Nematodes are microscopic roundworms that live in many habitats. At least 2,500 species of plant-parasitic nematodes have been described, characterized by the presence of a stylet, which is used for penetration of host plant tissue. Most of them attack roots and underground parts of plants, but some are able to feed on leaves and flowers. Plant-parasitic nematodes are of great economic importance. However, because most of them live in the soil, they represent one of the most difficult pest problems to identify, demonstrate and control [1]. Their effects are commonly underestimated by farmers, agronomists and pest management consultants, but it has been estimated that some 10% of world crop production is lost as a result of plant nematode damage [2].

Management of soil-borne plant pathogens, including parasitic nematodes, is one of the greatest challenges facing modern agriculture worldwide [3-7]. The importance of soil-borne pathogens in modern agriculture systems is made especially clear by the current concern worldwide to find alternatives to methyl bromide for pre-plant treatment of soils used to produce certain high-value crops. Losses caused by plant parasitic nematodes are estimated about US $100 billion annually [8].

The field of nanotechnology is one among the foremost important and active areas of research in modern science. Nanotechnology deals with the formulation of experimental processes for the synthesis of nanoparticles with different sizes and shapes [9]. The application of nanoparticles (usually ranging from 1 to 100 nm) is a developing and interesting area of nanotechnology.

The interesting physical properties of graphene, which consists of two-dimensional (2D) sheet of covalently bonded carbon atoms [10], have led to much excitement in recent years in material science and condensed-matter physics. There are potential applications of graphene for nanoelectronics. This is due to the excellent physical and chemical properties, and remarkable electronic properties of graphene which make it an ideal candidate for several applications [11].

The biological applications of graphene and the reduced graphene oxide r-GO remain unexplored and wide-open, however. There are several prerequisites for biological applications for a new study. First, rational functionalization chemistry is needed to impart graphene with aqueous solubility and biocompatibility. R-GO and its chemically converted derivatives form stable suspensions in pure water, but generally aggregate in salt or other biological solutions [12-16]. For that reasons this study aimed to examine the bioactivity of the reduced graphene oxide as control agent for nematode. The toxicity of the r-GO was examined to avoid any hazardous effect on the health of both plant and animal compared with the chemically synthesized nematocide.

Keywords: Meloidogyne incognita, carbon nanostructure, reduced graphene oxide r-GO, cytotoxicity and nematocide.
Experimental part

Material and methods

Root-knot nematode, Meloidogyne incognita was obtained from the Nematology Research Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University.

Preparation of reduced graphene oxide r-GO

Reduced graphene oxide r-GO was prepared as follows: 3.0 g of graphite flakes were added to a 9:1 mixture of concentrated H2SO4/H2O (360:40 mL) in ice bath. Then 18 g of KMnO4 were added very slowly to the mixture and heated to 50°C and stirred for 12 h. The mixture was cooled to room temperature and then poured onto ice (400 mL deionized water with 3 mL 30% H2O2). The obtained r-GO was filtered and then washed with a lot of deionized water by ultra-sonication. The obtained brown dispersion was then subjected to 30 min of centrifugation at 4000 rpm, to remove any un-exfoliated r-GO.

Characterization of r-GO

The crystal structure was determined by XRD analysis performed on Shimadzu X-ray diffractometer, operated at 40 kV and 30 mA with Cu Ka radiation. The morphologies of the prepared samples were investigated by Scanning Electron Microscopy (SEM, JEOL JSM 6360LA, Japan) and Transmission Electron Microscopy (TEM, JEOL JEM-2100 plus, Japan). Raman spectra of the samples were measured (using Bruker, Senterra, Germany). The physical properties of the synthesized r-GO, with different morphological structures, were investigated using different techniques. The morphological structure and the chemical compositions of the r-GO nano powder were examined.

Transmission Electron Microscopy (TEM)

TEM is used to scan a finely focused electron beam across the surface of a specimen. The reflected signals are collected, and their intensities are displayed on a cathode-ray-tube screen by brightness modulation. As already indicated, the method allows specimen magnifications to 300,000X, while maintaining a large depth of focus. The ease of sample scanning by scanning electron microscope (over large distances) is quite appealing, in that a large sample viewing area is first surveyed (at generally low magnification) to seek out particular areas of interest, followed by high magnification of those specific areas for subsequent detailed investigations. The TEM is also extensively employed for the generation of dimensional and spatial relationship details of structure elements.

Determination of cytotoxicity effect of r-GO and Commercial nematocide using MTT assay

Human peripheral blood mononuclear cells (PBMCs), human normal fetal lung cell line (WI-38), and normal adult African green monkey kidney cell line (Vero) were used to investigate the toxicity of r-GO and of the commercial nematocidal (Vydate® L and Nemaphos 40%), according to the method described by Mosmann [17]. PBMCs are the most available sources of human normal cells for investigating the toxicity of any compounds. Human PBMCs were isolated according to the Ficoll-Hypaque density gradient centrifugation method [18]. The heparinized blood was gradually added over an equal volume of the Ficoll-Hypaque solution (density=1.077 g/mL) and centrifuged at 2000 rpm for 30 min. The PBMCs at buffy layer were collected, suspended in PBS and centrifuged for 5 min at 1650 rpm. Cells were resuspended in RPMI 1640 medium (Lonza, USA) containing 10% fetal bovine serum (GIBCO, USA); then the viability was determined by staining of 50 µL of PBMCs with 0.5% trypan blue and counting on a hemocytometer.

Human WI-38 and mammalian Vero cells were maintained in DMEM medium (Lonza, USA), containing 10% fetal bovine serum. These cell lines were subcultured for 2 weeks before assay using trypsin EDTA (Lonza, USA). Their viability and counting were detected by trypan blue stain and hemocytometer. Human PBMCs, WI-38 and Vero were seeded in 96 well culture plates 10³, 10⁴ and 10⁵ cells per well, respectively, and incubated at 37°C in 5% CO₂ incubator. After 24 h, the cells were treated with the serial dilutions of graphene oxide and commercial nematocidal (Vydate® L and Nemaphos 40%) (0, 6.25, 12.5, 25, 50 and 100 µg/mL). After 72 h of incubation in 5% CO₂ incubator, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4h in 5% CO₂ incubator. MTT (Sigma, USA) solution was removed after centrifugation at 2000 rpm for 10 min and the insoluble blue formazan crystals trapped in cells were solubilized with 150 µL of 100% DMSO at 37°C for 10 min. The absorbance of each well was measured with a microplate reader (BMG Lab Tech, Germany), at 570 nm.

Determination half maximal inhibitory concentration (IC50) and safe dose (EC100) values

The half maximal inhibitory concentration (IC50) and safe dose (EC100) values were determined using GraphpadInstat software as the concentration of r-GO and commercial nematicidal (Vydate® L and Nemaphos 40%) that caused 50% and 100% cell viability, respectively; used data were calculated from the equation of cell viability. Additionally, morphological changes of r-GO and commercial nematicidal (Vydate® L and Nemaphos 40%) treated normal human and mammalian cells were investigated in comparison with untreated control cells, using phase contrast microscope supplemented with digital camera (Olympus, Japan).

Analysis of the cytotoxicity effect of r-GO and Vydate® L using fluorescence phase contrast microscope

Human PBMCs and normal cell lines were treated with 6.25 µg/mL of each tested r-GO and commercial nematicidal product (Vydate® L and Nemaphos 40%). After incubation for 72 h, the cells were stained with 100µg/mL of double fluorescent nuclear dyes; ethidium bromide and acridine orange (Sigma, USA) and then investigated using fluorescence phase contrast microscope (Olympus, Japan).

Statistical analysis

All data obtained from laboratory bioassay and pots experiment were analyzed using analysis of variance (ANOVA). The significant differences among treatments were determined according to the least significant differences (LSD), at p<0.05 level of probability using CoStat software.
Results and discussions

1-Reduced Graphene synthesis and characterization

Chemical oxidation is the best method for graphene oxide and the morphology of the synthesized reduced graphene oxide may be graphene oxide (GO) or graphene nanoplatelet [19]. Oxidized graphene could be used in different applications especially as nematicide [20-22]. Some authors used the graphene oxide in nematode control and they succeeded to identify many of microRNA which plays an important role in biocontrol of such pests [23,24].

1-1-Using Scanning and Transmission Electron Microscopes

The obtained materials were characterized using SEM, TEM, and the results presented in figures 1 and 2 revealed that the r-GO appeared as nano-sheets. The size of the obtained nanoparticles ranged from 20 to 100 nm.

1-2- X-Ray Diffraction

Data obtained by the XRD (presented in fig. 2) showed a broad diffraction peak observed at 2θ = 24.38, which indicated that the obtained material is graphene r-GO. This result was previously confirmed by other studies [25,26]; moreover, it was also confirmed using Raman analysis.

1-3- Raman

Data presented in figure 3 demonstrated that the obtained Raman spectra (which displayed the two main bands: D band at 1350 cm⁻¹ and the G band at 1590 cm⁻¹) are the characteristics bands of graphene and/or the reduced graphene oxide [10]. This result confirmed the graphene structure obtained by XRD pattern.

The cytotoxicity of the produced r-GO

The largest IC₅₀ and EC₁₀₀ values indicate the highest safety of the two nematicides compared with the r-GO. Table (1) illustrates that three tested compounds had the highest IC₅₀ (362.1, 86.2, and 203.9 µg/mL) and EC₁₀₀ (130, 52.1, and 117.7 µg/mL) doses against normal PBMCs, Wi-38, and Vero cells. Moreover, there is no morphological differences were observed between untreated control cells in comparing with the treated cell lines. The IC₅₀ and IC₁₀₀ of r-GO were significantly (p<0.001) higher than that of Vydate® L, Nemaphos 40%. Cytotoxicity of Vydate® L was intermediate between r-GO and Nemaphos 40%. Nemaphos 40% is the most toxic compound on all tested normal human and mammalian cells (with the lowest IC₅₀ and EC₁₀₀ values, less than 17 µg/mL against PBMCs and 3 µg/mL against Wi-38 and Vero cell lines).

Table (1) THE IC₅₀ AND EC₁₀₀ (µg/mL) OF VYDATE® L, NEMAPHOS 40% AND r-GO AGAINST Wi-38, PBMCs AND VERO CELLS

| Nematicides   | IC₅₀  | EC₁₀₀  | IC₅₀  | EC₁₀₀  | IC₅₀  | EC₁₀₀  |
|---------------|-------|--------|-------|--------|-------|--------|
|               | WI-38 | PBMCs  | Vero  | cells  | cells  | cells  |
| Vydate®       | 49.7±3.4  | 25.8±2.0  | 126.7±5.0  | 52.8±3.5  | 57.8±2.9  | 28.9±0.5  |
| Nemaphos 40%  | 6.7±0.03  | 0.14±0.09  | 16.7±0.48  | 5.3±0.37  | 2.6±0.36  | 0.13±0.09  |
| r-GO          | 86.2±1.4  | 52.0±1.0  | 362.1±0.5  | 130±2.1  | 203.9±3.5  | 117.5±1.2  |

*aAll values are expressed as mean± SEM. Different letters of the same column indicate significantly differences at p<0.05.
The highest cytotoxicity of Nemaphos 40% was confirmed by figure 4 (I, II and IIIB) that demonstrated severe alterations in normal shape of all investigated cells while r-GO and Vydate® L treated cells showed normal morphology (fig. 4, I, II, IIIA and C). Moreover, nuclei of Nemaphos 40% treated normal human cells exerted orange and red fluorescences when were stained with Ethidium Bromide-Acridine orange dyes that assured late stage of apoptosis was occurred (fig.5 I, II, and IIIB). 

Vydate® L treated PBMCs exhibited green color while Vydate® L treated Wi-38 and Vero cells emitted green with limited area of bright green or yellow fluorescence (fig.5.1, II, and IIIIA). r-GO treated human cells have green nuclei like that of untreated human cells. This indicates the highest safety of r-GO towards human cells compared to other two investigated nematicide (fig.5 I, II, and III). On the contrary, Liao et al reported that r-GO has cytoxicity through oxidative induction, suppression of cell division which leads to the cell death [27]. Several studies postulated that r-GO has immunotoxicity [21, 28,29]. Moreover, the cytotoxicity of the r-GO was examined in vivo either on rat or mice and it was observed that r-GO causes pulmonary and reproductive toxicity [22, 30-33]. Different reports on the toxicological studies on the environment and on the nematodes (Caenorhabditis elegans) as animal models and concluded that r-GO has toxicity on the nematode [34-37].

Results presented in table 2 revealed that the nematode galls were controlled by the r-GO and Vydate L with percentage of 94 and 93 respectively, whereas the nematode egg mass was controlled in equal manner. On the other hand, reduction in the number of J2 was recorded as 67 and 80% with Vydate L and r-GO respectively. 

Data presented in table 3 showed that there is an increase in shoot and root (dry and fresh) and in chlorophyll as well with percentage ranged from 20 to 285%. Wu et al. [9] used different concentrations of r-GO and they found that concentrations of 10-100 mg/L showed control activity against the J2. The same observation was reported by Yang et al. [20,22] that r-GO at the concentration of 100 mg/L reduced the lifespan of the larvae and inhibit their locomotion activity. The results obtained by Zhi et al. [28] confirm the findings in this study and also that obtained by Yang et al. [20,22] that r-GO is capable to control the nematode through inhibiting the ROS of its biological system. Other authors [38,39] confirm the results obtained in this study and they added that many regulatory genes of the treated nematode by r-GO could be affected and resulted in the death of the animal. Data represented in table 4 showed that the J2 mortality after 6 hrs with the
Table 3

THE EFFICACY OF r-GO AND VYDATE L® 5 24% ON GROWTH PARAMETERS AND CHLOROPHYLL CONTENTS OF TOMATO PLANTS INFECTED WITH M. INCognITA (MI) AND INCREASE % (I)

| Treatment      | Fresh weight (g) | Dry weight (g) | Chlorophyll (U) | I |
|----------------|------------------|----------------|-----------------|---|
|                | Shoot  | Root | Shoot | Root |                | |
| Check          | 13.7%  | -    | 5.94% | -    | 3.73%          | - |
| Vydate® L      | 15.4%  | -    | 8.0%  | -    | 3.75%          | - |
| r-GO           | 20.4%  | -    | 30.0% | 22.9% | 8.4%           | - |

Data are means of 5 replicates; means with the same letter(s), in each column, are not significantly different at (p<0.05). Check - plants inoculated with Meloidogyne incognita. r-GO - reduced Graphene oxide.

Table 4

EVALUATION OF THE NEMATICIDAL EFFECTS OF REDUCED GRAPHENE OXIDE ON J2 MORTALITY OF M. INCognITA AND REDUCTION % (R) AFTER 6 AND 48 h

| Type of the treatment | 6 hrs | 48 hrs |
|-----------------------|-------|--------|
|                       | S     | R/2    | R     | S     | R/2    | R     |
| Water                 | 100%* | -      | 100%* | -     | 230%* | -     |
| Vydate® L             | 96.2% | -      | 98.2% | 3.0%  | 95.2%  | 8.9%  |
| r-GO                  | 18.5% | 69.3%  | 89.5% | 8.4%  | 89.5%  | 9.8%  |

Data are means of 5 replicates; means with the same letter(s), in each column, are not significantly different at (P<0.05).

Vydate® L was of 96% followed by a decrease r-GO 89%, respectively.

On the other hand, the J2 mortality with 50% Vydate® L (96.2%) decreased, followed by 50% r-GO 89.5% decrease. Moreover, it was observed that there is no difference in the results obtained either r-GO or Vydate® L. Some authors used the biosynthesized nanosilvers as nematocide and they postulated that the green synthesis nano metal could be a safe substitution to the chemical pesticides [40,41]. Different studies reported that r-GO has cytotoxicity against the nematode and they postulated that the r-GO kill the nematode through activating the oxidative stress in the animal [30,41,42]. We assume that the r-GO has cell specificity, because it has no toxicity on the human cells, but it has high toxicity on the nematode cells.

Conclusions

Reduced graphene oxide sheets could be used as nematocide with efficacy more than 90%. In addition, it has no toxicity on human cells which recommend this product as a new pesticide without hazards on human being. Also, it is the main constituent of soil composition, and it has no harmful effects on the soil chemistry.

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