Using near-infrared laser activated Chitosan-Ashwagandha nanoparticles as tumor Targeting

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Abstract: In this study, three types of nanocomposites (Chitosan –TPP nanoparticles (C-TPP NPs), Chitosan-Ashwagandha nanoparticles (Ash-C NPs), and chitosan-Ashwagandha-TPP nanoparticles (C-Ash–TPP NPs)) were synthesized for targeting cancer colon (Caco-2) cell line. Fourier transform infrared spectroscopy (FTIR) analysis was applied to get the IR bands of the functional groups for each of Ashwagandha root water extract (ASH R WEX), Chitosan (c), and the numerous synthesized nanocomposites. Nanoparticles (NPs) were characterized by using Dynamic Light Scattering (DLS), Zeta Potential (ZP) and Transmission Electron Microscope (TEM). Two Groups of cancer therapy were applied to Caco-2 cells. In the first Group, the effectiveness of the synthesized NPs was observed without laser irradiation. the second Group, the effectiveness of the synthesized NPs was observed in the case of laser irradiation (660 nm diode laser, 5 min). On the other hand, the cell viability for Caco-2 cells exposed to low-level laser therapy was observed. The Cytotoxicity was analyzed by MTT assay and the cell viability was examined by the trypan blue method. The MTT assay showed increased cytotoxicity for each group of the treatment. Also, the results showed that the synthesized nanocomposites caused a decrease in cell viability in the two treatment groups and, in the case of laser irradiation lonely.

Key words: nanoparticles, optical radiation, herbal product, cancer cell line , NIR, nanocomposite, Ashwagandha , Chitosan.

1. Introduction

Cancer is one of the leading causes of death universal. Colon cancer remains the third most common cancer diagnosed in both sexes in the United States[1]. Colon cancer treatment is based largely on the cancer stage. In general: Current therapeutic strategies for most cancers involve a combination of surgical resection, radiation therapy, and chemotherapy [2]. Surgical excision usually fails to remove all cancerous cells resulting in serious morbidity without affecting the normal cells. In addition, surgery is limited to large numbers of tumors which are adjacent to critical tissue structures. Furthermore, the severe side effects of chemotherapy and radiotherapy make the patients have lots of sufferings [3]. Radiotherapy has an impact on the colon cancer; but it causes skin irritation, problems with wound healing, diarrhea, painful bowel movements, or blood in the stool, Fatigue/tiredness and sexual problem [4]. Chemotherapy is considered the main treatment options signify in cancer therapy. On the other hand, chemotherapeutic agents are wreak havoc [5] and highly toxic for normal cells [6]. Also, the chemotherapeutic agents kill both dividing cancer and normal cells and have a high incidence of life-threatening complications [7].

Over the past several decades, significant advancements have been made in fundamental understanding of cancer biology, which in turn , lead to better diagnostic and treatment options [5]. Natural products have a significant effect in cancer therapy [8] due to plant alkaloids and taxoids [9]. Withania somnifera (Ashwagandha) has a therapeutic effect in cancer therapy [10] [11]. The root extract of Ashwagandha has antioxidant activity, enhance the immune system [12], and inhibit the angiogenesis [13]. The pharmacological activity of the Ashwagandha root is attributed to the alkaloids and steroidal...
lactones [14]. The chitosan culture system provides a platform for the research of cancer stem cells biology and screening of anticancer drugs [15]. Chitosan is one of the most typical biodegradable and biocompatible polymers. on the other hand, chitosan has been approved by both the Food & Drug Administration (FDA) and European Medicine Agency, signifying their transition from the laboratory to clinical oral and parental administration [16] [17].

Many reasons lead to limit the cancer treatment with the conventional drug. Some of these reasons are the small distance of the interstitial space that separates tumor cells, and blood vessels embedded and crossed by the conventional anticancer drugs within the stroma of tumor cells. The concentration of drug reaching tumor cells is decreased due to the poor blood supply and the absence of the lymphatic network. The development of NPs (designed especially for diagnostic imaging purposes and as drug-carriers for therapy [18]) with a high-pressure gradient in the interstitial space assist the advection flow of drug to the tumor cells [19].

Nanotechnology overcomes all these reasons glitches by targeting cancerous cells directly with its beneficial properties as a nanoscale dimension [20]. Nanocomposite materials are suitable for cancer treatment due to their small size, varied composition, surface functionalization and stability which provide unique opportunities to interact and target the tumor microenvironment, such kind of interaction between nanoparticles and tumor aids in small molecule transport to the intracellular organelles to induce the greatest cytotoxic effect [5]. Thus, NPs with a high-pressure gradient in the interstitial space assist the advection flow of drug to the tumor cells.

NIR laser with wavelengths between 650 and 930 nm penetrates the external surface of tissue [21] and the emergent light is measured along the tissue boundaries. OBAYASHI, T. [22] proves the effectiveness of treatment using NIR radiation and its ability to promote apoptosis in pancreatic cancer cells. On the other hand, Dees, C. [23] treats the murine cutaneous melanoma with NIR light. Nanoparticles assist NIR laser to control therapeutic agents selectively to the targeted sites without adverse effects on the normal tissue [24]. They are suitable for many applications, especially optical imaging and drug delivery as reported by Boschi, F [18].

The present study deals with a novel method for synthesis of chitosan nanoparticles, employing the Egyptian ASH R WEX to target Caco 2 cell. In this novel study, we assessed the effectiveness of the synthesized natural nanocomposites in targeting cancerous cell in the presence and the absence of NIR laser to limit the utilization of traditional toxic chemicals drugs and the radiation.

2. Material and methods
2.1. Material

Egyptian Ashwagandha root water extract (ASH R WEX) was purchased from the National Cancer Institute, Cairo, Egypt. The chitosan with medium Molecular weight 161.1. was purchased from Oxford company, India. diode Laser (660 nm, 10 mW) was purchased from laser land company, China.

2.2. Preparation of nanoparticles:

Three different types of NPs were synthesized in this work: the first was chitosan nanoparticles (C-TPP NPs), the second was Ashwagandha–chitosan Nanoparticles (ASH-C NPs) and the third was Ashwagandha-chitosan-TPP Nanoparticles (Ash-C-TPP NPs) as shown in Scheme (1).

2.2.1. Chitosan nanoparticles (C-TPP NPs) preparation:

The cationic solution of chitosan was obtained by dissolving 100 mg of chitosan in 100 mL of 1% diluted acetic acid under continuous magnetic stirring for 2 hours at 2000 rpm. PH of the solution was raised to 4.7 using NaOH solution. The anionic solution of TPP was obtained by dissolving 33.3 mg of TPP into 10 mL of distilled water, and then the TPP solution dropped into the cationic solution of chitosan under magnetic stirring. NPs were formed instantly under continuous magnetic stirring after 45 min at 2000 rpm at room temperature and the clear solution gradually became milky (NPs formed). The NPs solution was dialyzed with 12000 rpm centrifuge for 20 min. The sponges were prepared by the direct freeze-dry of C-TPP NPs dispersion, as shown in Scheme (1.A).
2.2.2. Ashwagandha-chitosan nanoparticles (Ash-C NPs) preparation:

The formulation of Ashwagandha-chitosan nanoparticles (Ash-C NPs) were obtained by dissolving 150 mg of chitosan in 150 mL of 1% diluted acetic acid under continuous magnetic stirring for 1.5 hours at 2000 rpm. pH of the solution was raised to 4.7 with NaOH drop by drop. Fifty milligrams of ASH R WEX was dissolved in 10 mL of distilled water. ASH R WEX solution was dropped into chitosan solution drop by drop. NPs formed instantly under continuous magnetic stirring after 45 min at 2000 rpm at room temperature and the clear solution gradually became milky (NPs formed). The (Ash-C NPs) solution was dialyzed for 20 min with 12000 rpm centrifuge. The sponges were prepared by the direct freeze-dry of Ash-C NPs dispersion, as shown in Scheme (1.B).

2.2.3. Chitosan-Ashwagandha-TPP (C-Ash-TPP NPs):

The formulation of Ashwagandha-chitosan nanoparticles (Ash-C NPs) was obtained by dissolving 150 mg of chitosan in 150 mL of 1% diluted acetic acid under continuous magnetic stirring for 1.5 hours at 2000 rpm, and then the pH of the solution was raised to 4.7 with NaOH solution. ASH R WEX (33.3 mg) was incorporated into TPP solution (33.3 mg of TPP was dissolved into 10 mL of distilled water). The ASH-TPP solution was dropped into chitosan solution under continuous magnetic stirring for 45 min at 2000 rpm at room temperature and the clear solution gradually became milky (C-Ash-TPP NPs formed). The (C-Ash-TPP NPs) solution dialyzed for 20 min with 12000 rpm centrifuge. The sponges were prepared by the direct freeze-dry of C-Ash-TPP NPs dispersion, as shown in Scheme (1.C).

![Scheme 1. Three different types of NPs were synthesized in this work: (A) C-TPP NPs, (B) ASH-C NPs, (C) Ash-C-TPP NPs](image-url)
2.3. Physicochemical characterization of the nanocomposite:

2.3.1. Fourier transform infrared (FT-IR) spectroscopy:

The FT-IR spectra were taken using a Bruker Vertex 70 FT-IR spectrometer in the 600–4000 cm\(^{-1}\) region. Each of Chitosan, of ASH R WEX, C-TPP NPs, Ash-CNPs, and C-Ash–TPPNPs were characterized by FT-IR spectroscopy.

2.3.2. Dynamic Light Scattering (DLS) and zeta potential (ZP):

Nanoparticle size was determined by DLS from the NPs suspension, ZP was used to indicate the dispersion stability, using Zeta sizer Nano series instrument (Malvern, UK) with a range of 0.6:6000 nm and Zeta potential range (mV) from -200 to 200 mV.

2.3.3 Transmission Electron Microscope (TEM)

High-resolution transmission electron microscope (HR-TEM, Tecnai G20, FEI, Netherland) was used for the purpose of imaging, crystal structure revelation, and elemental analysis "qualitative and semi-quantitative analysis. Two different modes of imaging were employed; the bright field at electron accelerating voltage 200 kV using lanthanum hexaboride (LaB6) electron source gun and the diffraction pattern imaging. Eagle CCD camera with (4k*4k) image resolution was used to acquire and collect transmitted electron images. TEM Imaging & Analysis (TIA) software was used to spectrum acquisition and analysis of EDX peaks.

2.4. Treatments of cells

2.4.1 Cell culture and the treatment

Caco-2 cell line (colon cancer) was obtained from the VASCERA, Cairo, Egypt. The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) cells (1 \(\times \) 10\(^6\) cells/mL). Two Groups of cancer therapy were applied on Caco-2 cells. In the first Group: the treatment effect of the synthesized nanoparticles (CNPs, Ash-CNPs, and C-Ash–TPPNPs) with a range of concentrations of (6.25 mg/mL–100 mg/mL) was performed without laser irradiation. In the second Group: low-level laser therapy was applied after 24 h of cell incubation with the synthesized nanoparticles (CNPs, Ash-CNPs, and C-Ash–TPPNPs) with a range of concentrations of (6.25 mg/mL–100 mg/mL) with five minutes of irradiation using 660 nm Diode laser.

![Scheme 2: Schematic illustrate of nanoparticles killing a cancer cell upon NIR laser irradiation](image)

2.4.2 In vitro Cytotoxicity Assay:
The treatment methods were divided into two Groups: in the first Group, the therapy was performed without laser irradiation, only with the synthesized nanoparticles (C-TPP NPs, Ash-C NPs, and C-Ash–TPP NPs). In the second Group, low-level laser therapy was applied after 24 h of cell incubation with a range of concentrations of the synthesized nanoparticles (C NPs, Ash-C NPs, and C-Ash–TPP NPs) with 660 nm Diode laser. The effect of each treatment method on cell viability was estimated using a3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously. GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA) was used to calculate the half-maximal inhibitory concentration (IC50) of different types of treatment. The Caco 2 cells were examined after treatment in the two Groups and the morphological changes were observed by the inverted microscope (Zeiss Axiol Vert.A1; Zeiss, Gottingen, Germany) at 40 magnification. Cells were photographed using the digital camera. The viability percentage was examined using the trypan blue method.

3. Result and Discussion

3.1. FTIR spectroscopy:

The FTIR analysis (figure 1) of Chitosan, Egyptian ASH R WEX, and the synthesized nanoparticles were carried out to analyze environment of capping ligands responsible for the stability of biosynthesized polymeric nanoparticles and to determine the presence of the characteristic IR bands.

For chitosan, a strong band in the region 3421–524 cm⁻¹ was appeared and corresponded to N-H and O-H stretching. The peak at 3432 cm⁻¹ was attributed to the symmetric stretching vibration of OH. The peak at 2920 cm⁻¹ was referred to the symmetric and the asymmetric stretch of CH3 and CH2, respectively. The peak which appeared at 1631 cm⁻¹ was attributed to C-O secondary amide stretch. The peak at 1382 cm⁻¹ was referred to δs(CH3) in NHCOCH3 group. The peak around 1032 cm⁻¹ was attributed to ν(C–O) in secondary OH group. All bands found in the spectra of chitosan were reported [25] [26] [27].

For the ASH R WEX, the FTIR spectrum shown peaks at 3433, 2924, 1631, 1422, 1158 and 1024 cm⁻¹ which attributed to the symmetric stretching vibration of OH, C-H alkane stretching, C–O secondary amide stretch, C-H alkane bending (δ(CH2) inCH3OH group), C-O stretching of carboxylic acid and (C–O) in secondary OH group, respectively. All bands which are found in the spectrum of Ashwagandha were reported by [28].

The FTIR spectrum of C-TPP NPs shown a peak at 3429 cm⁻¹ which attributed to symmetric stretching vibration of OH. The peak at 2923 cm⁻¹ was referred to CH3 symmetrical stretch and CH2 asymmetric stretch. Absorption peak which was appeared at 2923.88 cm⁻¹ indicates the presence of CH stretch. The peaks at 1636, 1412 and 1153. 1323 cm⁻¹ were attributed to secondary amide stretch, CH2 bending and CH3 symmetrical deformations and vs(C–O–C) (glycosidic linkage), respectively. The signal at 899 cm⁻¹ was corresponded to the CH bending out of the plane of the ring of monosaccharides.

The spectrum of C-TPP NPs shown new peaks at around 890 and 1240 cm⁻¹ which were assigned to the characteristic bands of TPP polyns (P-OH and P=O stretches) [27], Where the formulation of C-TPP NPs by ionic gelation method (ionic crosslinking technology) is based on the formation of complexation between the positively charged amine group of chitosan and negatively charged polyanion such as tripolyphosphate (TPP) [29], as shown in the scheme (1. A).

The FTIR spectrum of ASH-CNPs presented peaks at 3431, 2925, 1632 and 1382 cm⁻¹ were assigned to symmetric stretching vibration of OH, C-H alkane stretching, C–O secondary amide stretch and δs (CH3) in NHCOCH3 group, respectively. The spectrum of Ash-C-TPP NPs which shown peaks at 3430 and 1565 cm⁻¹ were attributed to symmetric stretching vibration of OH and (NH3) in NHCOCH3 group as amid II group. CH2 bending and CH3 symmetrical deformations were confirmed by the presence of bands at around 1412 cm⁻¹. The peak at 1154 cm⁻¹ was assigned to C-O stretching of carboxylic acid. The new peak which appeared at 890 cm⁻¹ were attributed to new composite formation [30].

Nagaonk, D. [31] reported that the force of O-H (stretching), and C-O (stretching) functional groups of the phytoconstituents were Additionally, C-O stretch of alcohols and carboxylic which suggests capping of the synthesized NPs by nitro compound as shown in scheme 1(B, C). On the other hand, SUMITHTRADEVI, S. [32] reported that ASH R WEX encompasses several alkaloids, withanolides, withaffierins, and withanolides have their effeteness in the cancer treatments.
3.2. Dynamic Light Scattering and zeta potential

In aqueous solutions, each of DLS and ZP was measured. The average diameter for C-TPP NPs, Ash-CNPs and ASH-C-TPP NPs were estimated by DLS and they were 331, 188 and 357 nm, respectively, as shown in the table (1). Additionally, ZP of the CNPs, ASH-C NPs and ASH-C-TPP NPs were measured to be 49.8, 51.2 and 37.2 mV, respectively, as shown in the table (1). Most commonly Chitosan NPs were formed immediately upon mixing TPP and chitosan solution due to the molecular linkage between TPP and chitosan amino groups [33]. Our study indicated a decrease in the size of ASH-C NPs (the biosynthesized chitosan NPs using ASH R WEX in absence of TPP) compared with other development NPs in this study. ZP reflects the electrical superficial charge of particles and their influence by the particles composition and the dispersion stability [26]. Large values of zeta potentials predict a more stable dispersion. In the current study, ZP values which were recorded for the synthesized NPs showed strong positive ZP and documenting their surface cationic charge with stable nature. NPs with ZP above ±30 mV have shown to be stable in suspension, as the surface charge prevents aggregation of the particles [34][35].

<table>
<thead>
<tr>
<th>Nanoparticles Drug</th>
<th>DLS (nm)</th>
<th>ZP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-TPP NPs</td>
<td>331</td>
<td>49.8</td>
</tr>
<tr>
<td>Ash-C NPs</td>
<td>188</td>
<td>51.2</td>
</tr>
<tr>
<td>C-Ash–TTP NPs</td>
<td>357</td>
<td>37.2</td>
</tr>
</tbody>
</table>

3.3. Transmission Electron Microscope characterization

Figure 3 shows the TEM measurements of the as-prepared C-TPP NPs. ASH-C NPs, and ASH-C-TPP NPs. The mean diameter size of C-TPP NPs was found to be 75 nm (± 50 nm) with a spherical shape as revealed in figure 3A. ASH-C NPs were prepared by dropping the Ashwagandha solution to chitosan solution and after purification, the mean diameter size was observed with 20 nm (± 10 nm) in uniform regular and circular shape as shown in figure 3B. The TEM image revealed the average diameter of ASH-C-TPP NPs nanoparticles are 50 nm (±25.5 nm) in irregularly shaped as revered in Figure 3C. The irregular counter of C-TPP NPs and ASH-C-TPP NPs was due to the Oswald ripening [31]. However, the instability of C-TPP NPs and ASH-C-TPP NPs showed the insignificant effect of capping ligands on their surface [31]. The decrease in the particle size diameter of ASH-C NPs...
compared to that of C-TPP NPs and ASH-C-TPP referred to the effect of TPP [33]. The slight decrease of the particle size diameter determined by TEM in comparison to that by DLS was due to the shrinking of the NPs in the dry state that incurred during TEM measurements [36].

Table 1: IC of Caco2 cells treated in two groups.

<table>
<thead>
<tr>
<th>Nanoparticles Drug</th>
<th>Group 1 Without laser irradiation</th>
<th>Group 2 With laser irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-TPP NPs</td>
<td>300 µg/mL**</td>
<td>200 µg/mL</td>
</tr>
<tr>
<td>Ash-C NPs</td>
<td>52 µg/mL</td>
<td>25 µg/mL</td>
</tr>
<tr>
<td>C-Ash–TPP NPs</td>
<td>125 µg/mL</td>
<td>58 µg/mL</td>
</tr>
</tbody>
</table>

Legend:
*Chitosan nanoparticles (C-TPP NPs),
Chitosan-Ashwagandha nanoparticles (Ash-C NPs)
Chitosan-Ashwagandha-TPP nanoparticles (C-Ash–TPP NPs)
** IC50

3.4. Cytotoxicity Assay

For the biomedical application, the NIR spectrum region is a proper optical window for deep tissue screening. When appropriately adapted, the nanoparticles show the abilities of rapid imaging and early detection of cancer and provide helpful guidance in cancer therapy, reducing morbidity and mortality [37].

In this novel therapeutic study, we assessed the effectiveness of the synthesized NPs in targeting cancerous cell in the absence (Group 1) and the presence (Group 2) of NIR laser, for the first Group, the half maximal inhibitory concentration (IC50) of (C-TPP NPs, Ash-C NPs, C-Ash–TPP NPs) was found (300, 52 and 125) µg/mL, respectively. While, in the second treatment Group: in case of laser irradiation, the IC50 dose of (C-TPP NPs, Ash-C NPs, C-Ash–TPP NPs) was found to be (200, 25, 58) µg/mL, respectively as revealed in Table 2.

Our results revered that the lowest IC50 was observed when the Caco2 cells treated with Ash-C NPs while the highest IC50 was observed when the treatment was with C-TPP NPs. In the presence of laser, the thermal effect of the NIR reduced the IC50 dose to the half value approximately.

The effectiveness of the synthesized NPs in targeting cancerous cells agrees with the previous studies for Aruna, U. [5] and Ahmed, W.A. [38] who show the anticancer effect of Chitosan and Ashwagandha against many types of cancer, respectively. For examples, the last study of Ahmed, W. [10] Shows, an efficient cytotoxic effect of Ashwagandha on MCF-7 breast cancer cells. Medina, L.
S.[35] shows, the effect of Chitosan–TPP nanoparticles as Arrabidaea extract against many cancer cell lines.

In the present study: The morphological changes for the treated cells using the IC_{50} dose for each nanocomposite was observed by inverted microscope and was photographed using the digital camera to compare the photomicrographs of the control and the treated cells in presence and absence of NIR laser as shown in figure (3,4) The results revealed a strong effect with various synthesized NPs which appeared as shrinkage as well as accumulation of dead Caco 2 cells compared to the control and the treated groups.

Figure (3). Representative photomicrographs for the first group of the treatment and shows the viability of Caco-2 cells treated with different types of NPs in 37 °C in a 5% CO_{2} incubator for 24 h. (a) Control untreated cells, (b) cells treated with 300 µg/ml of CNPs, (B') optical tomography of Caco cells by using the 660 nm laser cells treated with 300 µg/ml, (C) cells treated with 52 µg/ml of Ash-CNPs, (C') optical tomography of Caco cells by using the 660 nm laser cells treated with 52 µg/ml, (D) cells treated with 125 µg/ml µg/ml of C-Ash–TPP-NPs and (D') optical tomography of Caco cells by using the 660 nm laser cells treated with 125 µg/ml of C-Ash–TPPNPs.

Figure (4). Representative photomicrographs of the second group of the treatment and shows the viability of Caco-2 cells treated with different types of NPs in 37 °C in a 5% CO_{2} incubator for 24 h. (A) Control untreated cells, (B) cells treated with 200 µg/ml of CNPs, (B') optical tomography of Caco cells by using the 660 nm laser cells treated with 200 µg/ml, (C) cells treated with 25 µg/ml of Ash-CNPs, (C') optical tomography of Caco cells by using the 660 nm laser cells treated with 25µg/ml, (D) cells treated with 58 µg/ml µg/ml of C-Ash–TPPNPs, (D') optical tomography of Caco cells by using the 660 nm laser cells treated with 58 µg/ml of C-Ash–TPPNPs, (E) treated Caco 2 cell with five min of 660 nm of laser irradiation and (E') optical tomography of Caco2 cells by using the 660 nm laser cells treated with five min of laser irradiation.
The viability of the first group of treatment with (CNPs, ASH-C NPs, ASH-C-TPP NPs), the viability of the second group of treatment with (CNPs, ASH-C NPs, ASH-C-TPP NPs), in the case of laser irradiation as well as the viability of low level laser therapy The cells viability percentage (using the IC50 dose for each synthesized NPs) was measured by the trypan blue method for the two treated groups. As presented in figure (5), the viability test using the IC50 dose for each NPs in the presence and absence of NIR show a significant difference compared to control. Also, the combination of NPs and NIR laser causes a dramatic change in the viability as it shows decreasing of viable cells compared with non-irradiated cells for all the synthesized NPs. On the other hand, in case of the laser irradiation only (low-level laser therapy), the thermal effect of NIR laser decrease the cell viability to the half (50%) compared with the control as represented in figure 5. Previous observations finding agreed with Wang, J. J. [20] who reported a decrease in the viability as well as the inhibition of growth of cancerous cells when treated with chitosan nanoparticles, also the results agree with Yi, X [37] who uses NIR fluorescent probes in cancer imaging and therapy, the results agree with Lai,B [41] who found the accumulation of NIR and NPs in tumor sites drastically increase the efficiency of treatment through effective conversion of light energy to heat.

Effective delivery of chemical drugs to tumor sites is particularly appealing for the enhancement of the tumor-killing effect and the reduction of systemic toxicities [39]. Using the natural and the herbal products give the chance to improve the quality of therapy. Consequently, development of nanoparticles from the natural products provides the opportunity to target the tumor cells without any drawback effect on normal tissues. Ashwagandha is a well-known herbal medicine and has anti-inflammatory, anti-oxidant and anti-tumor as well as neural protective properties. Additionally, Ashwagandha inhibits nuclear factor kappa-B (NF-κB) which is a target gene involved in inflammation, angiogenesis, cell cycle, metastasis, apoptosis and multidrug resistance [32].

On the other hand, Figure (3,4) show the effect of NIR laser exposure during the imaging, we observed that the NIR laser causes topographical enhancement (appear on the exposed cells compared with the non-exposed cells). This result agrees with Yi, X [37] who conclude the effect of Near-infrared fluorescent probes in cancer imaging and therapy.

Our work avoids all toxic dyes such as heptamethine neocyanine dyes such as IR-780 iodide, IR-783 , MHI-148, and porphyrin derivatives P 247, are identified with preferential accumulation in cancer tissues, displaying great advantages over the common fluorescent dyes, like ICG [40] From the therapy side, our study avoids all toxic materials such as graphene and metal nanoparticles and, the laser absorbing agents or dyes were used before to increase laser induction thermal damage in the tumor [42] are neglected.

**Conclusion**
In our opinion, biosynthesis of chitosan nanoparticles from the traditional herbal product using biological crosslinking agents is a great achievement. The combination of synthesized NPs with NIR laser showed a surprise cytotoxic effect and inhibition on the viability. The result gives a promise for future medicine.

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