Original Research Article

Crude and Chitosan Nano-particles Extracts of Some Maggots as Antioxidant and Anticancer Agents

Abstract: The antioxidant effects besides anticancer activities of *Musca domestica*, *Lucilia sericata* and *Chrysomya albiceps* maggots extracts against human liver carcinoma (HepG-2) and human colon carcinoma (HCT-116) were investigated. Two kinds of extracts, crude and chitosan nanoparticles (CNPs) were prepared. The antioxidant activity of different tested extracts was performed by DPPH radical scavenging method, the results obtained revealed that, the highest levels of DPPH scavenging activity were exhibited by the crude extracts of tested maggots with preference to *C. albiceps* extract, which exhibited a much more potent activity followed by *L. sericata* and *M. domestica* in crude and CNPs extracts. Crude extracts have lower anticancer activity than the CNPs extracts; however, the lowest percentage of cell viability (6.7±0.7%) was recorded by *L. sericata* crude extract against HCT-116, followed by *C. albiceps* crude extract (7.57±1.25%) against HepG-2 at the highest used concentration 100 µg/ml. The strongest anticancer activity was observed with CNPs extracts and it was recorded at concentrations of 80, 90 and 100 µg/ml against cell lines tested. Depending on Median inhibitory concentrations (IC_{50}) of maggots crude and CNPs extracts, the IC_{50} values were in the range of 37.3 to 74.3µg/ml and the highest anticancer activity was obtained by *C. albiceps* CNPs extracts against cell lines tested. In conclusion, both tested extracts have optimistic antioxidant activity. CNPs extracts have great therapeutic potential due to its anticancer inducing activities.

Keywords: Antitumor; Antioxidant; DPPH; *M. domestica*; *L. sericata*; *C. albiceps;* CNPs.

Introduction

Oxidative stress is caused by free radicals inducing many chronic and degenerative diseases including, heart disease, aging, diabetes and cancer [1]. Reduction of unstable and reactive free radicals can be achieved via antioxidants that protect cells from free radical attack. One of the main objectives of this study was to find natural origins antioxidants that replace synthetic antioxidants, which are limited by their carcinogenicity and have been suspected to cause negative health effects.

Despite considerable progress in medical research, cancer is still one of the high-ranking causes of death in the world. It is the second most common cause of death according to World Health Organization and by 2020 it will be caused death for more than 10 million people. Surgical therapy still promising and widely accepted cancer treatments, much attention also received for nonsurgical cancer treatments that aimed to reduce complications of surgical treatments. Also, cancer chemotherapy and radiations showed serious side effects; therefore, it is important to find new, powerful anticancer agents that are highly effective and biodegradable.

Maggot therapy has been traditionally practiced for debridement of necrotic wounds as well as for curing infections at the wounds site; maggots promote wound healing, stimulate granulation and promote the formation of human fibroblasts [2].

Insects offer a tremendous potential as a natural resource for chitin production. Even chitosan is a derivative of chitin; it has its own unique functions, chitosan is a natural nontoxic polysaccharide that has been widely used due to its various biological functions such as antioxidant [3] and antitumor activity [4]. However, because of its high molecular weight and water-insolubility, the application of chitosan is severely limited; therefore, nanoparticle formulation enhancing the therapeutic efficacy of chitosan [5]. CNPs exhibit more superior activities than chitosan and have been reported to boost anticancer activity than those of chitosan. In addition, nanoparticles possess a stronger surface curvature; this produces more dissolution pressure with a corresponding increase in saturation solubility [6].

Since not much data are available concerning the antioxidant and anticancer activities of insects, especially flies' maggots, therefore, the objectives of the present study were to evaluate the antioxidant and anticancer activity of the crude and CNPs maggots extracts of *M. domestica*, *L. sericata* and *C. albiceps*.

Materials and Methods

1- Tested species.

Common species of medical importance in many parts of the world, including Egypt, used in this study were *Musca domestica* (Diptera: Muscidae), *Lucilia sericata* and *Chrysomya albiceps* (Diptera: Calliphoridae) maggots. They were obtained from the susceptible laboratory-reared strains continuously raised in the institute of medical entomology, Dokki, Egypt.

2- Tested extracts.

Two kinds of extracts, crude and CNPs extracts from each species with serial concentrations were prepared as the following:

2-1- Crude extracts preparation.

The extraction was performed according to [7] as the following: 3rd larval instar (100 larvae) were washed with 70% methanol and sterile double distilled water (ddH_2O) then incubated overnight at 30 °C, excess water was removed by using filter paper. Ten grams of each species was thoroughly homogenized. The homogenate was centrifuged at 13,000 rpm for 30 min. at 4 °C. After centrifugation the supernatants were decanted, filtrated with filter paper, dried in a rotary evaporator at 40^{0}C for 40 min. The dry extracts were weighed and dissolved in methanol and used as methanol extract.

2-2- CNPs extracts preparation.

Extracts *preparation* as CNPs was done as the following.

2-2-1- Extraction of chitin.

Chitin was isolated from the tested maggots as the following: 3rd larval instar (500 larvae) from each species were washed with 70% ethanol and sterile ddH_2O, dried for 48 h and crushed with a mortar to create the maggots powder. The prepared maggots powder

were weighed, deproteinized using NaOH 2N with a ratio of 12ml/1g (v/w). The treatment was carried out for 30 min., the residue was collected with filter paper, washed with distilled water and dried in an oven at 50 °C. Deproteinized products were weighed; demineralization was carried out with a diluted HCl solution 1M for 30 min at room temperature [8]. Decolorization was done by treatment of the precipitate with 1% potassium permanganate solution 100 ml for 1 h then, chitin was washed with distilled water and dried at 50 °C.

2-2-2- Preparation and characterization of chitosan.

The product from decolorization was N-deacetylated using NaOH 12.5N with ratio of 1g/20ml (w/v). The residue was then washed with distilled water, collected with filter paper and dried in an oven at 50 °C. Infrared radiation by Fourier transform infrared spectroscopy (FT-IR) with different wavelengths released on the sample was used for chitosan characterization. An infrared spectrum represents a fingerprint of a sample with absorption peaks. Spectrum formed showing the absorption and transmission of the sample molecule. The spectrum is unique for the material as it has the unique combination of atoms and no other compound can produce the same spectrum. FT-IR spectrum was recorded on Jasco4100 spectrometer at Egyptian Petroleum Research Institute (EPRT). Samples were prepared as potassium bromide (KBr) pellet and scanned against a blank KBr pellet background at wave number range 4000–400 cm^{-1} with a resolution of 4.0 cm^{-1}.

2-2-3- Preparation of CNPs.

The CNPs was prepared using a ball milling (RETSCH Planetary Ball Mills Type PM 400, Germany) at EPRT. Chitosan powder was charged and dry mixed into 250 ml stainless steel agar with 8 grinding balls at 400 rpm for 8 hours.

2-2-4- Characterization of CNPs

Transmission electron microscopy: TEM was used to image the CNPs. The CNPs were suspended in water for 3 min sonication to obtain a dilute suspension. A drop of this suspension was deposited onto a glow discharged carbon-coated microscopy grid and allowed to dry. The sample was investigated and imaged using Hitachi H-7000 TEM at EPRT.

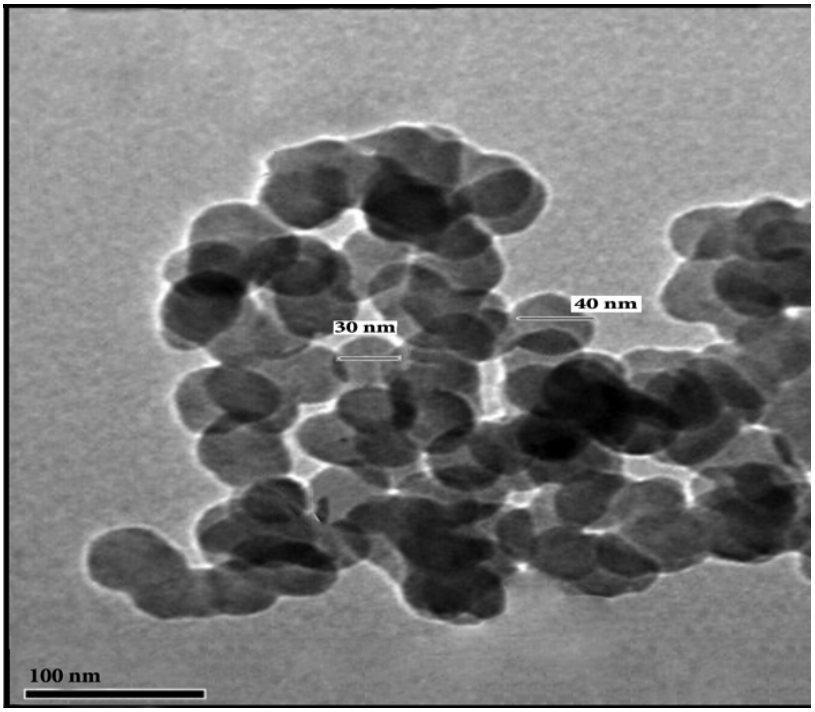

Fig. (1): TEM image of the CNPs.

The chitosan nanoparticles were spherical in shape and homogenously distributed with a particle size lower than 50 nm (Fig. 1). The CNPs powder later was dissolved in 0.1% acetic acid solution with a concentration ratio of 1g/100ml (w/v) for being applied as CNPs extracts. Fresh solutions were only prepared when required.

3- DPPH scavenging activity.

Free radical scavenging activity of different treatments were measured by 1,1-diphenyl-2-picryl hydrazyl (DPPH), 0.1 mM solution of DPPH was prepared. This solution was added to 3 ml of each treatment. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. then, absorbance was measured at 517 nm, using spectrophotometer [9]. The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using Log dose inhibition curve. Therefore serial concentrations were prepared for each treatment. Lower absorbance of the reaction indicates higher free radical activity [10]. The percent of DPPH scavenging effect calculated using the following equation: DPPH scavenging (%)= $(A_0-A_1)/A_0\times100$. Where A_0 control absorbance and A_1 sample absorbance, the control used in this study was the antioxidant and free radical scavengers Eugenol [11].

4-Cytotoxicity.

Human Liver Carcinoma cell line HepG-2, Human Colon Carcinoma cell line HCT-116 and skin normal human cell line (BJ-1) were obtained from VACSERA-Cell Culture Unit, Cairo, Egypt. These cell lines originally obtained from the American Type Culture Collection, and cultured in RPMI medium-1640 supplemented with 10 % inactivated fetal bovine serum (FBS). The reagents RPMI-1640 medium, SulphoRhodamine-B (SRB), dimethyl sulfoxide and fluorouracil (5-FU) were purchased from (Sigma Co., St. Louis, USA). Fetal bovine serum was obtained from (GIBCO, UK). The cell lines were used to

determine the inhibitory effects of different extracts on cell growth using the SRB assay. This colorimetric assay is based on the ability of SRB to bind to protein components of cells that have been fixed in tissue culture plates by trichloroacetic acid (TCA).

5- Cytotoxicity screening.

The cancer cells were cultured in RPMI-1640 medium with 10% FBS. Antibiotics were added 100 units/ml penicillin and 100µg/ml streptomycin at 37 °C in a 5% CO_2 incubator. The cells were seeded in a 96-well plate at a density of $1.0x10^4$ cells/well at 37 °C for 48 h in incubator. After incubation, the cells were treated with crude and CNPs extracts and incubated for 48 h, medium discard, fixed with 10% TCA 150 µl/well for 1 h at 4 °C and washed 3 times by water. Wells were stained by SRB 70 µl/well for 10 min at room temperature in dark place then washed with acetic acid 1% to remove unbound dye. The plates were air-dried for 24 h. The dye solubilized with 50 µl/well of 10 mM tris-base (PH 7.4) for 5 min. The optical density of each well measured at 570 nm with an ELISA microplate reader (EXL 800 USA). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated sample) X100 [12]. The IC_{50} values were calculated using multiple linear regressions [13]. The BJ-1 cells were used as a normal cell model to compare HepG-2 and HCT-116 cells. The IC_{50} value or the 50% cytotoxicity was determined from the linear equation obtained from the relation between the cell cytotoxicity % and the concentrations tested.

6- Statistical analysis.

The statistical analysis of the obtained data was done according to [14, 15]. The analysis was revised and graphics were drawn by SigmaPlot. The obtained data were assessed by calculation of the mean (M), standard deviation (SD) and student t-test.

Results

1- Antioxidant activities

1-1- Crude extracts.

The antioxidant activity of maggots crude extracts of *M. domestica*, *L. sericata* and *C. albiceps* and eugenol as a control were examined in the context of DPPH scavenging as a representative of antioxidant activity. Data obtained in (Table 1) showed that, *C. albiceps* extract exhibited the highest levels of DPPH scavenging activity; followed by *L. sericata* and *M. domestica*, the IC_{50} recorded 37.18, 72.28 and 81.5 µg/ml; respectively, compared to 4.05 µg/ml for eugenol.

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

Samples	Linear equation	R^2	IC_{50} µg/ml
M. domestica	Y= 0.5157× -3.1846	0.93503	81.5
L. sericata	Y= 0.7061× -3.1561	0.97068	72.28
C. albiceps	Y= 0.9267× +15.557	0.80864	37.18
Eugenol	Y=10.921× +5.5	0.9758	4.05

1-2- CNPs extracts.

The CNPs extracts of *M. domestica*, *L. sericata* and *C. albiceps* maggots and eugenol as a control were examined for their antioxidant activity. Data obtained in (Table 2) revealed

that, *C. albiceps* extract exhibited the highest levels of DPPH scavenging activity; followed by *L. sericata* and *M. domestica*, the IC_{50} recorded 103.13, 75.9 and 60.02 µg/ml for *M. domestica*, *L. sericata* and *C. albiceps*; respectively, compared to 4.05 µg/ml for eugenol.

Table (2): IC_{50} of DPPH scavenging activity of maggots CNPs extracts.

Samples	Linear equation	R^2	IC_{50} µg/ml
M. domestica	Y= 0.6522× -3.1648	0.96485	103.13
L. sericata	Y= 0.6962× -2.864	0.97068	75.9
C. albiceps	Y= 0.7988× +2.054	0.80864	60.02
Eugenol	Y=10.921× +5.5	0.9758	4.05

Comparing the results, on the basis of IC_{50}, *C. albiceps* extract exhibited the highest levels of DPPH scavenging activity; followed by *L. sericata* and *M. domestica* for both crude and CNPs extracts. The highest levels of DPPH scavenging activity were exhibited by the crude extracts of tested maggots with preference of *C. albiceps*, which exhibited a much more potent antioxidant activity than other tested species.

2- Anticancer activities

2-1- *Crude extracts.*

The anticancer activity of *M. domestica, L. sericata* and *C. albiceps* maggots crude extracts were examined against two human tumor cell lines. The anticancer activity was evaluated by determining the cell viability average percentages of cancer cells in the test cultures. The cell viability was variable among tumor cells tested. Data given in (Table 3, Figs. 2,3) showed that, at the highest used concentration 100 µg/ml the lowest percent of cell viability (6.7±0.7%) was recorded by *L. sericata* against HCT-116, followed by (7.57±1.25%) for *C. albiceps* against HepG-2. The cell viability percent was decreased as the concentration used increased. At the lowest concentration used 10 µg/ml, cell viability percentages recorded (88.9±0.78; 95.8±0.43%) for *L. sericata and M. domestica*; respectively against HCT-116, and it was 93.3±0.58% for *C. albiceps* against HepG-2 tumor cell line. BJ-1 was almost inactive at the highest concentration tested.

Table (3): Cytotoxicity effect *M. domestica, L. sericata* and *C. albiceps* maggots *crude extracts* against liver and colon carcinoma cell lines.

Concentrations µg/ml	Cell Viability average percentages %					
	HepG-2 cell line			HCT-116 cell line		
	M. domestica	*L. sericata*	*C. albiceps*	*M. domestica*	*L. sericata*	*C. albiceps*
100	25.9±0.9	14.43±1.5	7.57±1.25	30.1±0.11	6.7±0.7	14.1±1.0
90	37.5±0.62	19.2±1.06	11.17±1.25	33.37±0.4	10.4±0.5	19.87±0.2
80	42.17±1.12	25.2±1.59	19.07±1.16	42.03±1.82	13.93±1.0	23.87±0.8
70	51.9±1.97	36.1±1.04	29.4±0.58	52.8±1.4	26.1±1.2	36.8±0.2
60	66.07±0.2	49.57±1.4	44.7±1.4	60.13±0.23	38.5±0.6	51.23±1.6
50	70.9±1.8	61.93±1.7	53.6±1.7	69.63±0.4	51.46±1.4	63.27±0.64
40	81.97±2.0	77.0±2.6	62.67±3.8	81.93±1.95	62.7±3.8	79.0±1.0
30	87.8±0.72	95.7±2.2	69.07±1.2	86.63±1.6	69.63±1.58	94.3±1.2
20	100.0±0.0	98.78±0.0	89.67±1.5	91.43±0.47	75.3±2.0	97.8±0.3
10	100.0±0.0	100.0±0.0	93.3±0.58	95.8±0.43	88.9±0.78	100±0.0
0.0	100	100.0	100.0	100.0	100.0	100.0
BJ-1	92.9±2.01	91.8±2.5	89.3±1.3	88.9±4.7	88.9±1.1	98.0±0.8

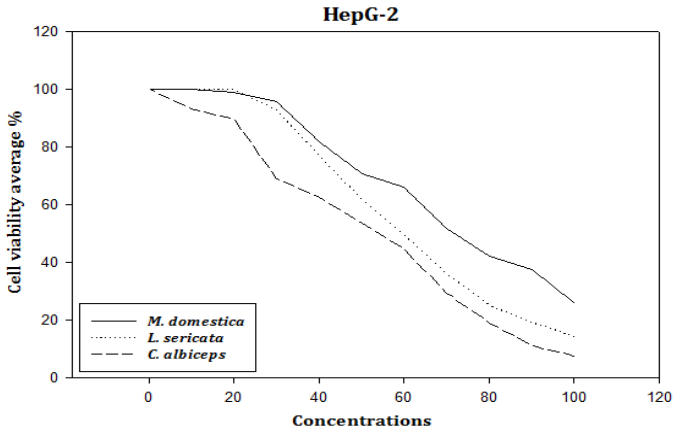

Fig. (2): Cytotoxicity curve of *M. domestica, L. sericata* and *C. albiceps* maggots crude extracts against liver carcinoma cell line.

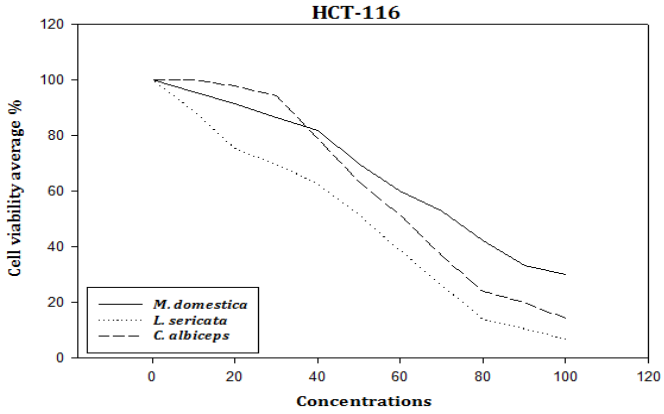

Fig. (3): Cytotoxicity curve of *M. domestica, L. sericata* and *C. albiceps* maggots crude extracts against colon carcinoma cell line.

2-2- CNPs extracts.

The anticancer activity of maggots CNPs extracts of *M. domestica, L. sericata* and *C. albiceps* were examined against HepG-2 and HCT-116 cell lines. Data given in (Table 4, Figs. 4,5) showed that, the cell viability percent was decreased as the concentration increased, the CNPs extracts revealed optimistic results in both tested cell lines, and it was highly effective. The cell viability ranged from (77.18±0.3) to (93.0±1.5) at the lowest concentration used 10 µg/ml and it was concentration dependent. Meanwhile, the highest anticancer activity was recorded at the concentrations of 80, 90 and 100 µg/ml of the different tested CNPs extracts against HepG-2 and HCT-116 cell lines tested, where the cell viability was at its lowest recorded values. BJ-1 was almost inactive at the highest concentration tested.

Table (4): Cytotoxicity effect *M. domestica, L. sericata* and *C. albiceps* maggots CNPs extracts against liver and colon carcinoma cell lines.

Concentrations µg/ml	Cell Viability average percentages %					
	HepG-2 cell line			HCT-116 cell line		
	M. domestica	*L. sericata*	*C. albiceps*	*M. domestica*	*L. sericata*	*C. albiceps*
100	1.87±0.35	1.99±0.3	1.6±0.36	1.87±0.11	1.4±0.23	1.61±0.26
90	4.3±0.36	4.3±0.47	4.03±0.15	7.3±0.2	7.5±0.18	7.9±0.3
80	9.07±0.45	9.0±0.26	9.6±0.67	10.9±0.3	11.3±0.3	11.1±0.11
70	14.4±0.47	14.8±0.26	14.1±0.15	15.53±0.5	14.7±0.3	13.2±0.41
60	21.6±0.49	19.1±0.95	17.7±1.1	22.6±0.5	20±0.7	18.87±0.5
50	28.73±0.95	31.6±1.3	27.2±1.4	32.7±0.4	30.4±0.8	28.4±1
40	43.7±1.15	45.0±0.57	39.6±0.58	43.9±1.1	41.3±0.5	40.3±0.2
30	52.0±0.46	55.0±1	49.8±0.46	56.35±0.6	55.9±0.9	53.27±0.8
20	73.6±1.8	75.0±0.2	73.0±1.2	70.9±1.1	69.9±0.3	66.8±0.43
10	89.43±0.5	93.0±1.5	86.0±0.58	88.6±0.5	85.9±0.5	77.18±0.3
0.0	100.0	100	100	100	100	100
BJ-1	96.9±2.02	95.8±1.1	96.3±1.3	98.9±1.7	98.9±3.6	98.0±5.0

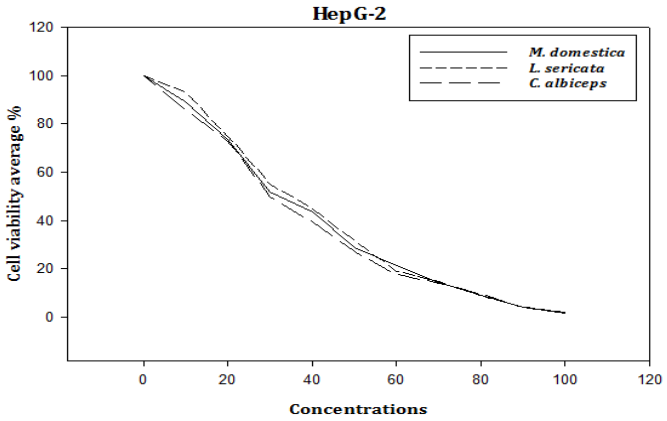

Fig. (4): Cytotoxicity curve of *M. domestica, L. sericata* and *C. albiceps* maggots CNPs extracts against liver carcinoma cell line.

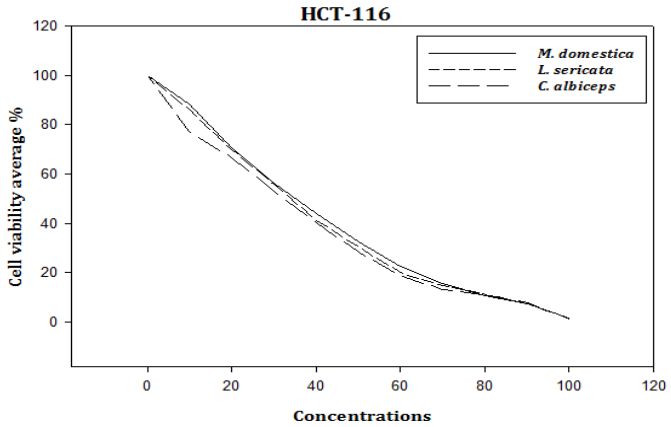

Fig. (5): Cytotoxicity curve of *M. domestica, L. sericata* and *C. albiceps* maggots CNPs extracts against colon carcinoma cell line.

Comparing the results, the cell viability was concentration dependent and it was highly affected by the CNPs treatments. The highest anticancer activity was recorded at the concentrations of 80, 90 and 100 µg/ml of different tested CNPs extracts against HepG-2 and HCT-116 cell lines.

Table (5): IC_{50} values of *M. domestica, L. sericata* and *C. albiceps* maggots crude and CNPs extracts against human Liver and Colon carcinoma cell lines.

Tested species	IC_{50} Concentrations µg/ml ± SD			
	Crude extracts		CNPs extracts	
	HepG-2	HCT-116	HepG-2	HCT-116
M. domestica	74.3±4.2	73.2±3.2	40.1±4.6	40.95±2.6
L. sericata	61.4±3.1	49.4±3.2	41.3±2.1	39.7±4.1
C. albiceps	52.8±4.8	61.8±4.1	38.5±4.8	37.3±2.1
Fluorouracil (5-FU)	28.3±2.1	19.8±2.6	28.3±2.1	19.8±2.6

Median inhibitory concentrations of *M. domestica, L. sericata* and *C. albiceps* maggots crude and CNPs extracts against tumor cells tested are summarized in Table (5). The IC_{50} values were in the range of 37.3 to 74.3µg/ml. The highest anticancer activity was obtained by *C. albiceps* CNPs extracts against cell lines tested when compared to the anticancer agent fluorouracil (5-FU).

Discussion

A primary component of insect cuticle is chitin; therefore, insects are an alternative chitin and consequently chitosan source. The production of chitin from insect has drawn increased attention because insects possess enormous biodiversity and represent 95% of the animal kingdom. Furthermore, insect cuticles have lower levels of inorganic material compared to crustacean shells, which makes their demineralization treatment more convenient [16].

Chitosan and CNPs are biopolymers that have unique structural possibilities for chemical and mechanical modifications to generate novel properties and functions. These biopolymers are biocompatible, biodegradable and nontoxic, and their chemical properties allow them to be easily processed, due to these unique properties, they are excellent candidates for cancer cure or cancer diagnosis [17].

The antioxidant effect of chitosan has been documented in several reports; the protective role of chitosan nanoparticle against oxidative stress in rat model was studied [17]; the antioxidative effect of chitosan on chronic hepatic injury in rats was also investigated [18] and the authors found that chitosan has strong antioxidative effects. Results obtained in this study may be in harmony with the previous findings, where high levels of DPPH scavenging activity were exhibited by both crude and CNPs extracts with preference to crude extracts which exhibited a much more potent antioxidant activity; followed by the CNPs extracts, indicating the overall antioxidant activity maybe due to antagonistic effect of free radicals by its antioxidant nature.

CNPs have been widely used due to its biological functions and antitumor activity; in this study, the CNPs extracts exhibited much more anticancer activity against cell lines tested than crude extracts, that might be due to a difference in the mechanism of cytotoxicity. The stronger cytotoxic effect of CNPs on tested cell lines might be related to the highly positive charged amino group that attracted to the cancer cell membrane that had a greater negative charge than normal cells. These results may be in harmony with [19] who found that chitosan display notable antitumor activity against sarcoma tumors in BALB/C mice, and [20] against Meth-A solid tumor in BALB/C mice.

Conclusion

The crude and CNPs extracts induced antioxidant and anticancer activities; the highest antioxidant activity was induced by *C. albiceps* extract as demonstrated by DPPH scavenging activity. The cell viability was concentration dependent, crude extracts have lower anticancer activity than CNPs extracts. The highest anticancer activity was recorded at concentrations of 80, 90 and 100 μg/ml of different CNPs extract tested against HepG-2 and HCT-116 cell lines. Both tested extracts have optimistic antioxidant activity. CNPs extracts have great therapeutic potential due to its anticancer inducing activities.

Conflict of Interest

The author declares that there is no conflict of interests regarding the publication of this article.

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