Chemical composition of some *Sargassum* spp. and their insecticidal evaluation on nucleopolyhedrovirus replication *in vitro* and *in vivo*

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Purpose
The application of natural products as well as biological control-specific bioagents, especially the nucleopolyhedrovirus, is considered a very important tool to ensure that pest control does not affect the environment.
Methods
Chemical constituents screening and determination of pharmacopoeial constants, and minerals and trace elements of the brown algae *Sargassum asperifolium*, *Sargassum dentifolium*, *and Sargassum linifolium* from the Red Sea (Hurghada, Egypt) were carried out.
Results and conclusion
It was found that *Sargassum* spp. have a high ash content and were positive for subliminal matter, volatile constituents, carbohydrate content, sterols, and/or triterpenes. In addition, they had high Ca, K, Mg, and Fe contents. The protein content as well as the amino acid composition of the three algae species mentioned were assessed using the Kjeldahl method and an amino acid analyzer, respectively. The hydroalcoholic extracts (70%) of *Sargassum* spp. under investigation were examined for insecticidal activities *in vitro* on *Spodoptera littoralis* (Sl52 cells) and *Spodoptera frugiperda* (Sf9 cells) and *in vivo* on *S. littoralis* nucleopolyhedrovirus replication. The results showed that the three algae species tested had various insecticidal and antiviral activities.

Keywords:
insecticidal activity, nucleopolyhedrovirus, *Sargassum asperifolium*, *Sargassum dentifolium*, *Sargassum linifolium*

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Introduction

Baculoviruses are a large group of arthropod double-stranded DNA viruses (almost 1000 species have been described) [1]. The majority have been isolated from a few insect orders: Lepidoptera, Diptera, Hymenoptera, and Coleoptera. Baculoviruses are well known because of their potential as agents of biological control of pests in agriculture and forestry.

They infect arthropods and do not replicate in vertebrates, plants, and microorganisms. Although they do not replicate, they may, under certain conditions, enter animal cells. This unexpected characteristic has made them a valuable tool in the last few years for studies of the transient expression of foreign genes in vertebrate promoters introduced into the baculovirus genome [2,3]. The accumulation of genotypic variations by serial passage in a cell culture prevents its large-scale production. One of the most important effects of the viral passage is the change from the parental, many polyhedra per cell phenotype to the few polyhedra per cell phenotype. However, the passage effect results in reduced occlusion and loss of virulence of the occluded virus [4]. The in-vitro commercial production of baculoviruses depends on the development of new techniques to sustain the production of many polyhedra through passages in cell cultures from small flasks to large-scale commercial fermentors. Future developments of the formulations of brighteners and other additives as biological materials such as algae on the basis of our work may lead reduced costs of baculovirus production. Inactivation of baculoviruses may also be caused by plant metabolites such as peroxidases, which generate free radicals [5]. The inactivation can be reduced by the addition of free radical scavengers such as mannitol, which can be found in the brown algae, or enzyme superoxide dismutase to baculovirus preparations [6]. Baculoviruses have enveloped rod-shaped virions and two distinct phenotypes in a single cycle of infection. First, the budded virus is responsible for cell-to-cell transmission of the virus. Second, the occlusion-derived virus is occluded in a proteinaceous occlusion body and is responsible for horizontal insect-to-insect transmission of the virus [7].

Seaweeds have been identified as a rich source of bioactive compounds with complex chemical structures and different

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biological activities. The seaweed extracts contain plant growth hormones, regulators, promoters carbohydrates, amino acids, antibiotics, auxins, gibberellins, and vitamins, which can be used to improve the yield of crops, seed germination, resistance to frost, and fungal and insect attacks [8]. In recent years, marine natural products from algae have been screened for larvicidal activity [9,10]. Different extracts of *Sargassum dentifolium*, collected from the Mediterranean coast of Egypt, showed potential insecticidal activity against *Spodoptera littoralis* at different stages of the life cycle [11]. *Sargassum* spp. were widely distributed on the Egyptian coasts. Therefore, marine algal products may be a suitable alternative to synthetic insecticides in the future as they are relatively safe, biodegradable, and are easily available worldwide.

The aim of the present investigation is to assess the potential of a hydroalcohol extract of the three mentioned algae species and to screen their insecticidal activities *in vitro* on *S. littoralis* (Sl52 cells) and *Spodoptera frugiperda* (Sf9 cells) and *in vivo* on *S. littoralis* nucleopolyhedrovirus replication.

Materials and methods

Thallus material

The three brown algae *Sargassum asperifolium* (Hering & G. Martens ex J. Agardh), *S. dentifolium* (Agardh), and *Sargassum linifolium* (C. Agardh) (Family: Sargassaceae) were collected at about 2–4 ft under the water surface on the Red Sea coasts in Hurghada (Egypt) during May 2007 and authenticated by Prof. Dr S.A. Shaalan (Faculty of Science, Alexandria University). The harvested algae were cleaned and washed with distilled water to remove epiphytes and encrusting materials, air dried in the shade, and finely powdered.

Preparation of crude extract

The air-dried, powdered thallus (100 g) from each collected sample was exhaustively extracted several times with 70% ethanol. Each extract was filtered and the solvent was distilled off under vacuum.

Preparation of successive extracts

The air-dried, powdered thallus (100 g) from each sample was extracted exhaustively with petroleum ether (40–60°C), ether, chloroform, ethyl acetate, and methanol in a Soxhlet apparatus. The solvent was distilled off under vacuum and weighed. Each extract of the algae under investigation was weighed and the yield percentages are presented in Table 1.

Determination of pharmacopoeial constants

Moisture contents: Each air-dried algal powder (10 g) was dried in an electric oven at 105 ± 2°C for 5 h; thereafter, it was cooled in a desiccator and weighed. The processes were repeated until there was no difference between two successive weightings [12]:

$$\text{Moisture percentage for air dried powder algae} = 100(W_1 - W_2)/W_1 - W,$$

Table 1 Pharmacopoeial constants and yields % of the brown algae *Sargassum* spp.

	Mean ± SEM (%)		
Fraction	S1	S2	S3
Moisture constant	5.549 ± 0.028	5.98 ± 0.005	6.308 ± 0.008
Total ash	69.98 ± 0.012	69.85 ± 0.012	65.31 ± 0.015
Acid-insoluble ash	34.22 ± 0.012	34.63 ± 0.017	37.90 ± 0.035
Water-insoluble ash	29.73 ± 0.039	29.02 ± 0.017	25.79 ± 0.023
Petroleum ether	0.33 ± 0.012	0.38 ± 0.011	1.08 ± 0.015
Ether	0.38 ± 0.018	0.18 ± 0.005	0.26 ± 0.008
Chloroform	0.17 ± 0.008	0.45 ± 0.011	0.13 ± 0.015
Ethyl acetate	0.15 ± 0.005	0.14 ± 0.00	0.18 ± 0.003
Methanol	17.29 ± 0.074	13.27 ± 0.020	18.80 ± 0.028

S1, *Sargassum asperifolium*; S2, *Sargassum dentifolium*; S3, *Sargassum linifolium*.

where W_1 is the weight in grams of the crucible with the sample before heating, W_2 is the weight in grams of the crucible with the sample after heating, and W is the weight in grams of the empty crucible.

The procedure was carried out in triplicate and the average percentages are presented in Table 1.

Ash contents: The ash content of demoisturized algal samples was determined by calcinations in a muffle furnace at 550°C until a constant weight was attained [13]:

$$\text{Ash}\,\% = (W_1/W_2) \times 100,$$

where W_1 is the weight of ash and W_2 is the initial weight of the sample dried at 105°C.

The average percentages of the three determinations are presented in Table 1.

Screening of the chemical constituents: The air-dried powdered thalli of *Sargassum* spp. were subjected to a chemical screening test for crystalline sublimate [14], alkaloids [15], anthraquinone glycosides [15], saponin [15], cardiac glycoside [15], coumarin, and essential oil [15], Liebermann–Bürchard's test for steroids [15], Molisch's test for carbohydrates [16], and Shinoda's test for flavonoids [17].

Determination of minerals and trace elements

Sample preparation: Each of the air-dried powdered sample of *Sargassum* spp. (1 g) was dry ashed in a muffle furnace at 550°C for 6 h. The ash was digested in 3 N HNO_3 and the residue was then suspended in 3 N HCl [18]. Bidistilled water was added to the extract up to 50 ml volume in a glass-measuring flask.

Measurements: Major mineral elements (P, Ca, Mg, Na, K) and trace elements (Fe, Mn, Zn, and Cu) were determined using a Perkin-Elmer 1100B atomic absorption spectrophotometer (Norwalk, Connecticut, USA) equipped with a single hollow cathode lamp for each element and an air–acetylene burner [19] against mineral elements standards (Merck, Germany). The results are presented in Table 2.

Determination of protein and amino acids

Determination of protein contents: Determination of the protein content was carried out by the micro Kjeldahl

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method using a Markham distillation apparatus [20]. The percentage of proteins in the extract was determined by multiplying the total nitrogen by a conversion factor of 6.25.

Determination of free and total amino acids: The amino acid content was determined as described by Moore *et al.* [21]. The analysis was performed in the Central Service unit, National Research Centre, Egypt, using an LC 3000 amino acid analyzer (Eppendorf-Biotronik, Maintal, Germany). The technique involved the separation of amino acids using strong cation exchange chromatography, followed by the ninhydrin color reaction and photometric detection at 570 nm. Standard amino acids were used for the comparison of the resulting profile, allowing quantification of amino acid residues. The defatted powdered algae under investigation were hydrolyzed with 6 N HCl at 110°C in teflon-capped vials for 24 h. After vacuum removal of HCl, the residues were dissolved in a lithium citrate buffer, pH 2.2. Twenty microliters of the solution was loaded onto a cation exchange column (pre-equilibrated with the same buffer). Then four lithium citrate buffers with pH values of 2.2, 2.8, 3.3, and 3.7, respectively, were successively applied to the column at a flow rate of 20 ml/min. The ninhydrin flow rate was 10 ml/h under these conditions and a typical analysis required 160 min. The amino acid contents are presented in Table 3.

Insecticidal activity

Preparation of a different concentration

Each 70% alcoholic extract of the residue (100 mg) of *S. asperifolium*, *S. dentifolium*, and *S. linifolium* was dissolved in 3 ml of ethyl alcohol (stock solution). A dilution of 1×10^{-2} was prepared from the stock solution diluted with ethanol.

Cell line preparation and treatment for insecticidal investigation

The Sf9 cell line is a cloned cell line from the ovary of *S. frugiperda* and the cell line Sl52 is a cloned cell line from the ovary of *S. littoralis* pupae, which were provided by Dr G. Croizier (IRNA, St Christol, Les Alès, France). The cell lines Sf9 and Sl52 were maintained at 19 and 27°C, respectively, as an attached cell line in Grace's modified media [22] with 15% fetal bovine serum.

To test the influence of extracts on viral multiplication, viral inoculation was carried out with 250 µl of 0.45 µm filtered virions/dish of SlNPV (Egyptian isolate, multiplied, purified, and cloned *in vivo* and *in vitro*). Treatments were conducted each in three replicates using two concentrations, stock

Table 2 Minerals and trace element contents in the thallus of *Sargassum* spp.

	Mean ± SEM (%)								
	Macronutrients (mg/100 g)					Micronutrients (mg/100 g)			
Algal sample	P	K	Mg	Na	Ca	Fe	Mn	Zn	Cu
S1	200 ± 0.88	3670 ± 0.88	760 ± 1.20	74 ± 0.57	7760 ± 1.66	0.0772 ± 0.002	0.0060 ± 0.003	0.0020 ± 0.005	0.00064 ± 0.0005
S2	160 ± 0.76	4410 ± 3.17	800 ± 2.08	128 ± 0.73	5910 ± 2.96	0.0385 ± 0.003	0.0024 ± 0.008	0.0021 ± 0.00	0.00056 ± 0.001
S3	180 ± 0.66	430 ± 1.15	760 ± 2.00	32 ± 0.33	7830 ± 2.20	0.0712 ± 0.006	0.0036 ± 0.005	0.0039 ± 0.012	0.00100 ± 0.002

S1, *Sargassum asperifolium*; S2, *Sargassum dentifolium*; S3, *Sargassum linifolium*.

Table 3 Amino acid composition of the brown algae of *Sargassum* spp. as determined by high-performance liquid chromatography

		Free amino acids (mg/100 g dry weight powdered)			Total amino acids (mg/100 g dry weight powdered)		
Amino acids	Algal samples	S1	S2	S3	S1	S2	S3
Essential amino acids	Threonine	16.008	16.395	157.862	0.093	0.091	0.057
	Valine	21.801	16.574	26.066	0.096	0.100	–
	Methionine	–	–	10.717	–	–	0.145
	Isoleucine	22.832	7.002	23.146	0.071	0.079	–
	Leucine	56.407	26.403	59.059	0.349	0.378	0.055
	Phenylalanine	13.400	36.917	41.751	0.242	0.254	0.045
	Lysine	27.236	14.29	21.355	0.117	0.125	0.082
	Total	157.684	117.581	339.959	0.968	1.027	0.384
Nonessential amino acids	Aspartic acid	56.060	102.598	85.175	0.191	0.177	0.286
	Glutamic acid	61.495	165.651	–	0.281	0.529	0.297
	Serine	24.707	68.151	–	0.139	0.172	0.090
	Glycine	14.78	41.400	55.764	–	–	0.134
	Histidine	–	12.586	18.733	0.124	0.117	0.255
	Arginine	–	16.365	–	0.051	0.050	–
	Alanine	53.640	–	–	0.281	0.299	0.181
	Proline	39.033	148.537	51.779	0.325	0.233	0.471
	Tyrosine	20.581	47.133	46.303	0.179	0.200	0.181
	Cystine	–	167.655	–	–	–	–
	Total	270.296	770.076	257.754	1.571	1.777	1.895
Total contents of amino acids		427.98	887.657	597.713	2.539	2.804	2.279

S1, *Sargassum asperifolium*; S2, *Sargassum dentifolium*; S3, *Sargassum linifolium*.

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solution (1.1 mg/250 μl) and a 1×10^{-2} dilution of the ethanolic prepared extract. Sl52 and Sf9 cells were harvested from late log-phase growth from f30 T flasks and treatments were carried out on C35 culture dishes (3.5 cm diameter small plastic Petri dishes) containing 2 ml media 2×10^5 cells/dish. Cells were observed daily and counted five times within 15 days. Cell viability was detected using viability staining (trypan blue). The cytopathic effect of cells (polyhedra in the nucleus) was observed and photographed under a phase-contrast inverted microscope at $\times 450$. 250 μl of 1×10^{-2} dilution and extract stock solution were added separately to the cell dishes to a final solvent concentration of 0.5% ethanol [23,24]. Sl52 cells were incubated at 27°C and Sf9 cells were incubated at 19°C. Cells were observed daily and counted 4 h after treatments and then five times over 15 days using a Thoma hemocytometer (Marienfeld GmbH, Marienfeld, Germany). The observations are presented in Table 4.

Assay method for insecticidal activity in vivo

Three groups of 10 third instar larvae of *S. littoralis* were fed on treated discs of semi-synthetic media with 250 μl of extract stock solution. In the other two groups, one was infected with the baculovirus (SlNPV) and treated with 250 μl of extract stock solution. The other group, used as a control, was infected with the virus without the alcoholic extract and was used as a positive control. Each treatment was carried out in three replicates. The percentage of mortality is presented in Table 5.

Results and discussion

Pharmacopoeial constants of the brown algae *Sargassum* spp.

The average percentages of moisture and ash values of *Sargassum* spp. under investigation are shown in Table 1. These constants could be used as the criteria for the determination of the identity and purity of the thallus of *Sargassum* spp. The high total ash content reflects the high mineral content.

The chemical screening of *Sargassum* spp.

The chemical screening indicated that the three algal samples contained crystalline sublimate, essential oil, sterols, carbohydrates, alkaloids, and/or nitrogenous bases. These chemicals may be responsible for their insecticidal properties [25]. The presence of terpenoids indicated that the algae can act mainly as an antifeedant and growth disruptor and may be considerably toxic toward insects [26].

Minerals and trace elements

Analysis of the total ash content of *Sargassum* spp. under test indicated the presence of the macroelements P, K, Mg, Na, and Ca and microelements Fe, Mn, Zn, and Cu

Table 4 Effect of crude extract of the brown algae *Sargassum* spp. on the division of *Spodoptera littoralis* Sl52 and *Spodopterafrugiperdae* Sf9 cell lines *in vitro*

		Observation			
		Spodoptera littoralis Sl52		*Spodoptera frugiperda* Sf9	
Algal sample	Concentration	Without NPV	With NPV	Without NPV	With NPV
S1	Stock solution	Overmultiplication	Overmultiplication and no viral symptoms	Cells were granules and agglomerated. Number of cells was clearly less than those in the control	Highly damaged cells and chance to developed virus
	1×10^{-2} of stock solution	Overmultiplication	Overmultiplication and no viral symptoms	Cell damage	Vacuoles and damaged cells-some fibroblasts
S2	Stock solution	Destroyed cells	Healthy cells and viral symptoms are clear	Detached cells	Granulated cells were healthy and contained virus
	1×10^{-2} of stock solution	Destroyed cells	Overmultiplication	Healthy cells and number less than that in the other treatment	Damaged cells
S3	Stock solution	Destroyed cells	Overmultiplication and no viral symptoms	Completely destroyed and with a clear cytopathic effect	–
	1×10^{-2} of stock solution	Overmultiplication	Overmultiplication and a huge number of viruses	Overmultiplication	Healthy cells of several viral polyhedra

NPV, nucleopolyhedrovirus; S1, *Sargassum asperifolium*; S2, *Sargassum dentifolium*; S3, *Sargassum linifolium*.

Table 5 Effect of alcoholic extract of *Sargassum* spp. on third instar larvae of *Spodoptera littoralis* and on the replication of the baculovirus (nucleopolyhedrovirus), *in vivo*

	Mortality (%)		
Treatment	S1	S2	S3
Control (healthy *Spodoptera littoralis*)	2.3	2.3	2.3
Control (*S. littoralis* inoculated with NPV)	62	62	62
S. littoralis with alcoholic extract alone	0	10	10
S. littoralis-infected NPV and treatment with alcoholic extract	38.6	74	46.3

NPV, nucleopolyhedrovirus; S1, *Sargassum asperifolium*; S2, *Sargassum dentifolium*; S3, *Sargassum linifolium*.

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(Table 2). Calcium was the major element in the three algal samples. Therefore, *S. asperifolium*, *S. dentifolium*, and *S. linifolium* can be used as nutritive fertilizers as well as supplements for minerals. The mineral content of seaweeds is higher than that of land plants [27].

Protein content

The protein contents of *S. asperifolium*, *S. dentifolium*, and *S. linifolium* were found to be 3.59, 4.13, and 3.52% w/w, respectively, on a dry weight basis.

Amino acid composition

High-performance liquid chromatography analyses of the free and bounded amino acid contents of the algae examined are presented in Table 3; 13 and 14 free and total amino acids were found in *S. asperifolium*. *S. dentifolium* had 15 and 14 amino acids, whereas *S. linifolium* had 12 and 13 free and total amino acids.

Glutamic acid, leucine, and aspartic acid were the major free amino acids found in *S. asperifolium*, whereas cysteine, glutamic acid, and proline were the main free amino acids found in *S. dentifolium*. However, threonine was the major free amino acid found in *S. linifolium*. The proteins of *S. asperifolium* and *S. dentifolium* were characterized by high contents of essential amino acids leucine and phenylalanine. The high levels of aspartic and glutamine acid are responsible for the special flavor and taste of seaweeds [28]. Furthermore, methionine (S-containing amino acid), which is deficient in beans [29], was found only in the free and bounded amino acid of *S. linifolium*. This is the first report of the amino acid contents of *S. asperifolium*, *S. dentifolium*, and *S. linifolium*.

Figure 1

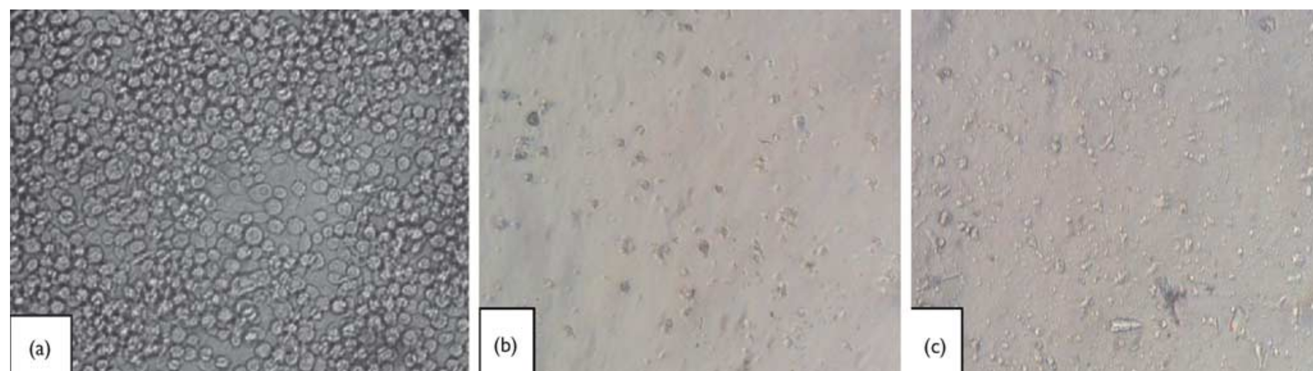
Comparison of control nontreated Sl52 cells with treated cells with a stock solution of *Sargassum asperifolium*. The cells showed over-multiplication.

Insecticidal activity of *Sargassum* spp.

The results in Table 4 show the *in-vitro* treatments of the crude extracts of *S. asperifolium*, *S. dentifolium*, and *S. linifolium*. Two concentrations of stock solutions and 1×10^{-2} dilution were tested in both types of cell cultures (Sl52 and Sf9) alone and in the presence of NPV. For all treatments *in vitro*, it was found that the treatment with stock solutions of all extracts considerably damaged the cultured cells, but the treatment of Sl52 cells with the *S. asperifolium* extract resulted in the multiplication of cells (Fig. 1).

The *in-vitro* treatments of both concentrations of *S. asperifolium* on the two types of cells Sl52 and Sf9 resulted in an antiviral effect despite the positive effect on cells that was found in the multiplication of cells when compared with the control nontreated cells. These results confirmed the result obtained *in vivo* using third instar larvae of *S. littoralis* (Table 5). No mortality was found in third instar larvae of (*S. littoralis*) when treated with 250 μl of stock solution alone of *S. asperifolium*, but in the presence of viral infection, it caused 38.6% mortality; 62% mortality was found in the larvae infected with NPV. However, treatment with the *S. dentifolium* extract using two concentrations, a stock solution and 1×10^{-2} dilution, resulted in the complete destruction of the Sl52 cells, no division was observed, and cells were granulated. Furthermore, effect of the same extract with viral treatment on the same type of cells was different: healthy, different cells and over-multiplied cells were observed, and the division rate was high compared with the nontreated control cells. In addition, no viral symptoms or cytopathic effects were observed. Meanwhile, the treatment of cells Sf9 with a stock solution of *S. dentifolium* induced the appearance of detached cells (Fig. 2b). The use of a 10^{-2} dilution of *S. dentifolium* extract resulted in healthy cells and they were almost the same as control nontreated cells. However, viral treatment with the stock solution exerted a clear

Figure 2



(a) Control nontreated Sf9 cells. (b) Treated cells Sf9 with *Sargassum dentifolium* note the affected detached cells. (c) Treated cells Sf9 with *Sargassum linifolium* were completely destroyed and there was a clear cytopathic effect.

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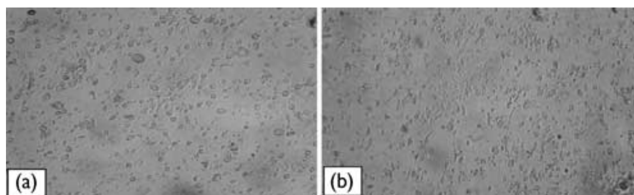
Figure 3



Sf9 cells treated with a stock solution and 1×10^{-2} dilution of *Sargassum dentifolium* extract with viral treatment; note that granulated cells contained the virus and cells in (a) seem to be more healthy compared with the damaged cells in (b).

cytopathic effect and cells were granulated and polyhedral (Fig. 3a). However, the 10^{-2} dilution induced damage and no viral cytopathic effect was observed (Fig. 3b). These results are in agreement with and confirmed the results obtained *in vivo* with the use of third instar larvae of *S. littoralis* (Table 5), which shows that *S. dentifolium* extract has insecticidal activity, but does not exert an antiviral effect. The dilution of a 250 μl stock solution of *S. dentifolium* extract led to 10% mortality of *S. littoralis* larvae and 74% mortality of *S. littoralis* larvae infected with NPV.

Moreover, the stock solution of *S. linifolium* extract damaged the Sl52 and Sf9 cells with high cytopathic effect (Fig. 2c), whereas the 10^{-2} dilution of *S. linifolium* promoted cell division and viral multiplication. These results are in agreement with the results obtained *in vivo* because the percentage of mortality and infectivity of viral treatment alone was 62%, but in the presence of *S. linifolium* extract, it was 46%, which shows that *S. linifolium* extract exerts an antiviral effect. Also, the insecticidal effect of *S. linifolium* extract at a dilution of 250 μl was 10% of larval mortality.

Conclusion

The present study showed that the hydroalcoholic extract of *S. dentifolium* has potent insecticidal activity against *S. littoralis* and *S. frugiperda*, alone or in combination with NPV, and may be used as a natural insecticide. In addition, *Sargassum* spp. have significant amounts of micronutrients and amino acids that may promote plant growth.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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