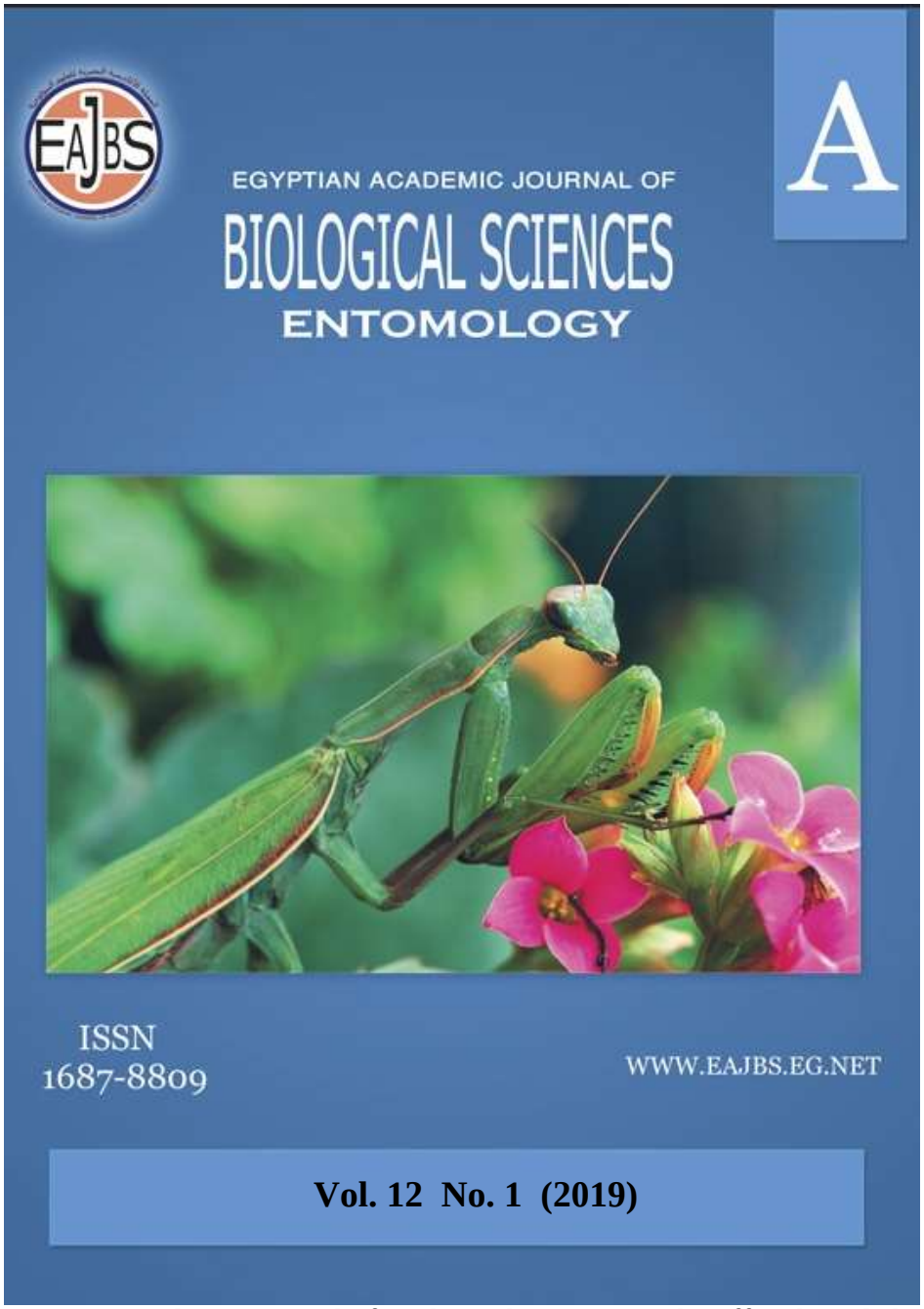

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Antioxidant and Anticancer Activities of Some Maggots Methanol Extracts

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ABSTRACT

Insects offer a source of novel natural compounds that may have therapeutic utility in cancer and other diseases. Antioxidant activity of these extracts was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl radical), and total antioxidant capacity assays. The antioxidant and anticancer effects of the crude extract of maggots namely; *Musca domestica*, *Lucilia sericata* and *Chrysomya albiceps* against the human colon carcinoma cell line (Caco-2), were investigated. The antioxidant activity of tested extracts revealed that the highest DPPH scavenging activity levels were exhibited by methanolic extract of *C. albiceps*, followed by those of *L. sericata* and *M. domestica*. Also, the highest effect was recorded against human colon carcinoma cell line (Caco-2) with *L. sericata* maggots methanolic extract, followed by those of *C. albiceps* and *M. domestica* at the highest concentration used (25 mg/ml). The IC_{50} values of tested extracts ranged from 0.27 to 1.91 mg/ml. In conclusion, data suggest that tested extracts possess antioxidant and anticancer properties that may further explored as a potential source for treatment of cancer and other diseases involving oxidative stress.

INTRODUCTION

Despite considerable progress in medical research, cancer is still the second most common cause of death according to the World Health Organization 2018 factsheet report and by 2020 it will have to cause the deaths of more than 10 million people. Surgical management of tumors is a widely accepted cancer treatment for many tumor types, and there have also been advances in the development of targeted therapies for cancer. However, surgery, chemotherapy and radiation treatment have serious side effects; therefore, there is a need for new, powerful, and highly effective anticancer agents.

Oxidative response plays a vital role in human health. An antioxidant is a molecule capable of slow/prevent the oxidation of other molecules and so prevent such pathogenic changes, free radicals are ubiquitous in our body and are generated by some physiological processes to eliminate invading these pathogenic microorganisms. Many diseases, including neurodegenerative diseases, autoimmune skin diseases, cardiovascular diseases, and chronic renal failure (Zhu *et al*., 2013), Cancer (Roy *et al*., 2015) are due to the oxidative stress activation and the deficiency of intracellular antioxidant defenses. Therefore, unstable and reactive free radicals

can be reduced by antioxidants that protect cells from free radical attack (Souri *et al*., 2008).

One source of novel natural compounds that may have therapeutic utility in cancer and other diseases are insects. Most available antioxidant and antitumor agents are derived from plant, microbial and animal secondary metabolites (Oliver *et al.,* 1994; Cragg *et al*., 1997), however, few data are available on insect-derived molecules. Insects comprise approximately 55% of total biodiversity and approximately 85% of all animal diversity (Chernysh *et al*., 2002). Insects are present in many types of ecological systems, from waterways to septic environments, stimulating scientists to look for a cheap and abundant supply of therapeutics in this arthropod class (Roy *et al*., 2015). In this study, the therapeutic potential of maggot extracts was examined as previously there have been few studies investigating the utility of maggot-derived compounds as cancer therapeutics.

Since few data are available regarding the antioxidant and anticancer activities of insect-derived materials, especially flies' maggots, the objectives of this study were to investigate some maggots crude extract from three representative species, *M. domestica*, *L. sericata* and *C. albiceps,* as a potential source for novel cancer agents as well as giving protection and preventing from diseases.

MATERIALS AND METHODS

Tested Species:

Musca domestica (Diptera: Muscidae), *Lucilia sericata* and *Chrysomya albiceps* (Diptera: Calliphoridae) larvae were obtained from Medical Entomology Insectary, Animal House, Department of Zoology, Faculty of Science, Al-Azhar University and reared under controlled conditions of temperature, relative humidity and photoperiods. A standard rearing procedure (Busvine, 1962; Queiroz and Milward-de-Azevedo, 1991) was applied to provide maggots needed for the bioassay.

Preparation of Crude Maggots Extract:

Crude maggots extract was prepared according to Hassan *et al.,* (2018) as the following; 500 of *M. domestica*, *L. sericata* and *C. albiceps* third instar maggots were washed with ethanol 70% as a disinfectant then with deionized water; the excess of water removed using filter paper. Each maggot species was completely homogenized in 50ml of 40 mM tris-HCl (pH 7.4). The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant was used as buffer extract. Pure methanol was added to the residue and centrifuged again. The supernatant was taken and considered as methanolic extract. Extracts from tested species were dried and kept in the freezer at -4°C until needed for treatments.

Antioxidant Assays :

1. DPPH Radical Scavenging Activity:

The free radical scavenging activity of the extracts was assayed using the stable free radical diphenyl-2-picryl hydrazyl (DPPH) 0.1 mM -DPPH solution, was obtained from Sigma Aldrich GmbH, Sternheim, Germany, it was added to 3 ml of each maggot extract. The mixture was shaken vigorously and incubated at room temperature for 30 min. The absorbance was measured at 517 nm using a Bausch and Lomb spectrophotometer 710 (Widowati *et al*., 2011). The IC_{50} values were calculated using a log dose inhibition curve, and serial concentrations were prepared for each extract. A lower reaction absorbance indicates higher free radical activity (Choudary *et al*., 2006). The experiment was achieved in triplicates. The DPPH scavenging activity was calculated from this equation: DPPH scavenging (%) = (A_0-

$A_1)/A_0 \times 100$, where A_0 is the control absorbance and A_1 is the sample absorbance. The antioxidant and free radical scavenger eugenol was used as a positive control at concentration 50 µg/ml (Shekhar and Anju, 2014).

2. Total Antioxidant Capacity:

The total antioxidant capacity of the tested extracts was determined by Abdel-Hady *et al.* (2018). An aliquot of 0.1 mL of sample (200 µg/mL) solution was mixed with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Blank was contained 1 mL of the reagent solution and nearly volume of the same solvent used for the samples. The tubes were capped and incubated at 95°C for 90 min. After the samples had cooled to 25-27°C, the absorbance of the mixture was measured at 695 nm versus the blank and ascorbic acid was used as a standard. The experiment was repeated for 3 times. The antioxidant activity of the extracts was expressed as mg AAE eq./g extract.

Cytotoxic Assay:

1- Cell Culture:

Colon cancer (Caco-2) cell lines were obtained were obtained from the VACSERA-Cell Culture Unit, Cairo, Egypt. These cell lines were originally obtained from the American Type Culture Collection.

***2- In Vitro* Cytotoxicity by MTT Assay:**

The MTT [3- (4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay is based on the conversion of MTT into formazan crystals by living cell, which determines mitochondrial activity (Hansen *et al*., 1989). The tested extracts were tested at concentrations ranged from 25 to 0.048 µg/mL. Colon cancer (Caco-2) cell lines were cultured in RPMI-1640 medium (Sigma Co., St Louis, USA) supplemented with 10% inactivated fetal bovine serum (FBS; Gibco, UK), 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were seeded in a 96-well plate at a density of 1.0×10^4 cells/well at 37 °C for 48 h in a 5% CO_2 incubator and humidified atmosphere in a sterile environment until half-confluent monolayer formed. The 200 µL of treatment medium (serum-free medium) was added in an independent manner to the cells. 100 µL of MTT dye was added to each well then incubated for 2 hrs. Cells were washed by 100 µL of PBS and 150 µL of MTT destaining solution was added on microtiter shaker for at least 10 min or until all MTT dye that has been extracted formed the homogeneous solution. The optical density of each well was measured at 570 nm with an ELISA microplate reader (EXL 800 USA). The relative cell viability percentage was calculated by (A570 of treated samples/A570 of the untreated sample) X100, (Skehan *et al*., 2003). The results represent IC_{50} is the percentage of inhibitory concentration of cell viability.

Statistical Analysis:

Statistical analysis was performed per the method described by (Armitage, 1974; Lentner *et al*., 1982). The analysis was done, and graphics were constructed using SigmaPlot. Data were assessed by calculating the mean (M), standard deviation (SD) and standard error (SE).

RESULTS

Antioxidant Activity of Crude Maggots Extract by DPPH:

DPPH is commonly used to test the ability of compounds as free-radical scavengers or hydrogen donors. Results of antioxidant activity using DPPH proved that the methanolic extracts of *C. albiceps* has the highest antioxidant activity than other extracts (37.18 ± 1.06 µg/mL) followed by *L. sericata* (75.28 ± 1.64 µg/mL)

finally *M. domestica* extract has the lowest activity (103.13 ± 0.97 µg/mL) compared to 4.05 mg/ml for eugenol (Table 1).

Table (1): IC_{50} values of the DPPH scavenging activity of the crude maggots extract.

Samples	Linear equation	R^2	IC_{50} mg/ml
M. domestica	Y= 0.5157× -3.1846	0.93503	103.13
L. sericata	Y= 0.7061× -3.1561	0.97068	75.28
C. albiceps	Y= 0.9267× +15.557	0.80864	37.18
Eugenol	Y=10.921× +5.5	0.9758	4.05

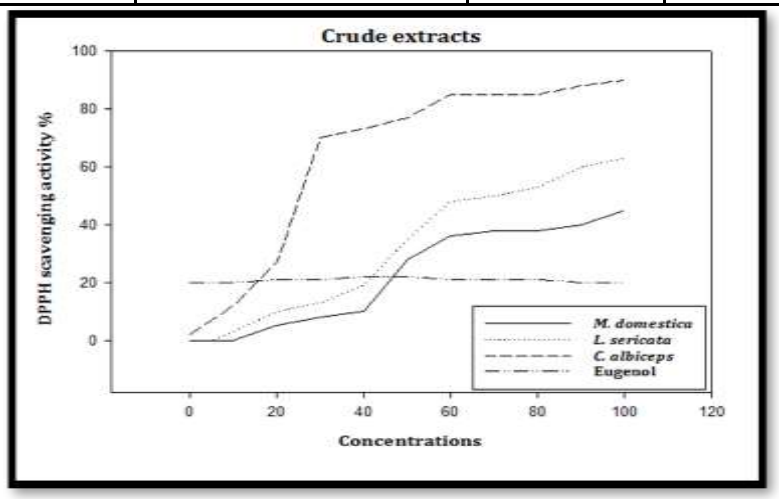

Fig. 1: DPPH scavenging activity of crude maggots extract.

1- Total Antioxidant Capacity:

Results in table (2) demonstrated that methanolic extracts of *C. albiceps* has higher antioxidant activity (203.56 ± 1.07mg AAE/g extract) followed by *L. sericata* (155.59 ± 1.03 mg AAE/g extract), and *M. domestica* (130.33 ± 1.03 mg AAE/g extract); while the lowest antioxidant activity was recorded by water residue (110.43 ± 1.09 mg AAE/g extract).

Table (2): Total antioxidant capacity of different tested extracts.

Samples	Total antioxidant capacity [mg AAE/g extract]
M. domestica	203.56 ± 1.07
L. sericata	155.59 ± 1.03
C. albiceps	130.33 ± 1.03
Water residue	110.43 ± 1.09

The data were expressed as mean ± standard deviation

2- MTT Assay for Cytotoxicity with Caco-2 Cells:

The activity of *M. domestica, L. sericata* and *C. albiceps* maggots' methanolic extract on tumor cell viability was evaluated by treating Caco-2 colon cancer cells with tested extracts using MTT assay. Data given in (Table 3, Figs. 2-5) showed that, at the highest concentration (25 mg/ml), the lowest percentage of cell viability (2.41±0.01) recorded from treatment with the *C. albiceps* extract followed by *L. sericata* (3.64±0.03), and the least potent extract was from *M. domestica* (13.86±0.07). The IC_{50} value of Doxorubicin (positive control) was 0.14±0.03. A

concentration-dependent effect of tested extracts was observed. At a concentration of 0.195 mg/ml, cell viability percentages were (51.52±0.06; 70.11±0.02 for *C. albiceps* and *L. sericata*, respectively, while it recorded (100.49±0.04) for *M. domestica*. Overall, tested extracts possess significant cytotoxic activity against Caco-2 cell line.

Table (3): Anticancer activity of crude maggots extract against Caco-2 line.

Concentrations mg/ml	*M. domestica*		*L. sericata*		*C. albiceps*		Doxorubicin	
	Viability	IC_{50}	Viability	IC_{50}	Viability	IC_{50}%	Viability	IC_{50}%
25	13.86±0.07		3.64±0.03		2.41±0.01		2.46±0.2	
12.5	17.21±0.03		2.655±0.06		2.56±0.03		2.45±0.4	
6.25	20.45±0.01		3.74±0.03		2.85±0.08		2.55±0.09	
3.125	24.49±0.03		3.74±0.03		3.34±0.03		3.14±0.07	
1.562	32.55±0.02	1.91	4.52±0.03	0.28	4.13±0.06	0.27	3.93±0.59	0.14
0.781	98.13±0.02	mg/ml	10.23±0.04	mg/ml	6.49±0.02	mg/ml	7.57±0.01	mg/ml
0.390	100±0.01		23.30±0.03		26.35±0.02		20.1±0.06	
0.195	100.49±0.04		70.11±0.02		51.52±0.06		29.1±0.05	
0.097	--		99.41±0.09		100±0.05		69.9±0.01	
0.048	--		100.89±0.07		103.83±0.03		98.6±0.03	

Median inhibitory concentrations of *M. domestica*, *L. sericata* and *C. albiceps* crude maggots extract are also summarized in table (3). The IC_{50} values ranged from 0.27 to 1.91 mg/ml. The highest anticancer activity was obtained from the *C. albiceps* extract, which was more potent after the anticancer agent Doxorubicin.

The morphological changes in Caco-2 cell line exposed to various concentrations of different crude maggots extract are shown in (Figs. 3-5). Results showed that Caco-2 cell lines exposed to 25, 12.5, 6.25, 3.12 and 1.56 mg/ml concentrations reduced the normal morphology of the cells and cell adhesion capacity of different tested extracts as compared to the control. Most of the cells at concentration 25 mg/ml lost their typical morphology and appeared smaller in size, shrunken and rounded. However, at concentration 0.78 mg/ml and lower, extracts did not cause any effects on the morphology of the cells (Fig. 3).

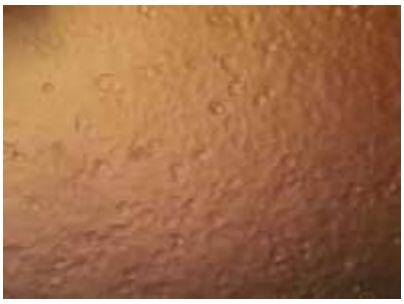

Fig.2: Human colon normal cell line FHC (ATCC CRL-1831).

Comparing the results, the cell viability was found to be concentration-dependent and it was highly affected by the crude extracts tested. The highest anticancer activity was recorded by *C. albiceps* extract, followed by *L. sericata* and *M. domestica*.

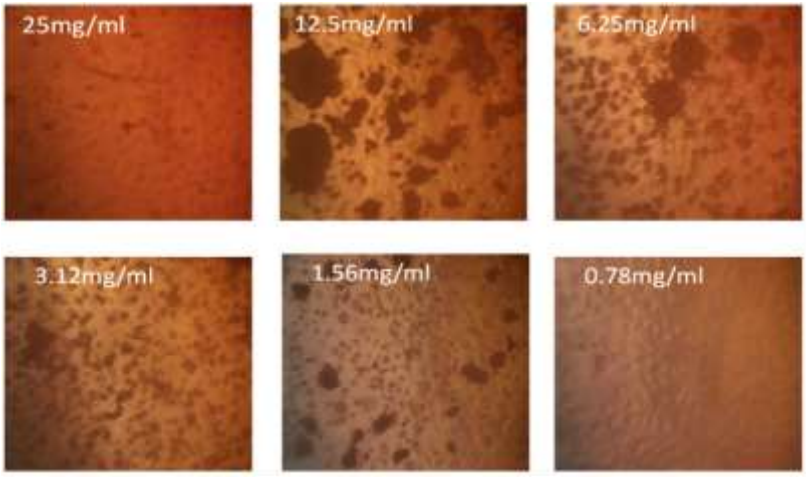

Fig.3: Morphological changes in Caco-2 cell line exposed to various concentrations of *M.domestica* crude maggots extract. X20.

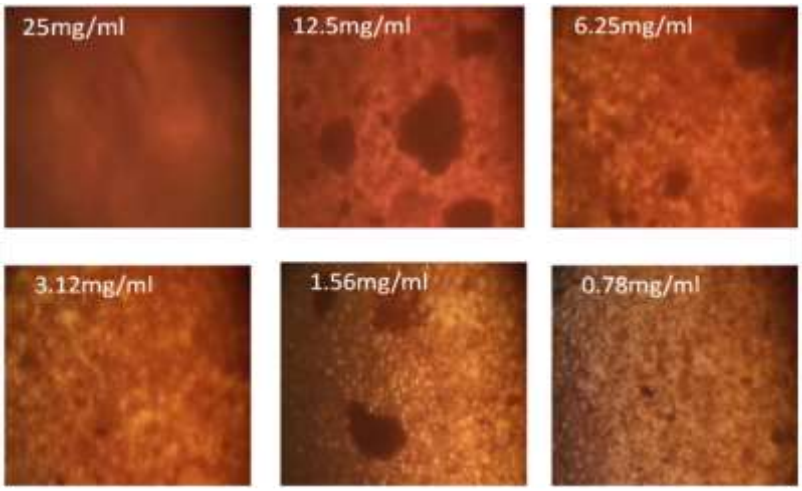

Fig.4: Morphological changes in Caco-2 cell line exposed to various concentrations of *L. sericata* crude maggots extract. X20.

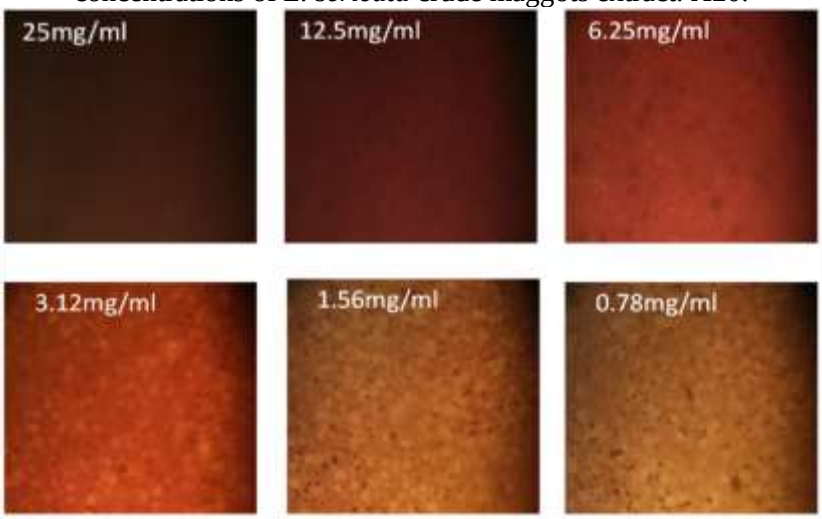

Fig.5: Morphological changes in Caco-2 cell line exposed to various concentrations of *C. albiceps* crude maggots extract. X20.

DISCUSSION

The present study reports the capability of maggots' whole body extracts as antioxidant and anticancer agents. Interestingly, these extracts were found to have therapeutics properties especially *C. albiceps* extract. Insects have been providing for years with many natural products, including (silk, honey, beeswax, propolis and royal jelly). Also, insects extract traditionally used in many diseases treatments, including arthritis treatment with pseudomyrmex ant venom and diabetics (Lockhart, 2007), larval grounds up bodies have been also used in folklore medicine in many parts of the world e.g. (Srivastava *et al*., 2009; Dossey, 2010). Dipterous flies are important insects, and their larvae are a source of protein, polyunsaturated fats, vitamins, minerals and other nutrients (Hwangbo *et al*., 2009). Despite the fact that only few insects have undergone clinical trials to provide their efficacy, scientists recently developing new potential medicines for treatment of many diseases such as cancer (Ratcliffe *et al*., 2014; Shehata *et al.,* 2016), in addition to the use of maggots to advance the human health through its application in pharmacotherapy (Sherman and Cooper 2018).

Free radicals such as hydroxyl groups, peroxyl radicals, and single oxygen have a harmful effect due to their ability to oxidize cell components and causing different diseases (Bozin *et al*., 2008). The antioxidant property plays an important role in reducing chronic diseases like cancer and cardiovascular diseases by scavenging free radicals. Several synthetic antioxidants have been used to reduce the biological toxicity by exerting several deleterious effects. Therefore, there is a great demand of replacing synthetic antioxidants with natural oxidizing agents. Naturally, origin substances such as ground-up bodies have been widely used in medical industries and generally considered safe to use due to the presence of many compounds with antioxidant and anticancer activities (Suh *et al*., 2010). The extracts tested in this study revealed that *C. albiceps* extract displayed more potent scavenging ability, while scavenging ability of *M. domestica* was weaker than *L. sericata.*

Results obtained in this study showed also that Caco-2 cell exposed to different concentrations of tested extracts reduced the normal morphology of cells and cell adhesion capacity with preference to *C. albiceps* crude maggots extract that decreased the cell viability in a concentration-dependent manner, which may indicate the same principles involved in other anticancer agents. These results support those of previous studies, which established that *M. domestica* maggots could be used clinically to alleviate gastric cancer (Hou *et al*., 2007), and that alloferon -a peptide from *Calliphora vicina*- had cytotoxic effects on cancer cells at a dose of 25µg (Chernysh *et al*., 2002). However, the relationship between the order of potency with reference to antioxidant effects, and the order of potency against Caco-2 cells still unknown.

Conclusion

It could be concluded that the tested extracts showed relatively high concentration-dependent antioxidant effects, and *C. albiceps* extract recorded the highest levels of DPPH scavenging activity, followed by *L. sericata* and *M. domestica*. The different tested extracts inhibited the proliferation of tumor cells and the highest anticancer activity was recorded by *C. albiceps* crude maggots extract followed by *L. sericata* and *M. domestica,* and these results could be the first demonstration that larvae from these three particular insect species may have antioxidant and anticancer activities.

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ARABIC SUMMERY

"دراسة تأثير مستخلصات الميثانول لبعض اليرقات كمضادات أكسدة ومضادات للسرطان"

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تعتبر الحشرات مصدرا طبيعيا للمركبات الجديدة والتى من الممكن ان يكون لها أهمية/فائدة علاجية ضد السرطان او ضد الأمراض الأخرى. في هذه الدراسة تم تقييم نشاط هذه المستخلصات كمضادات للأكسدة بواسطة استخدام طريقة (DPPH) وأيضا بطريقة القدرة الكلية لمضادات الأكسدة .

تم إختبار تأثير مستخلصات يرقات الحشرات التالية ؛ *مسكا دومستيكا* ؛ *ليوسليا سيريكاتا* ؛ و*كريزومايا ألبيسبس* كمضادات للأكسدة ومضادات للسرطان حيث تم تطبيق اختبار مضادات السرطان على خلايا القولون المسرطنة (CACO-٢). أظهرت النتائج عند قياس تأثير هذه المستخلصات كمضادات للأكسدة أن أعلى تأثير مضاد للأكسدة كان ناتجا من مستخلص الميثانول ليرقات حشرة *كريزومايا ألبيسبس* يليه مستخلص يرقات حشرة *ليوسليا سيريكاتا* ثم *مسكا دومستيكا*. من ناحية أخرى تم تسجيل أعلى تأثير مضاد للسرطان على خلايا القولون المسرطنة (CACO-٢) من مستخلصات الميثانول ليرقات حشرة *ليوسليا سيريكاتا* يليها *كريزومايا ألبيسبس* ثم *مسكا دومستيكا* وذلك عند أعلى تركيز مستخدم (٢٥ملغ \مل). تراوحت قيم التركيزات المثبطة لنصف العينة من ٠,٢٧ ملغ\مل الى٩١ .١ملغ \مل. الخلاصة، تقترح نتائج الدراسة الحالية ان المستخلصات محل الأختبار تتميز بخصائص علاجية يمكن استكشافها منها مضادات الأكسدة والمضادات السرطانية مما يجعلها مصدر محتمل لعلاج السرطان والأمراض الأخرى.