Cytotoxicity and Antioxidant Activity of *Beta vulgaris* Extract Released from Grafted Carbon Nanotubes Based Nanocomposites

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Summary: This work deals with preparation and characterization of novel grafted multi-walled carbon nanotubes (MWCNTs) with dodecenyl succinic anhydride (DSA) in presence of plant extract (*Beta vulgaris)* and hydroxyapatite nanoparticles (HA). Moreover, cytotoxicity and antioxidant activity of the prepared nanocomposites using *Ehrlich ascites* carcinoma cells (EACC) were examined and evaluated. The prepared nanocomposites were characterized using Fourier transform infrared spectroscopy (FTIR) and transmission electron microscope (TEM). In addition to the particle size and zeta potential were recorded. The grafting technique was significantly enhanced the nanotube dispersibility and long term stability in the aqueous solution. The results indicated that the conjugation between *B. vulgaris* extract, HA nanoparticles and functionalized MWCNTs exhibited great antioxidant activity through the scavenging of the free radicals using DPPH assay. Also, the encapsulated *B. vulgaris* extract within the grafted MWCNTs based nanocomposites exhibited acceptable effect on the viability of EACC in compared with the standard (Vincrestine®). In other words, these novel functionalized MWCNTs based nanocomposites could be used as antioxidant and antitumor agents in the future after further studies.

Keywords: antioxidant; *B. vulgaris* extract; carbon nanotubes; cytotoxicity; dodecenyl succinic anyhydride; *Ehrlich ascites* carcinoma cells

Introduction

Carbon nanotubes (CNTs) are currently regarded as ideal materials for use in a growing range of applications. In addition to their outstanding combination of electrical and thermal properties, CNTs-based materials have proven to be highly biocompatible that led to be used for molecular dilevery systems.[1] However, difficulties involved in dispertion and solubilization of CNTs, largely owing to strong force interactions between the nanotubes, continue to present obtacles to thier practical application. To improve the dispersion of CNTs, several approaches involving covalent and non-covalent functionalization methods have been applied.[2,3] The ideal drug-delivery vehicle would be biocompatible and biodegradable, able to navigate the circulatory system to reach the target and capable of releasing its therapeutic cargo only to the desired cells. In an effort to meet this goal avariety of nanoscal structures have been developed by using a variety of different materials and shapes.[4–10] Several *in vitro* studies have shown that functionalizaing CNTs, thus improving their aqueous solubility, improves their biocompatibility and their toxicity profile.[11–13] On the other hand, recent *in vivo* study found no severe inflammatory response, such as necrosis, tissue degeneration, or nutrophile infiltration, when oxidized-CNTs were

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intravenously administrated to rats.[14] Natural products from plants have been valuable sources for anticancer drug discovery.[15] Beta vulgaris rapacea (Chenopodiaceae) has been indicated in folk medicine as a hypoglycemic, anti-inflammatory and hemostatic herb. This beet root has anti-cancer activity and its hydroalcoholic extract contains several compounds such as: quercetin-7-glucuronide, apigenin-7-rutinoside, vitexin 2-*O*-rhamnoside and 2-xylosylvitexin.[16,17] Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives each year. An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents to block the development of cancer in human beings. The mechanism of action of antioxidants can include chelating of pro-oxidative metals, oxygen scavenging and free radical termination.[18] The main antioxidant capacity test procedures have been classified as either single electron transfer (ET) or hydrogen atom transfer (HAT) assays.[19] An example of an ET-based method is α,α-diphenyl-β-picryl hydrazyl (DPPH) assay, in which a more effective radical scavenger removes the purple coloured DPPH radical more rapidly and/or more completely.[20,21] In the present work, we carried out a comprehensive study on prepared nanocomposites based on grafted multi-walled carbon nanotubes (MWCNTs) with dodecenyl succinic anhydride (DSA) and hydroxyapatite nanoparticles in presence of *B. vulgaris* leaves extract. In addition to the cytotoxicity and antioxidant activity of the prepared nanocomposites were examined and evaluated using Ehrlich ascites carcinoma cells (EACC) and DPPH assay, respectively.

Experimental Part

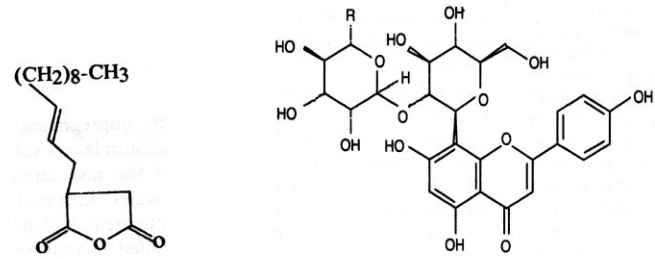
Materials

Multi-walled carbon nanotubes (MWCNTs), carbon content 95%, O.D L 6–9 nm ×5 μm and hydroxyapatite nanopowder (HA), particle size <200 nm, 97% were obtained from Aldrich. Dodecenyl succinic anhydride (DSA) 96% obtained by Merck. The leaves of *Beta vulgaris* collected from local resources. All chemicals and other reagents were used as received without further purification. Adult Female Swiss albino mice of 8–10 weeks old and 22–25 g weight purchased from the breeding unit of the Egyptian Organization for Biological Products and Vaccines were used in this study. A cell line of Ehrlich ascite carcinoma cells (EACC), was obtained from National Cancer Institute, Cairo University, Egypt.

Methods

Oxidation and Purification of MWCNTs[22,23]

Pristine MWCNT (3.0 g) was dispersed in mixed concentrated sulphuric and nitric acids (3:1, v/v) at ratio of 50 mL acid mixture per 10 mg of CNTs. The resulted



(a) (b)

Scheme 1.
Chemical structrues of (a) DSA and (b) Vitexin 2-*O*-rhamnoside (R= CH_3).

mixture was then refluxed at 110 °C overnight with continuous stirring to produce oxidized carbon nanotubes. The samples were washed with ultrapure water until the filtrate is neutral (pH 7.0). The collected solid was dried under vacuum at 70 °C for 12 h. The resulted materials denoted oxidized MWCNTs and kept for further investigation.

Extraction of B. Vulgaris Leaves

B. vulgaris dry leaves (0.5 kg) were cut in small pieces and extracted with 40% ethanol. The solvent was evaporated using rotary evaporator to reach 11% dry matter. This extract was dried and kept for further investigation.

Prepration of Grafted CNTs-Based Nanocomposites[24–26]

100 mg of oxidized MWCNTs was added to the reaction flask with the required amounts of DSA and ceric ammonium sulphate (100 mg and 30 mM, respectively) in presence of sodium dodecyl sulphate (0.1 mg) with or without *B. vulgaris* extract and/or HA nanoparticles (100 mg). The reaction mixture was carried out at 65 °C for 8 h with constant stirring under nitrogen atmosphere. The resulting materials (NC I and II) were filtered, extracted and washed with methanol/dist water (1:1), dried and kept for further investigation. (Table 1).

Characterization

The prepared materials were examined using Perkin-Elmer (FTIR) spectroscopy under certain condition such as: scan resolution: 4 cm^{-1}, scan rate: 2 mm sec^{-1}, range: 500–4000 cm^{-1} and mode: transmission. The morphologies and the nanocomposite particle size were carried out with a JEOL transmission electron microscope (TEM). The zeta potential (mv) was studied in buffer pH solution (7.4) using laser Doppler anemometer (Mastersizer S, Malvern Instruments, Orsay, France). Each sample was measured in triplicate.

Determination of Antioxidant Activity (DPPH Assay)

The free radical scavenging activity using 1,1-diphenyl-2-picryl-hydrazil (DDPH) reagent was determined according to Williams *et al.*.[27] The samples (0.01 g) were dissolved using different organic solvents such as methanol and dimethyl sulfoxide (DMSO). The freshly prepared methanolic DPPH solution (20 μg/mL) was added to 0.75 mL of each sample solution under stirring. After that, the decolorizing process was recorded after 1 h of the reaction at λ 517 nm in compared with the blank control (butylated hydroxyl toluene, BHT). The antioxidant activity (AO) was calculated according to the following equation:

$$\text{AO}\,(\%) = [(\text{control absorbance} - \text{sample absorbance}/\text{control absorbance})] \times 100 \quad (1)$$

In Vitro Antitumor Activity/Cytotoxicity Assay Using EACC

The tumor line was maintained in female Swiss albino mice by weekly intraperitoneal inoculation of 2.0×10^6 cells per mouse. The EACC cells were counted before intraperitoneal injection using the bright line hemocytometer. Dilution were made by physiological sterile saline solution and desired numbers of cells were injected in a volume of 0.2 mL. The

Table 1.
Chemical compositions of the prepared grafted MWCNTs-based nanpcomposites.

Sample Code	Chemical compositions (wt %)			
	MWCNTs	DSA	HA nanoparticles	*B. vulgaris* extract
Pure MWCNTs	50	0	0	0
NC I	50	49	1	0
NC II	49	49	1	1

investigated samples were incubated at 37 °C for 2 h. After that, the tubes were centrifuged at 1000 rpm for 5 min. For each sample 10 μl of cells suspension, 80 μl saline and 10 μl trypan blue were added and mixed. The number of living cells was calculated using a hemocytometer slide. Survived cells appeared as unstained bodies while non-viable cells stained blue.[28] The viability percentage of tumor cells was measured after incubation with the prepared nanocomposites (NC I and II) in compared with pure *B. vulgaris* extract, oxidized MWCNTs and Vincrestine® (standard drug, 50 μg).

Experimental Design

Sixty mice were divided into four groups as follows:

Group 1: Mice were served as the untreated control (negative control).

Group 2: Mice were intraperitoneal injected with 2.5×10^6 EACC/mouse (untreated tumor control).

Group 3: Oral treatment (3 doses/week) using the prepared nanocompsites.

Group 4: Intraperitoneal treatment (3 doses/week) using the prepared nanocompsites.

Antitumor activity was evaluated by increase in the life span (ILS) in compared with the untreated tumor control group according to Rajkapoor *et al.*[29] using the following equation:

$$\%\,\mathrm{ILS} = [(\mathrm{T} - \mathrm{C})/\mathrm{C}] \times 100 \quad (2)$$

Where T and C are the numbers of days of the treated and tumor control mice survived.

Tumor mass was measured at the end of the mice life span. The volume of the tumor mass (V) was calculated according to Ramnath *et al.*[30] using the following equation:

$$V = 4/3\,\pi r^2 \quad (3)$$

Where r is the mean of r_1 and r_2 which are two independent radii of the tumor mass and π is a constant = 3.142857.

Statistical Analysis

The data obtained were subjected to standard analysis of variance procedure, where the LSD0.05% values were obtained at $P \leq 0.05\%$. All values are the mean of three replicates.

Results and Discussion

Characterization of the Prepared Grafted CNTs-Based Nanocomposites

As reported in the literature, the oxidation of CNTs in a mixture of sulfuric acid and nitric acid introduces carboxy and other polar groups to the tips and the sidewalls of the MWCNTs and their zeta potential in water is negative.[31,32] It is found that the oxidized MWCNTs exhibited excellent stability in water after being neutralized by sodium bicarbonate. No precipitation is observed even after several months. On the contrary, precipitation is observed if the MWCNTs are not neutralized.

Figure 1 Shows FTIR spectra of oxidized MWCNTs and nanocomposites (NC I and II). It was observed that the peaks at 1570 cm^{-1} is attributed to the vibration of carbon skeleton (C—C stretch) of the bulk MWCNTs. For acid treated MWCNTs (oxidized MWCNTs) spectrum, obvious band can be observed at 1629 cm^{-1} corresponding to the stretching vibration of

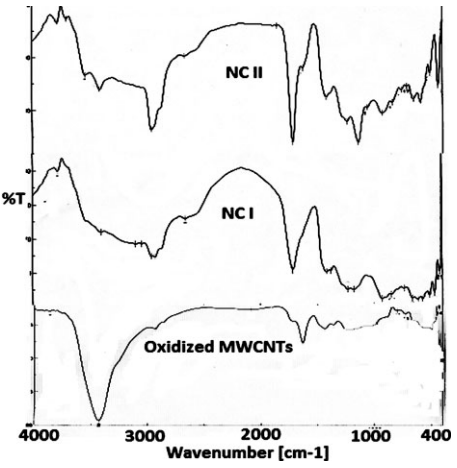


Figure 1.
FTIR spectra of oxidized MWCNTs, encapsulated and grafted *B. Vulgaris* extract within functionalized MWCNTs. (NC I and II).

C=O. This may be due to the introduction of the carboxylic acid groups (—COOH) on MWCNTs after treatment. A broad peak at approximately 3500 cm^{-1} is attributed to the O—H stretch. It is due to either moisture, alcohol or phenol, OH^- or carboxylic groups in the samples. The weak broad peaks at 1230 cm^{-1} could be due to C—O stretch. These results show clearly that the MWCNTs can be oxidized and that carboxylic, phenolic and lactone groups are likely obtained. On the other hand, in case of NC I and II spectra, the characteristic peaks of (C=C) groups in DSA were disappeared from the vibration region at 1629–1640 cm^{-1}, this may be due to the overlapping with the characteristic peak of the ester carbonyl group due to the interactions between DSA, *B. Vulgaris* (OH groups) and oxidized MWCNTs which appeared at 1714 cm^{-1}. Moroever, These spectra illustrated that some prominent peaks were observed at around 2925 and 2853 cm^{-1} corresponding to the stretching modes of functional groups CH_3 and CH_2, respectevily. Figure 2 represents typical TEM images of pure MWCNTs, NC II and NC II-after *B. vulgaris* extract released, it was observed that in case of pure MWCNTs, the bundles of carbon nanotubes are obviously observed, and most of the nanotubes lump together and tend to agglomerate, which resulted in a poor dispersion.

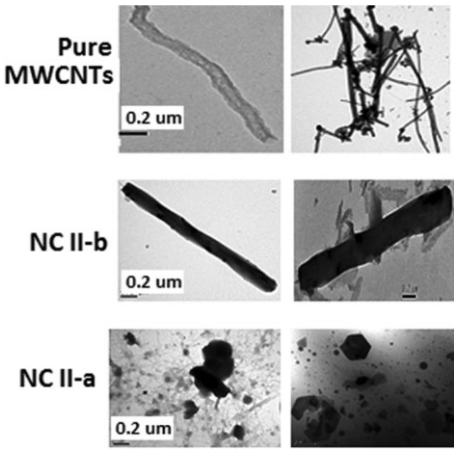


Figure 2.
TEM images of pure MWCNTs and encapsulated and grafted *B. Vulgaris* extract within functionalized MWCNTs. [before (NC II-b) and (NC II-a) after released].

While, in case of NC II, the phase separation occurs when the MWCNTs have negatively charged (DSA) particles and no MWCNT bundles agglomerates can be observed. Moreover, the short carbon nanotubes are adsorbed or anchored on the surface of the DSC particles, while the unfolded long tubes connect many DSA particles. On the contrary, after *B. vulgaris* extract released from NCII, the uniform distribution of the nanotubes was lost and dispersed quite randomly. This was observed for different areas of the samples with different nanotube loadings and no micrometer scale in-homogeneities.

From Table 2, it was observed that the pure extract (*B. vulgaris*) particles, (90 and 903 nm) had high negative zeta potential value (−7.25 mv) in compared with that in case of the pure MWCNTs particles (303 nm and −4.75 mv). Howevere, the encapsulated *B. vulgaris* extract particles (NC II, 205 and 1540 nm) had low zeta potential value (−0.126 mv). This may be due to the high tendency to floculate and the presence of the attractive forces between the different charges. From this results, it can be concluded that *B. vulgaris* extract could be successfully encapsulated and grafted within the functionalized MWCNTs.

Cytotoxicity and Antioxidant Activity

Cancer is a disease of misguided cells that have high potential of excess proliferation without apparent relation to the physiological demand of the prosses. According to

Table 2.
Particle size and zeta potential analysis of the prepared grafted MWCNTs-based nanpcomposites.

Sample Code	Particle size (nm) at pH 7.4	Zeta Potential (mv)
Pure MWCNTs	303	−4.75
Pure Plant extract	90, 495	−7.25
NC I	877, 903	−3.81
NC II	205, 1540	−0.126

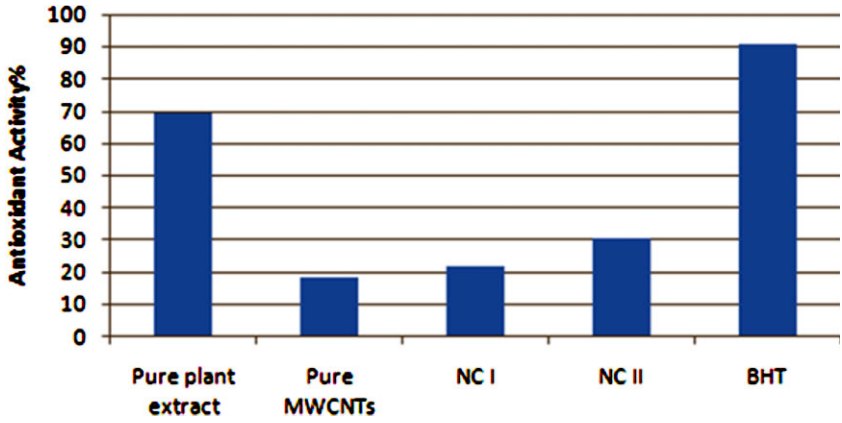


Figure 3.
Antioxidant activity of the encapsulated and grafted *B. vulgaris* extract within functionalized MWCNTs (NC I and NC II) in compared with butylated hydroxyl toluene.

Ninfali *et al.*[33] *B. vulgaris* hydroalcoholic extract exhibited the major polyphenolic component vitexin 2-*O*-rhamnoside (66%), in addition to some other minor compounds such as: 2-xylosyl vitexin, rutin, quercetin-7-glucuronide, isorhamnetin, apigenin-7-rutinoside and unknown flavonol-glycoside. Furthermore, it was found that this extract expressed only a slight toxicity to peripheral human lumphocytes and macrophages. On the other hand, they have remarkable antioxidant and DNA synthesis rate inhibition in MCF-7 cancer cells. Figure 3 shows that the pure *B. vulgaris* extract recorded high antioxidant activity (70%) in compared with that in case of the standard butylated hydroxyl toluene (BHT), pure MWCNTs, NC I and II (90, 19, 23 and 30%, respectively). This high antioxidant capacity may be due to the presense of high concentration of phenolics and flavonoids in the extract. As expected that the Phenolic and flavonoid compounds are common in the medicinal plants. These compounds are an important group of the natural antioxidants with possible beneficial effects on the human health.[34] Figure 4 illustrated that the effect of the encapsulated and grafted

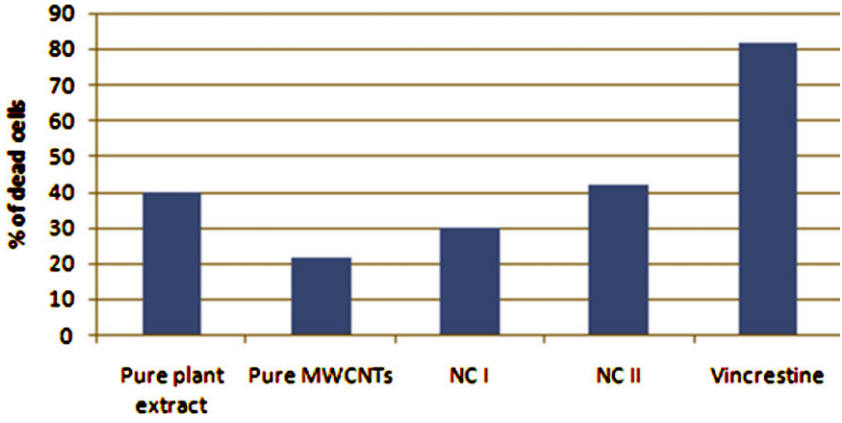


Figure 4.
Effect of encapsulated and grafted *B. vulgaris* extract within functionalized MWCNTs (NC I and NC II) on the viability of EACC in compared with Vincrestine®.

B. vulgaris extract within the functionalized MWCNTs on the viability of EACC in compared with pure plant extract, pure MWCNTs and standard Vincrestine®. Nanocomposite II (NC II) exhibited high effect on the viability of EACC in compared with NC I.

Tables 3, 4, 5 and 6 represent the change in tumor volums, mean survival day range and an increaing in the life span (%). The mean volume of control Ehrlich carcinoma (untreated tumor) and survival day range had 16.82 ± 0.257 mL and 13 days, respectively.

In case of pure *B. vulgaris* ectract, oral treatment of mice bearing Ehrlich carcinoma with 150 mg/kg/week reduced the mean of tumor volume (14.59 ± 0.792 mL) in

Table 3.
Effect of oral and intraperitoneal injection treatments using pure *B. vulgaris* extract on the survival of mice and tumor volume.

Treatment Groups	Mean of tumor volume (mL)	Relative % of tumor volume	Mean survival day range	Increase in Life span (%)
Group 1				
Negative control	–		25	–
Group 2				
Untreated tumor control	16.82 ± 0.257	100	13 ± 1	0.00
Group 3				
oral treatment	14.59 ± 0.792	86.74	16.33 ± 1.15	25.61
Group 4				
intraperitoneal injection treatment	15.54 ± 0.394	92.39	15 ± 1	15.38
L.S.D. (0.05)	1.062		2.105	

Table 4.
Effect of oral and intraperitoneal injection treatments using pure MWCNTS on the survival of mice and tumor volume.

Treatment Groups	Mean of tumor volume (mL)	Relative % of tumor volume	Mean survival day range	Increase in Life span (%)
Group 1				
Negative control	–		25	–
Group 2				
Untreated tumor control	16.82 ± 0.257	100	13 ± 1	0.00
Group 3				
oral treatment	13.33 ± 0.486	79.25	16.67 ± 0.577	28.23
Group 4				
intraperitoneal injection treatment	14.14 ± 0.106	84.06	14 ± 0.577	12.85
L.S.D. (0.05)	0.827		1.48	

Table 5.
Effect of oral and intraperitoneal injection treatments using NC I on the survival of mice and tumor volume.

Treatment Groups	Mean of tumor volume (mL)	Relative % of tumor volume	Mean survival day range	Increase in Life span (%)
Group 1				
Negative control	–		25	–
Group 2				
Untreated tumor control	16.82 ± 0.257	100	13 ± 1	0.00
Group 3				
oral treatment	14.33 ± 0.775	85.19	18.66 ± 0.577	29.85
Group 4				
intraperitoneal injection treatment	15.90 ± 1.04	94.53	15.33 ± 0.577	17.92
L.S.D. (0.05)	0.827		1.48	

Table 6.
Effect of oral and intraperitoneal injection treatments using NC II on the survival of mice and tumor volume.

Treatment Groups	Mean of tumor volume (mL)	Relative % of tumor volume	Mean survival day range	Increase in Life span (%)
Group 1				
Negative control	–		25	–
Group 2				
Untreated tumor control	16.82 ± 0.257	100	13 ± 1	0.00
Group 3				
oral treatment	12.726 ± 0.37	75.62	23.33 ± 2.08	79.46
Group 4				
intraperitoneal injection treatment	13.86 ± 0.237	82.40	18 ± 1	38.46
L.S.D. (0.05)	0.587		2.90	

compared with that in case of intraperitoneal one (15.54 ± 0.394 mL). While, in case of pure MWCNTs, oral treatment reduced the mean of tumor volume (13.33 ± 0.486 mL) in compared with that in case of intraperitoneal one (14.14 ± 0.106 mL). On the other hand, in case of NCI and NCII, oral treatment reduced the mean of tumor volums (14.33 ± 0.775 and 12.726 ± 0.37 mL, respectively) in compared with that in case of intraperitoneal one (15.90 ± 1.04 and 13.86 ± 0.327 mL, respectively). Also, in case of oral treatment, it can be noticed that the most promising sample was NCII that exhibited high life spane percentage (79.46%) relative to the other ones. Our results are as near as of those obtained by Kreuter *et al.*[35] which reported that an increase in the life span was observed after administration of the nanoparticles into Ehrlich ascites carcinoma bearing mice. In other words, The nanoparticles may affect the redox system in the tumor cells and enhanced of cells to enter the cell death (apoptosis), in addition oral administration of the nanoparticles may affect the frequencies of chromosomal abnormalities of EACC. The mechanism of the nanoparticles in reducing the tumor size may be through the long-circulating nanoparticulate carriers. They are able to efficiently deliver the chemotherapeutics to solid tumors by exploiting the enhanced permeability and retention effect and thus can significantly enhance the therapeutic index of the drug or improve reducing undesirable side effects.[36] Moreover, the synergestic effects of *B. vulgaris* extract and HA nanoparticles in NC II could be reduced EACC growth after transplantation.This was illustrated as a significant increasing in the extent of the mice life span. Oral treatment exhibited more effect than that in case of intraperitoneal injection with all the examined samples. It is known that the majority of the phenolic compounds and many types of tannins dissolve in both water and ethanol solutions. Therefore, these groups of compounds may contain the major active components for the destruction of leukemia and carcinoma cells. In other words, analysis of the transplanted animals after tumor transplantation showed that NC II delayed the death and inhibited the tumor growth of the transplanted animals in compared with NC I, pure *B. vulgaris* extract and MWCNTs.

Conclusion

Modification of MWCNTs with DSA resulted in highly versatile CNTs with amphiphilic side-chains. The pure extract of *B. vulgaris* plant exhibited high antioxidant activity using DPPH assay. Furthermore, *B. vulgaris* extract could be encapsulated and grafted with functionalized MWCNTs to form nanocomposites. The conjugation between *B. vulgaris* extract and functionalized MWCNTs led to an increasing the cytotoxcity using EACC in compared with pure plant extract.

Moreover, encapsulation of *B. vulgaris* extract within functionalzied MWCNTs reduced the EACC growth after transplantation. In spite of these novel nanocomposites could be induced an antitumor activity, they are still recommend further studies to optimize and to elucidate the mechanism of action in the future.

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