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Novel Trend in Colon Cancer Therapy Using Silver Nanoparticles Synthesized by Honey Bee

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Abstract

Recently, the biosynthesis of nanoparticles has been well explored which draws attention to its possible biomedical applications especially in cancer therapy. In the current study, the novelty in the biosynthesis of silver nanoparticles (AgNPs) using honey bee extract has been explained. This study was also aiming at investigating the anti-colon cancer activities of the biogenic AgNPs along with its capping biomolecules *in vitro*. The obtained biogenic AgNPs were well characterized by X-ray diffraction (XRD), energy dispersive X-ray (EDX), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). It was found that the formed AgNPs have spherical shape with size range from 12 to 18 nm embedded in honey bee biomolecules. The cytotoxicity results of AgNPs on peripheral blood mononuclear cells (PBMC) indicated that the obtained AgNPs could be used safely with concentrations upto 39 µg/ml. On the other hand, the potentialities of the biogenic AgNPs against colon cancer proliferation recorded 60% inhibition using its non-toxic dose with a down regulation of the expression of Bcl2 and survivin gene. By the extraction of AgNPs capping biomolecules to explain the exact fraction that is responsible for the anticancer properties, it was found that both AgNPs and its capping biomolecules have anti-proliferative effects with a priority to the naked AgNPs.

Keywords: Colon cancer; Biogenic AgNPs; Survivin; BCl2; Honey bee

Introduction

Nanomedicine refers to the use of precision-engineered nanomaterials in order to reveal novel therapeutic and diagnostic tools for human use [1]. The collaboration between nanotechnology and nanomedicine resulted in the emergence of new trend in both therapeutic and pharmaceutical fields [2]. The growing need to develop environmentally benign nanotechnology using microbes and nanomaterial has received great attention in the last few years [3]. Recent reports [3-5] recorded the use of coriander leaf, henna leaf and edible mushroom in the biosynthesis of Au and Ag nanoparticles. Also, Mendoza-Reséndez [6] described the eco-friendly synthesis of AgNPs mediated by various bee products (raw honey, royal jelly, honeydew honey and propolis). Nowadays, silver nanoparticles (AgNPs) have a wide spread of applications and the highest level of commercialization among the nanomaterials [7]. For instance, among different nanoparticles investigated, AgNPs are the most promising particles that are used in the field of nanomedicine for their antimicrobial activity against different microbes [1]. However, there is serious lack of sufficient information concerning the biological effects of AgNPs on human cells. In addition, there are limited studies on the potential of biogenic AgNPs as anticancer agents. Among these studies, there is a recent report on the anti-proliferative activities of AgNPs against human glioblastoma cells (U251) [2]. Besides, Yezhelyev et al. [8] revealed that AgNPs in the size range 1-1000 nm could be very beneficial for the diagnosis and treatment of cancers which may lead to the new discipline of nano-oncology. Due to the lack of efficiency in many of the available synthetic anticancer drugs, such as doxorubicin, cisplatin, and bleomycin, it is important to find novel therapeutic agents [9]. This report introduces a simple, cost-effective and environmentally benign synthesis of AgNPs using honey bee. In addition, the activity of the synthesized AgNPs against human epithelial colorectaladeno carcinoma was evaluated with also referring to its molecular action on cellular gene expressions.

Materials and Methods

Materials

Silver nitrate ($AgNO_3$) was purchased from Sigma-Aldrich. Cell culture media was provided by Lonza. Other chemicals were of analytical grade and used without any further purification.

Green synthesis of AgNPs using honey bees extract

About 50 mature honey bee workers were collected from honey bee colony (SRTA-City Farm), sterilized with 0.05% Clorox solution, and then washed three times with sterile H_2O. The insects were left to dry on a pre-sterilized laminar flow. The insects were then ground in a clean and sterile motor with sterile phosphate buffer (PBS; pH=7, 25 mM). Afterwards, the extract was filtrated through three layers of sterilized muslin and each 100 ml of filtrate was transferred into a sterile 250 ml flask. As a substrate for AgNPs production, $AgNO_3$ was added to the insects filtrate with final concentration of 1 mM in the presence of glucose (560 Mm) as electron donor. The mixture was

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incubated with shaking (220 rpm) at room temperature. At different time intervals, samples of 1.5 ml were taken from the reaction mixture and the absorbance of the resulting colloidal suspension of AgNPs was recorded immediately against the experimental blank at 450 nm with the aid of Shimadzu UV-vis spectrophotometer. The collection of the produced AgNPs was achieved by centrifugation at 10,000 rpm for 20 min followed by dissolving the formed pellet in 0.1 ml of 25 mM PBS at pH=7, and stored at 4°C until being used.

Characterization of the synthesized AgNPs

The morphology of AgNPs was analyzed using scanning electron microscopy (JEOL SEM, GSM-6610LV, Japan) at an accelerated voltage of 15 kV. The samples surfaces were vacuum coated with gold for SEM. Dimensional analysis and structural characterization of the obtained AgNPs were carried out by transmission electron microscopy (TEM) using high-resolution JEOL 2100-TEM, Japan operated at 200 kV, and with the aid of X-ray diffraction (Shimadzu XRD 7000 X-ray diffractometer, Japan) using a DRON 2.0 diffractometer with Cu tube (Kα radiation).

Cytotoxicity assay of the biogenic AgNPs on cell lines

Cell culture: $CaCO_2$ (human epithelial colorectaladeno carcinoma, American Type Culture Collection, ATCC; Manassas, VA, USA) were washed twice with DMEM media (Lonza) supplemented with 200 µM L-glutamine and 25 µM HEPES buffer (all chemicals and media were Cambrex). 2×10^5 cells were suspended in DMEM culture media with 10% fetal bovine serum (Gibco-BRL). The cells were left to adhere on the polystyrene 6 well plates for 4 h in an incubator (37°C, 5% CO_2, 95% humidity). The cells were washed twice from debris and dead cells by using RPM1 supplemented media.

Human blood lymphocytes separation: Peripheral blood cells mononuclear cells (PBMCs) were isolated by gradient centrifugation as reported by Lohr et al. [10]. Briefly, peripheral blood samples were diluted with 5 volumes of freshly prepared RBC lysis buffer (38.8 mmol/L NH_4Cl, 2.5 mmol/L K_2HCO_3, 1 mmol/L EDTA, pH 8.0), incubated at room temperature for 10 min, and then centrifuged at 1500 rpm for 5 min. The nucleated cells were precipitated at the bottom of the tube.

Cytotoxicity and proliferation assays: The safe dose of the biogenic AgNPs was determined on PBMC as normal cell model according to the methods described by Borenfreund and Puerner [11]. Briefly, 6×10^4 cell/ml was seeded in 96-well plates and incubated under 5% CO_2 at 37°C till semi-confluences. After incubation, about 100 µl of different AgNPs concentrations was added. After 3 days of incubation, both dead and living cells were treated with 100 µl of neutral red stain solution (100 µg /ml). Neutral red stained only the living cells via stain incorporation into liposomes providing a quantitative assay. The stain intensity was quantified using automated ELIZA microplate reader adjusted at 540 nm.

Assessment of antiproliferative activity: The antiproliferative activity of the biogenic AgNPs on $CaCO_2$ cell was confirmed using trypan blue assay. The dye exclusion test is used to determine the number of viable cells present in a cell suspension. Besides, the trypan blue stain is considered as a simple way to evaluate cell membrane integrity and thus assesses cell proliferation or death. Briefly, $CaCO_2$ cells were treated either with the non toxic doses of 5-Fluorouracil (5 FU, 0.9 µg/ml) or the biogenic AgNPs (39 µg/ml) for 48 hrs. At the end of incubation, the suspensions of treated cells were simply mixed with dye and then visually examined to determine whether cells take up or exclude dye with reference to untreated cells, as a control. In the protocol presented here, viable cells will have clear cytoplasm whereas dead cells will have a blue cytoplasm.

The anticancer signals of the biogenic AgNPs: Mechanisms of target gene regulation: The anticancer activity profile of the synthesized biogenic AgNPs was explained by studying their activity in controlling the expression of p53, Bcl2, caspase 3 and survivin genes in $CaCO_2$ cells. $CaCO_2$ cells were cultured in 12 well plates (6×10^3 cell/ml) for 2 days with the recorded non-toxic concentration of either biogenic AgNPs or 5FU (as a positive control). After incubation, total cellular RNA was extracted and subjected to RT-q PCR for genes expression quantification after and before treatment. Oligo-dT primer and the AMV reverse transcriptase (Promega Corp., Madison,WI)were used to synthesize the first-strand cDNA. GAPDH was used as internal control for standardization of PCR product [12]. The RT-PCR was done to the cDNA based on the SYBR Green dye (QuantiTect SYBR Green PCR Kits) and Light Cycler fluorimeter (BIO RAD S1000 ^{Tm} thermal cycler) (Table 1).

Extraction and identification of the biomolecules capping the AgNPs: Briefly, the possible biomolecules responsible for capping of the synthesized AgNPs were extracted with 3 volumes of absolute ethanol 3 times in succession. The extraction process was continued overnight at 4°C. Then, the formed pellet was obtained via centrifugation at 40,000 rpm for 10 min. The phosphate buffer solution (PBS)-washed pellet was then subjected to FTIR analysis for identification, in comparing with that of the complete particles.

The possible anticancer activities of the bio-synthesized AgNPs and its capping biomolecules: From a suspension of biogenic AgNPs at concentration of 39 µg/ml, both of AgNPs and their capping biomolecules were separated from each other by ethanol precipitation as previously mentioned. Semi confluent layer of $CaCO_2$ cells were treated for 48 hrs, with 100 µl of either AgNPs or the capping biomolecules. At the end of incubation, cellular viability was checked using trypan blue dye as previously described.

Results and Discussion

UV-Vis spectral analysis of the synthesized AgNPs

The formation of the AgNPs was confirmed through the color change in the reaction mixture ($AgNO_3$ solution + honey bee extract) which is due to the reduction of the Ag^+ ions into Ag atoms then into nanoparticles. Besides, as mentioned previously in the method section, the absorbance of AgNPs colloidal suspension was detected at 450 nm.

Primers	Sequence
Bcl2-forward	5'-TATAAGCTGTCGCAGAGGGGCTA3'
Bcl2-reverse	5'-GTACTCAGTCATCCACAGGGCGAT3'
P53-forward	5'-AACGGTACTCCGCCACC-3'
P53-reverse	5'-CGTGTCACCGTCGTGGA-3'
Caspase-3-forward	5'-TGCGCTGCTCTGCCTTCT-3'
Caspase-3-reverse	5'-CCATGGGTAGCAGCTCCTTC-3'
Survivin-forward	5'-TGCCCCGACGTTGCC-3
Survivin-reverse	5'-CAGTTCTTGAATGTAGAGATGCGGT-3'
GAPDH-forward	5'-GAA GGT GAA GGT CGG AGT
GAPDH-reverse	3'-GAA GAT GGT GAT GGG ATT TC

Table 1: List of primers.

	Day 1	Day 2	Day 3	Day 4	Day 5
OD (450 nm)	0.561	0.822	0.856	0.896	0.891

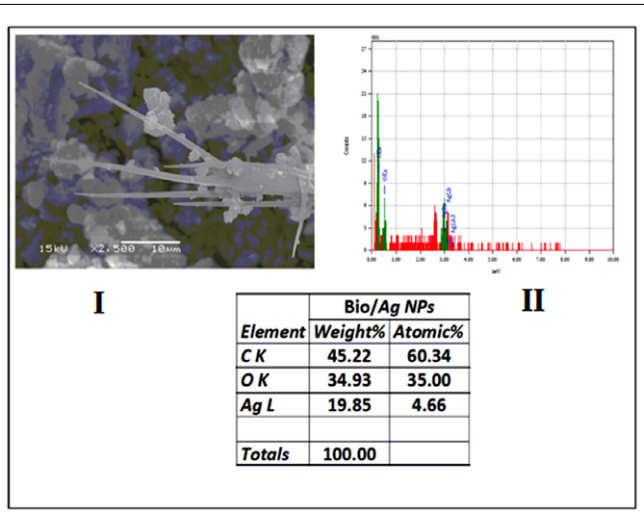
Table 2: UV-Vis spectral analysis of the biosynthesis of AgNPs.

This absorption is attributed to the surface plasmon resonance (SPR) of the nanoparticles. The UV-vis absorption corresponding to the bio-reduction of the Ag^+ ions in aqueous solution occurred at different time intervals is shown in Table 2. The results revealed that the reduction reaction progressed spontaneously with incubation time but this biosynthesis process of AgNPs was almost completed after four days.

Chemical and physical analysis of AgNPs

A scanning electron micrograph as well as the EDX analysis results of the AgNPs prepared from honey bee are shown in Figure 1I. It is apparent from the figure that the AgNPs obtained with raw honey bee extract are spherical. Elemental analysis of the AgNPs prepared by honey bee was carried out using SEM-EDX as also appeared in Figure 1II. The results showed that carbon, oxygen, and Ag were the principal elements of the resulting AgNPs. The EDX quantitative analysis confirms the nanostructure of the AgNPs which contains about 19.85 wt% Ag, 45.22 wt% carbon, and about 34.93 wt% of oxygen.

Figure 2 illustrates the XR diffractogram of the biosynthesized AgNPs. From the figure, the crystalline nature of the AgNPs was confirmed as elemental Ag (0). Besides, the calculation revealed that the size of the obtained AgNPs ranged from 20 to 60 nm. The diffraction peaks appeared at about 32.3°, 46.6°, and 76.8° are corresponding to the planes; (111), (200) and (311), respectively.



I

II

	Bio/Ag NPs	
Element	Weight%	Atomic%
C K	45.22	60.34
O K	34.93	35.00
Ag L	19.85	4.66
Totals	100.00	

Figure 1: (I) SEM image, and (II) the elemental distribution and stoichiometric ratios of AgNPs/ honey bee as determined by EDS.

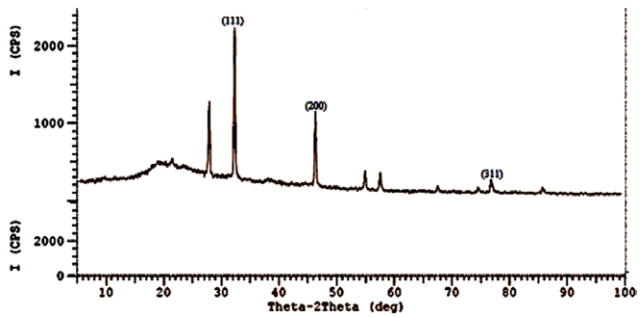


Figure 2: XR diffractogram of dried powder of AgNPs.

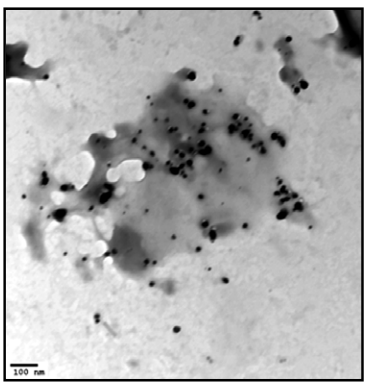


Figure 3: TEM micrographs of the AgNPs capped with the honey bee biomolecules.

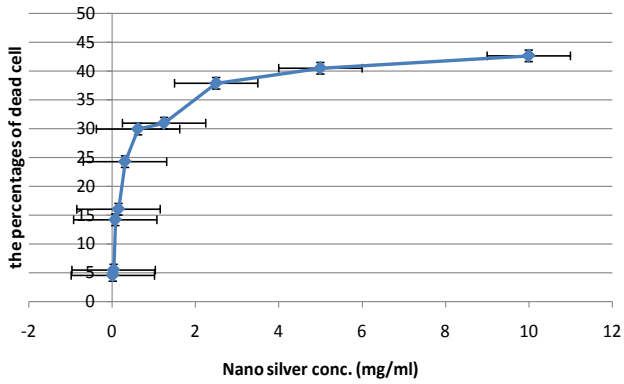


Figure 4: Cytotoxicity assay of the developed biogenic AgNPs on peripheral blood mononuclear cells.

The surface morphology of the developed AgNPs has been investigated using TEM as illustrated in Figure 3. From the figure, the AgNPs are dense and spherical with a size range between 12 and 18 nm. The TEM micrograph also demonstrates that the obtained AgNPs are embedded on the honey bee biomolecules.

Cytotoxicity assay of the biogenic AgNPs

Cytotoxicity test on PBMC was chosen as a reference to the treatments safety on normal cells. The cytotoxicity results shown in Figure 4 indicated that the recommended dose of the biogenic AgNPs is 39 µg/ml with a cellular viability inhibition percentage of 5.453. On the other hand, the non toxic dose of 5-Fluorouracil (5 FU), as the positive control has reached up to 0.9 µg/ml with inhibition percentage of 10.9 (Data is not shown). The comparison between the toxicity of the developed biogenic AgNPs and that of the standard anticancer drug, 5 FU proved that the biogenic AgNPs were safer on the PBMC.

Assessment of the antiproliferative activity

The results of antiproliferative activity of the biogenic AgNPs on $CaCO_2$ cells are shown in Figure 5. The results indicated that, at the end of AgNPs incubation with $CaCO_2$ cells, the inhibition of cellular viability reached up to 60% compared with 66.68% in the case of the cells treated with 5 FU.

Target	Sample	Mean Cq	Mean Efficiency Corrected Cq	Normalized Expression	Relative Normalized Expression	Regulation	Compared to Regulation Threshold
bcl2	5 Fu	29.36	29.36	1.61248	0.07412	13.49113-	Down regulated
bcl2	(-ve) Control	28.86	28.86	21.75417	1	1	No change
bcl2	AgNPs	32.44	32.44	0.46529	0.02139	46.75447-	Down regulated
caspase 3	5 Fu	N/A	N/A	N/A	N/A	N/A	No change
caspase 3	(-ve) Control	N/A	N/A	N/A	N/A	N/A	No change
caspase 3	AgNPs	N/A	N/A	N/A	N/A	N/A	No change
GAPDH	5 Fu	30.05	30.05	N/A	N/A	N/A	No change
GAPDH	Control	33.3	33.3	N/A	N/A	N/A	No change
GAPDH	AgNPs	31.34	31.34	N/A	N/A	N/A	No change
p53	5 Fu	23.51	23.51	93.18246	2.04442	2.04442	No change
p53	(-ve) Control	27.79	27.79	45.57886	1	1	No change
p53	AgNPs	27.36	27.36	15.80025	0.34666	2.88469-	No change
Survivin	5 Fu	27.53	27.53	5.75725	0.03707	26.97446-	Down regulated
Survivin	(-ve) Control	26.02	26.02	155.2988	1	1	No change
Survivin	AgNPs	34.17	34.17	0.14034	0.0009	1106.62206-	Down regulated

Table 3: Gene expression regulation via the biogenic AgNPs treatment.

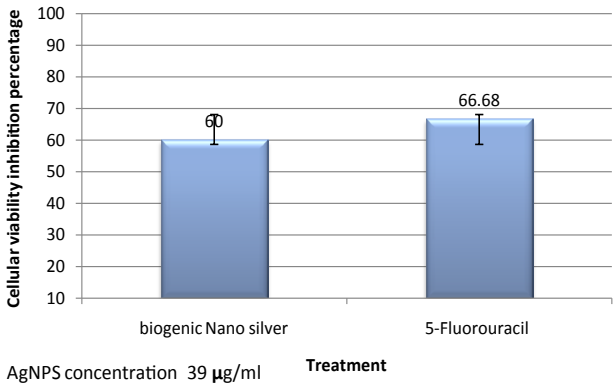


Figure 5: Antiproliferative activity of the biogenic AgNPs on $CaCO_2$ cell.

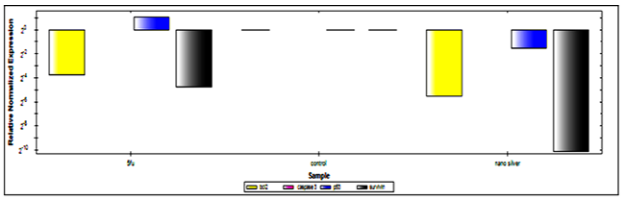


Figure 6: Gene expression regulation via the biogenic **AgNPs** treatment.

The mechanisms of gene regulation targeted by the biogenic AgNPs

The possible mechanisms of AgNPs-mediated cell death via the apoptotic regulators have been measured via gene expression patterns. In order to investigate the apoptotic pathway, RNA levels of Bcl2, survivin and caspase3 were quantitatively measured through RT-PCR with GAPDH expression normalization as shown in Figure 6. The expression of the used genes upon AgNPs treatment was also compared with the standard anticancer drug, 5 FU. The overall results indicated that AgNPs treatment could induce cellular apoptosis in human epithelial colorectaladeno carcinoma cells via down regulation of the expression of both survivin and bcl2 genes without affecting the expression of caspase3 gene. Also, treatment with 5 FU down regulates the expression of both Bcl2 and survivin gene but with regulatory values smaller than that with AgNPs treatment (Table 3).

FTIR analysis of the AgNPs capping biomolecules

FTIR spectrum of the AgNPs extracted capping biomolecules is shown in Figure 7a. The frequencies for the pristine biomolecules are indicated as follows: 3446 cm^{-1} due to the NH group stretching vibration, 1097 cm^{-1} and 854 cm^{-1} are due to the C-O-C asymmetric stretching and bending vibrations, respectively. The bands at 2467 cm^{-1} and at 841 cm^{-1} are attributed to the hydrogen-bonded OH stretching vibrations. On the other hand, as shown in Figure 7b, the FTIR spectrum of AgNPs with its capped biomolecules indicated the presence of large amount of carbohydrates as seen by the large peak at 3510 cm^{-1}, the N–H stretch band merges with this hydroxyl peak. The strong absorption peaks at 2890 cm^{-1} can be assigned to associate with the stretching vibration of C-H, and the strong absorption peaks were observed around 1652–1656 cm^{-1}, representing the stretching vibration of C=O and carboxyl group, the band at 1423 cm^{-1}, indicative of C=C stretching, (corresponding to carbohydrates linked with AgNPs). Further evidence of linking carbohydrates with AgNPs absorbance at 2165 cm^{-1} which is an indicative of -C≡C- stretching present in carbohydrates.

Anticancer activities of the AgNPs and their capping biomolecules

In order to detect which part of the biogenic AgNPs responsible for the anti-proliferative activity against $CaCO_2$ cells, the anticancer activities of both AgNPs and their capping biomolecules were tested as shown in Figure 8. The figure demonstrates that both AgNPs and their capping biomolecules showed anti-proliferative effects against colon cancer superior to the naked AgNPs that showed 58.6% inhibition. On the other hand, the green synthesized AgNPs with their capping biomolecules showed 60% inhibition, while the capping biomolecules showed 57.08% inhibition.

The green and eco-friendly approach for the synthesis of AgNPs is simple, amenable for large scale commercial production and pharmaceutical applications. Since ancient times, honey and other bee products have been widely used as therapeutic agents due to the unique health benefits they are providing which include, for instance, being antibacterial, antioxidant, antitumor, anti-inflammatory and

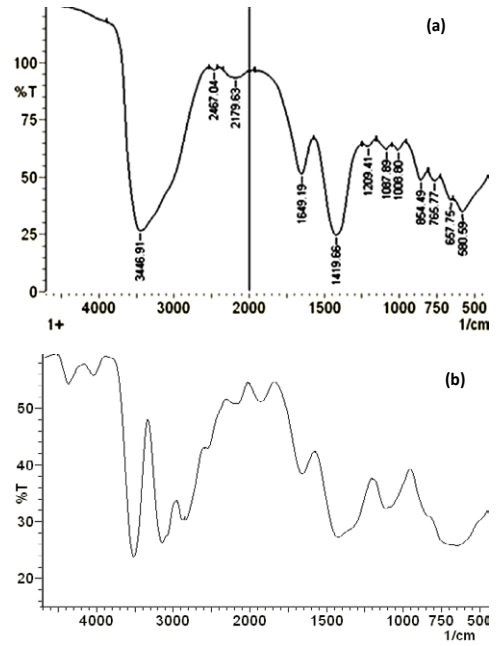


Figure 7: FTIR spectra of AgNPs (a) after extraction of capped biomolecules, and (b) with its capped biomolecules.

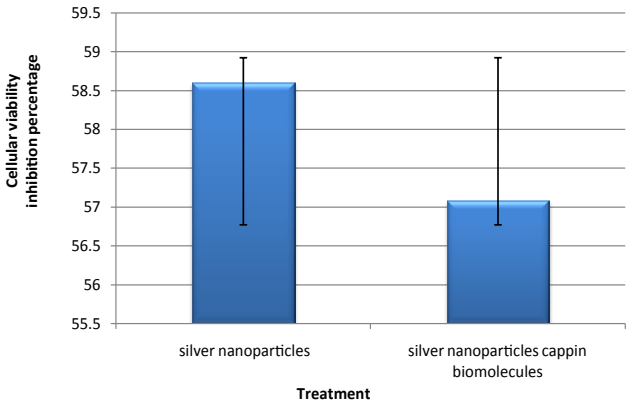


Figure 8: The anticancer activities of AgNPs and their capping biomolecules.

antiviral [13]. In the current study, bio-synthesis of AgNPs using honey bee workers was carried out rapidly and completed within few days with particle size range between 12-18 nm. EDX analysis provided direct evidence that Ag ions were embedded in the honey bee biomolecules and were well prepared without any chemical and structural modifications into the honey bee biomolecules. In addition, using XRD, the diffraction peaks appearing at 32.3°, 46.6°, and 76.8° correspond to the (111), (200), and (311) facets of the face centered cubic crystal structure, respectively [14,15]. The exact mechanism for the green synthesis of the nanoparticles still unknown. However, some recent studies have shown that biomolecules such as proteins, phenols and flavonoids present in the bio-systems play an important role in the reduction of metal ions and capping of the resulting nanoparticles [16]. Based on these reports and on parallel with Murugaraj [17] and the current results, we could clearly conclude that, part from honey bee biomolecules (may be proteins or polysaccharides) was bound with the AgNPs via free amino groups and/or carboxylic residues. These biomolecules were responsible for not only the reduction of Ag+ into Ag^{o} but also, they acted as stabilizing capping agents to Ag^{o} and could also enhance the biological properties of the formed AgNPs. It was noticed that, mostly, the amino groups are the key agents in Ag^{+} ions reduction and strongly adsorbed to them. In addition, the SEM micrographs confirmed the stability and the lower agglomeration tendency of the formed AgNPs due to the presence of these biomolecules.

Because the biomolecules interaction with noble metals may be helpful in cell biology and medicine [18], biogenic nanoparticles could be used as alternative or complementary agents in cancer treatment. In this study, the non-toxic dose of AgNPs on PBMC reached 39 µg/ml. This dose recorded 60% inhibition on CaCo2 cell proliferation. This data is specified particularly to $CaCO_2$ cells. Concerning other types of human cancer cells such as MCF7, they showed 100% cell death by treating with 50 µg/ml concentrations of the AgNPs [18]. Contrariwise, the mushroom obtained AgNPs showed sever cytotoxic effects against MDA-MB-231 cell lines at comparatively low concentration of 6 µg/ml [19]. Based on these findings, it is here envisaged that the cytotoxicity of nanoparticles is relied much on the cell types and nanoparticle size [19,20]. Cellular uptake of nanoparticles leads to generation of reactive oxygen species which provoke oxidative stress. In cells, inorganic nanoparticles easily cross the nuclear membrane and profoundly interact with intracellular macromolecules like proteins and DNA. The mechanism of nanoparticles interaction with DNA is still unknown [21]. Apoptosis-inducing agents that specifically target the tumour cells via gene expression regulation might have the potential to be developed as new anti-tumour agents without induction of inflammatory response. One of the most noticeable aspects in this study is the strong correlation between the survivin and bcl2 over expression in colorectal cancer in p53 independent manner. A similar association between survivin and bcl-2 was recorded also in neuroblastoma [22], gastric cancer [23], and breast cancer [24]. Unlike gastric cancer [23], there was no significant correlation between survivin expression and p53 abnormalities in colorectal cancer. Both survivin and bcl-2 genes are similarly regulated by TATA-less and GC-rich promoter sequence [25]. However, regardless of the pathway of simultaneous co-expression, survivin and bcl-2 have different and non-overlapping anti-apoptosis mechanisms. Moreover, unlike bcl2, survivin was undetectable in normal adult tissues but abundantly expressed in transformed cell types and a variety of human cancers *in vivo* [25].

Conclusion

This study reports that 12-18 nm sized biogenic AgNPs have been successfully synthesized using honey bee extract as a novel reducing agent. Both the obtained AgNPs and their capping biomolecules showed anti-colon cancer activities at both cellular and molecular levels comparing with 5 FU. In conclusion, the single-step and eco-friendly synthesized AgNPs could be a promising agent in colon cancer therapy or drug delivery.

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