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Green nanotechnology: Anticancer Activity of Silver Nanoparticles using *Citrullus colocynthis* aqueous extracts

Alaa M. Shawkey[1], Mohamed A. Rabeh[2,]*, Abeer K. Abdulall[3] and Ashraf O. Abdellatif [4]

[1]*Department of microbiology and immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt.*
[2]*Department of pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt.*
[3]*Department of microbiology and immunology, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt*
[4]*Department of microbiology and immunology, Faculty of Pharmacy, Karary University, Khartoum, Sudan.*

* *Corresponding author:* E-mail: mohamedabdelatty68@yahoo.com Tele: +20223639307, Fax: +2023628426

Abstract:

Green synthesis of metal nanoparticles is a growing research area because of their potential applications in nanomedicines. The green synthesis of silver nanoparticles (SNPs) is a convenient, cheap and environmentally safe approach compared to chemical synthesis. In the present study, we synthesized SNPs from $AgNO_3$ using aqueous extracts (AEs) of fruits, leaves, roots and seeds of *Citrullus colocynthis* as reducing and capping agents. The SNPs were early detected in the aqueous extracts by color change to the reddish brown, and further were confirmed by Transmission Electron Microscope (TEM) analysis. The TEM analysis of SNPs showed spherical nanoparticles with mean size between 7 to 19nm. The anticancer activity of SNPs has been assessed *invitro*. MTT assay on human cancer cell lines of colon (HCT-116), breast (MCF-7), liver (Hep-G2) and intestine (Caco-2) showed good anticancer activity which was negligible for the aqueous plant extracts. Regarding to the tested cell lines the Hep-G2 cell line and HCT-116 were the most sensitive cell line towards the cytotoxic activities of the tested SNPs, while the Caco-2 was the most resistant cell line towards the cytotoxic activities.

***Keywords*:** green synthesis, silver nanoparticles, *Citrullus colocynthis*, anticancer.

1. Introduction

Nanoparticles of free metals have been extensively studied because of their unique physical properties, chemical reactivity and potential applications in catalysis, biological labeling, biosensing, drug delivery, antibacterial activity, antiviral activity, detection of genetic disorders, gene therapy and DNA sequencing (Thirumurgan *et al.,* 2010).

Nanoparticles are particles with a maximum size of 100 nm. These particles have unique properties, which are quite different than those of larger particles. These new properties have been attributed to variation in specific characteristics such as size, shape and distribution (Nalwa, 2005).

Silver (Ag), as a noble metal, has potential applications in medicine due to its unique properties (Gurunathan *et al.,* 2009). There are various methods for SNPs preparation, for example; sol-gel process, chemical precipitation, reverse micelle method, hydrothermal method, microwave, chemical vapor deposition and biological methods, etc (Murthy *et al.,* 2010; Panáček *et al.,* 2006; Sharma *et al.,* 2009). However; biological methods are preferred for being eco-friendly, cost effective, and don't involve the use of toxic chemicals.

Nanoparticles green synthesis is not time consuming compared to other biological processes (Chandran *et al.,* 2006). Synthesis of SNPs using different medicinal plants for pharmaceutical and biological applications have been reported (Gardea-Torresdey *et al.,* 2003; Huang *et al.,* 2007; Leela and Vivekanandan, 2008; Li *et al.,* 2007; Shankar *et al.,* 2004; Song and Kim, 2009).

Cancer is an abnormal type of tissue growth in which the cells exhibit an uncontrolled division, relatively in an autonomous fashion, leading to a progressive increase in the number of dividing cell (Kanchana and Balakrishna, 2011). There is increasing demands for anticancer therapy (Unno *et al.,* 2005). *Invitro* cytotoxicity testing procedures reduces the use of laboratory animals (Abraham *et al.,* 2004) and hence use of cultured tissues and cells have increased (Byrd *et al.,* 2003).

The discovery and identification of new antitumor drug with low side effects on immune system has become an essential goal in many studies of immuno-therapies (Xu *et al.,* 2009). Despite many efforts, multi drug resistance is still considered as a major drawback in chemotherapy of cancer which has been the subject of exhaustive experiments recently (Gottesman *et al.,* 2002). With this aim, many attentions have been paid to

natural compounds in plants, marine organism and microorganisms. Many medically relevant nanoparticles such as AgNPs were investigated for their cytotoxicity aspect. AgNPs showed different degrees of in vitro cytotoxicity (Hsin *et al.*, 2008).

Citrullus colocynthis belongs to Family *Cucurbitaceae*. It grows widely in Sudan and Egypt and it has been used in folk medicine of Sudan and many other African countries for its anti-inflammatory, anti-diabetic, and antioxidant activities (Gurudeeban and Ramanathan, 2010; Kumar *et al.*, 2008).

In the current study, we are showing the synthesis of SNPs using AEs of *C. colocynthis* in a cheap simple method. Additionally, we are verifying the possible upgrading of cytotoxic action of SNPs on HCT-116, MCF-7, Hep-G2 and Caco-2 human cancer cell lines relative to normal AEs of *C. colocynthis*.

2. Materials and methods

2.1 Plant material:

C. colocynthis fruits, seeds, leaves and roots were collected from Omdurman, Sudan (15°39′N 32°29′E), The plants were authenticated by Dr. Mohammed El-Gibali; senior botanist for plant identification at El-Orman botanical garden, Giza, Egypt. Voucher specimens have been deposited in the herbarium of the El-Orman botanical garden.and were used to prepare the AEs.

2.2 Cancer Cell lines:

Colon adenocarcinoma (HCT-116), breast adenocarcinoma (MCF-7), liver carcinoma (Hep-G2) and intestinal adenocarcinoma (Caco-2) cell lines were obtained from National Institute of Cancer, Cairo University, Cancer biology department, pharmacology unit, Cairo, Egypt. Cells were routinely cultured in DMEM (Dulbecco's Modified Eagle's Medium), which was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulphate, and 250 mg/ml Amphotericin B.

2.3 Preparation of plant aqueous extracts:

50grams of each plant organ (fruits, leaves, seeds and roots) were thoroughly washed in distilled water, cut into fine pieces, soaked in 200 ml distilled water for 24 hours at 40°C. The decoction obtained was then filtered through Whatman No.1 filter paper. The same procedure was repeated twice. The aqueous extracts of two successive extractions were pooled, concentrated under vacuum, and lyophilized.

2.4 Synthesis of silver nanoparticles using C. colocynthis AEs:

90 ml of 5.0 mM aqueous solution of silver nitrate **(**Sigma Aldrich, Egypt**)** were added to 10 ml of *C. colocynthis* AEs of concentration 2 mg/ml and kept at room temperature for 24 hours. Silver nanoparticles were gradually formed during the incubation period.

2.5 *Characterization of SNPs by TEM analysis:*

A drop of the silver nanoparticles solution was placed on a cupper grid and coated with carbon support film. After drying, the shape and size of SNPs were analyzed using Transmission Electron Microscope (TEM) JEOL model JEM-2000EX (100 keV).

2.6 Evaluation of invitro cytotoxic activity of the SNPs on tested cell lines:

MTT assay was performed to determine the cytotoxic property of synthesized SNPs against HCT-116, MCF-7, Hep-G2 and Caco-2 cell lines. Briefly cell lines were seeded in 96-well tissue culture plates. Appropriate concentrations of stock solution were added and incubated for 48 hours at 37^{o}C. Non-treated cells were used as control. Incubated cultured cell was then subjected to MTT (3-(4,5 Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide, a tetrazole) colorimetric assay. The tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) is used to determine cell viability in assays of cell proliferation and cytotoxicity. MTT is reduced in metabolically active cells to yield an insoluble purple formazan product. Cells were harvested from maintenance cultures in the exponential phase and counted by a hemocytometer using trypan blue solution. The cell suspensions were dispensed (100µl) in triplicate into 96-well culture plates at optimized concentrations of 1 $\times$ 10^{5}/well for each cell lines, after a 24 hours recovery period. Assay plates were

read using a spectrophotometer at 520 nm. The spectrophotometrical absorbance of the samples was measured using a microplate (ELISA) reader. The cytotoxicity data was standardized by determining absorbance and calculating the correspondent SNPs concentrations. Data generated were used to plot a dose-response curve of which the concentration of extract required to kill 50% of cell population (IC_{50}) was determined.

Cell viability (%) = Mean OD/ control OD × 100

3. Data analysis

The IC_{50} values (concentration at which 50% of cells were death) against colon adenocarcinoma (HCT-116), breast adenocarcinoma (MCF-7), liver carcinoma (Hep-G2) and intestinal adenocarcinoma (Caco-2) cell lines are reported as mean± standard deviation of three independent experiments. IC_{50} values that were greater than 30µg/ml considered insignificant, and vice versa.

The experimental work can be summarized in the following chart (Figure 1).

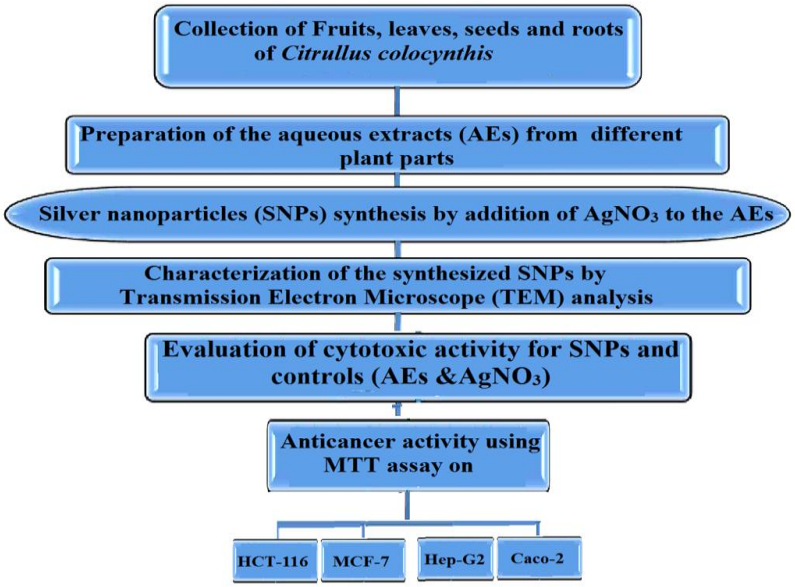

Figure 1: Experiment flowchart

4. Results

4.1 Synthesis and characterization of SNPs/C. colocynthis:

We treated the AEs of different organs of *C. colocynthis* with $AgNO_3$ which resulted in changing the color of AEs to reddish brown which is the early sign for SNPs formation as shown in figure 2, due to the excitation of surface plasmon vibrations in SNPs (Thirumurgan *et al.,* 2010). Further confirmation for SNPs synthesis was carried out using TEM analysis. The $AgNO_3$ solution and the AEs were used as a negative control throughout the study.

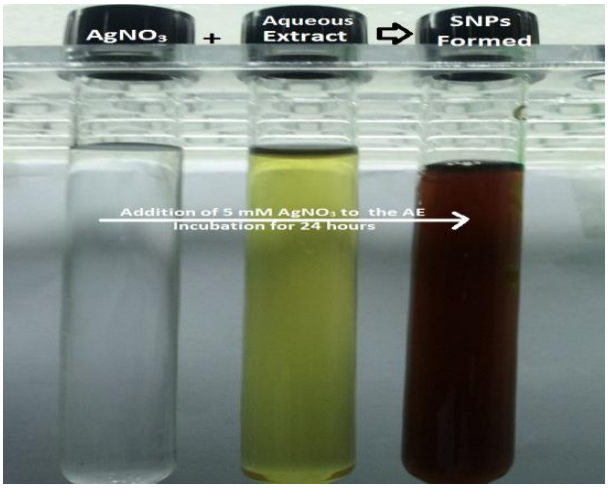

Figure 2: Early sign for SNPs formation observed as color change of *C. colocynthis* /AEs to reddish brown after 24 hours incubation with 5mM $AgNO_3$

Figure 3 shows (TEM) picture of 5 mM $AgNO_3$ solution**,** 5 mM $AgNO_3$ solution , particles exhibited irregular shapes with an average size 562.4 nm; whereas Figure 4 shows TEM images of the AEs from different parts of *C. colocynthis* before and after treatment with $AgNO_3$ formation; fruits AE (A1), fruits SNPs (A2), seeds AE (B1), Seeds SNPs (B2), leaves AE (C1), leaves SNPs (C2), roots AE (D1) and roots SNPs (D2). The average mean sizes of silver nanoparticles were 19.267 nm, 16.578 nm, 13.376.nm and 7.398 nm for fruits, seeds, leaves and roots SNPs respectively. All produced nanoparticles seem to be spherical in their morphology.

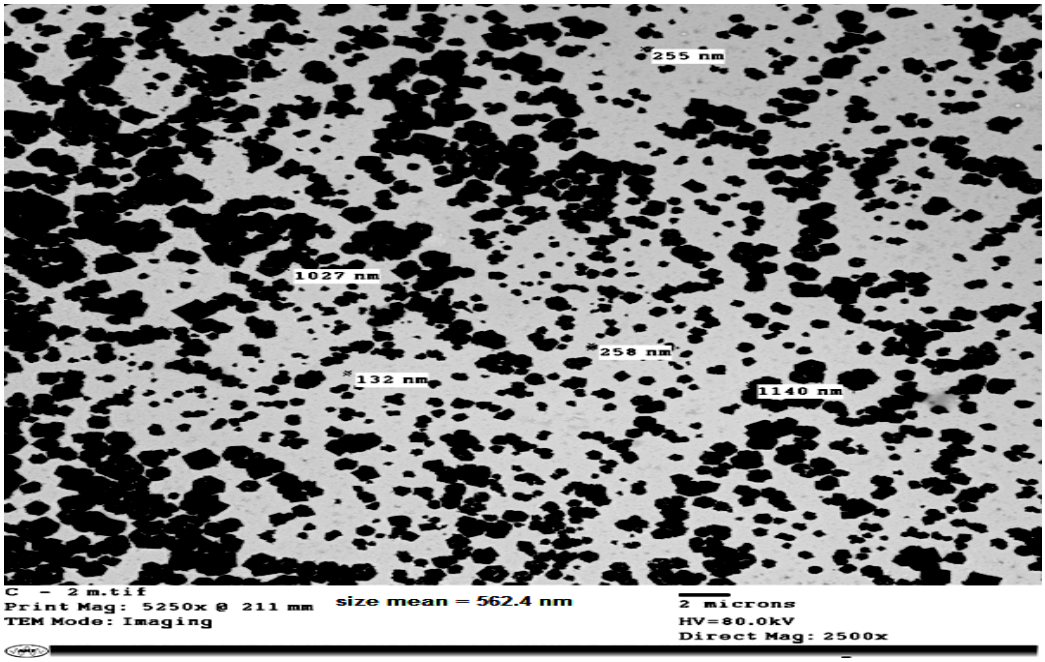

Figure 3: TEM photo showing 5 mM $AgNO_3$ solution . Particles exhibits irregular shapes with an average size 562.4 nm

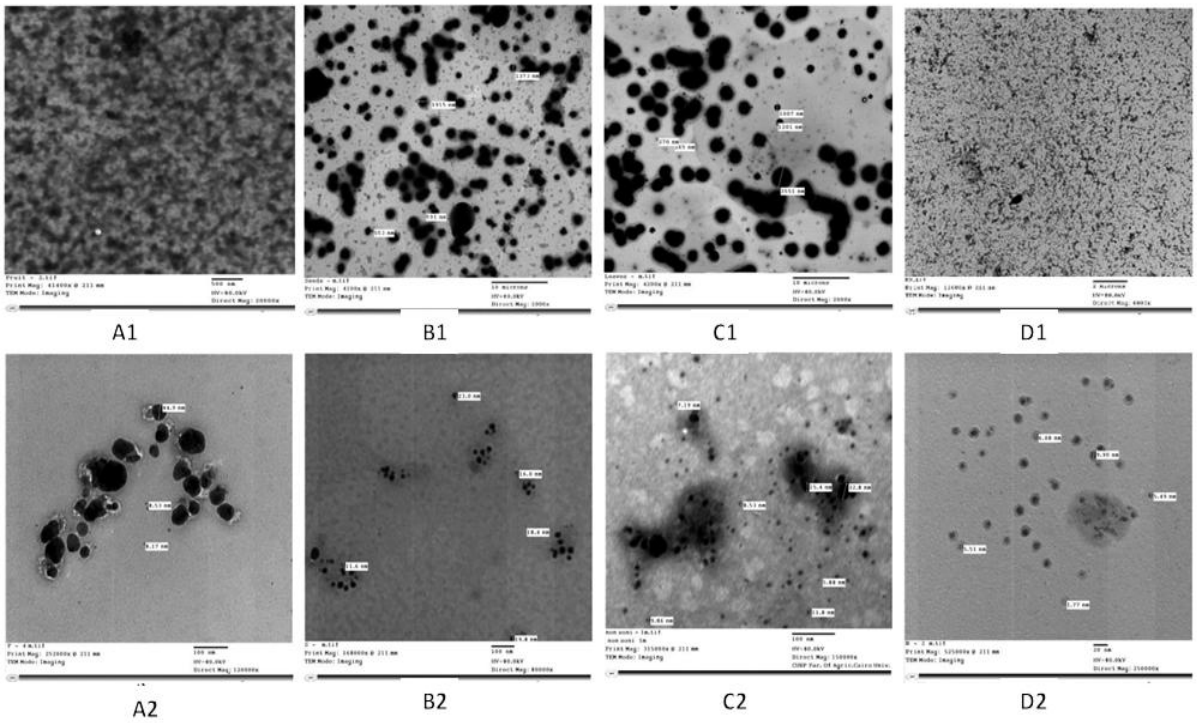

Figure 4: TEM photos for the aqueous extracts (AEs) from different parts of *C. colocynthis* before and after treatment with $AgNO_3$, showing the shape and size of produced SNPs after treatment process : fruits AE (A1), fruits SNPs (A2), seeds AE (B1), Seeds SNPs (B2), leaves AE (C1), leaves SNPs (C2), roots AE (D1), roots SNPs (D2).

4.2 Citrullus colocynthis /SNPs anticancer activity:

The cytotoxicity of the SNPs and AEs were studied invitro against HCT-116, MCF-7, Hep-G2 and Caco-2 cancer cell lines at different concentration (0, 5, 12.5, 25, 50 µg/ml).

The results obtained from MTT assay after 48hrs of incubation showed that fruits/SNPs showed significant effect on Hep-G2 and MCF-7 with IC_{50} = 17.2 and 22.4 µg respectively. Fruits/ SNPs cytotoxic effect on HCT-116 and Caco-2 showed insignificant anticancer activity with IC_{50} > 30µg/ml (Table 1, Figure 5).

Leaves/SNPs was effective only against Hep-G2 cancer cell line, as it led to inhibition in cell growth as concluded from IC_{50} values 10.2µg /ml as shown in (Table 1 , Figure 6).

Treatment of tested cell cancer cell lines with seeds/SNPs led to insignificant cytotoxic effect with IC_{50} values above 30 µg (Table 1, Figure 7).

At the same time treatment of HCT-116 and Hep-G2 cancer cell lines with roots/SNPs led to significant cytotoxic effects with IC_{50} 21.2 µg for HCT-116 and IC_{50} = 22.4 µg for Hep-G2 . Insignificant cytotoxic effect was observed on MCF-7 and Caco-2 cell lines as shown in (Table 1, Figure 8).

Table 1: *Invitro* cytotoxicity effect of silver nanoparticles (SNPs) from the fruits, leaves, seeds and roots of *C. colocynthis* on colon adenocarcinoma (HCT-116), breast adenocarcinoma (MCF-7), liver carcinoma (Hep-G2) and intestinal adenocarcinoma (Caco-2) cell lines represented as cell viability percentage ± standard deviation:

		Cell Viability % ± SD					
		Conc. (µg /ml)	**0**	**5**	**12.5**	**25**	**50**
Tested extracts	**Fruits SNPs**	HCT-116	100	83.1±0.41	82.3±0.18	66.8±0.56	41.3±0.73
		MCF-7	100	88.8±0.22	64.9±0.85	43.6±0.31	41.1±0.26
		Hep-G2	100	90.4±0.73	59.1±0.82	34.7±0.16	46.1±0.91
		Caco-2	100	84.1±0.26	80±0.35	55.2±0.11	61.6±0.56
	Leaves SNPs	HCT-116	100	54.3±0.11	50.5±0.96	52.7±0.42	58.5±0.23
		MCF-7	100	95.5±0.12	95.5±0.68	98.6±0.22	99.6±0.35
		Hep-G2	100	52.4±0.55	48.4±0.41	51.2±0.15	53.2±0.35
		Caco-2	100	66.4±0.34	63.1±0.53	68.6±0.60	61.1±0.87
	Seeds SNPs	HCT-116	100	91.6±0.13	77.4±0.27	51.5±0.17	48.1±0.45
		MCF-7	100	82.3±0.44	80±0.21	83.2±0.15	91.6±0.82
		Hep-G2	100	77±0.41	79.1±0.35	67.5±0.41	49.1±0.91
		Caco-2	100	70.4±0.25	63.8±0.17	52.3±0.35	67.6±0.46
	Roots SNPs	HCT-116	100	87.7±0.47	62.6±0.29	43.6±0.36	41±0.84
		MCF-7	100	94.1±0.73	100±0.33	97.6±0.19	100±0.14
		Hep-G2	100	76.6±0.23	62.8±0.57	44.4±0.62	42.3±0.33
		Caco-2	100	95.7±0.45	77.9±0.63	74.9±0.45	71.4±0.25

Conc. = concentration (W. /V. mg/ml H_2O), **SNPs** = Silver Nanoparticles, **SD =** Standard deviation, **HCT-116**= Colon adenocarcinoma cell line, **MCF-7**= Breast adenocarcinoma cell line, **Hep-G2**= Liver carcinoma cell line, **Caco-2**= Intestinal adenocarcinoma cell line.

From the obtained data no significant cytotoxic activities were observed against Caco-2 cancer cell line and with exception of fruits/SNPs, other SNPs didn't show any significant activity against MCF-7 cancer cell line. Fruits/SNPs showed good cytotoxic properties comparing to other SNPs and to less degree roots/SNPs whereas leaves/ SNPs were only active against Hep-G2 while seeds/SNPs cytotoxicity is the weakest one. Regarding to the tested cell lines the HCT-116 and Hep-G2 cell lines were the most sensitive cell lines towards the cytotoxic activities of the tested SNPs, while the Caco-2 was the most resistant cell line towards the cytotoxic activities.

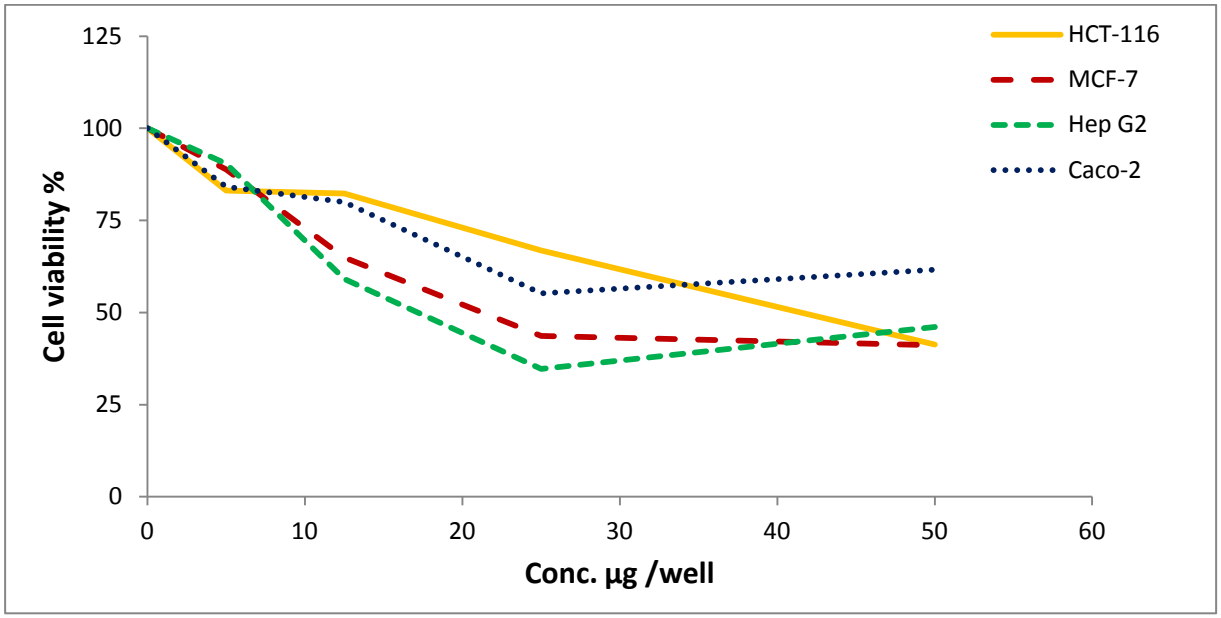

Figure 5: Cytotoxicity effect of fruits/SNPs on HCT-116, MCF-7, Hep-G2 and Caco-2 cancer cell lines using MTT assay; Fruits/SNPs showed significant effects on Hep-G2 and MCF-7 with IC_{50} = 17.2 µg 22.4 µg respectively . Fruits/SNPs Cytotoxic activity on HCT-116 and Caco-2 cell lines was non-significant (IC_{50} > 30 µg)

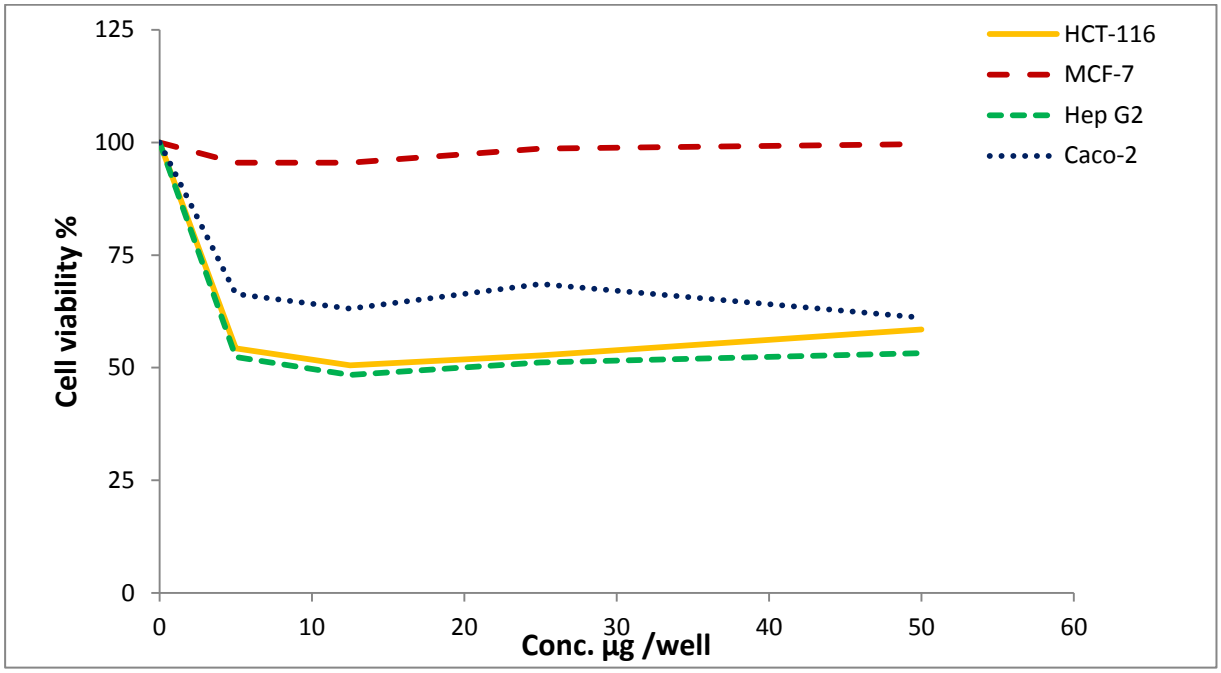

Figure (6): Cytotoxicity effect of leaves/SNPs on HCT-116, MCF-7, Hep-G2 and Caco-2 cancer cell lines using MTT assay; Leaves/SNPs showed significant effects only on Hep-G2 with IC_{50} = 10.2 µg. Leaves /SNPs anticancer activity on HCT-116, Cytotoxic activity on MCF-7 and Caco-2 cell lines was non-significant (IC_{50} > 30 µg)

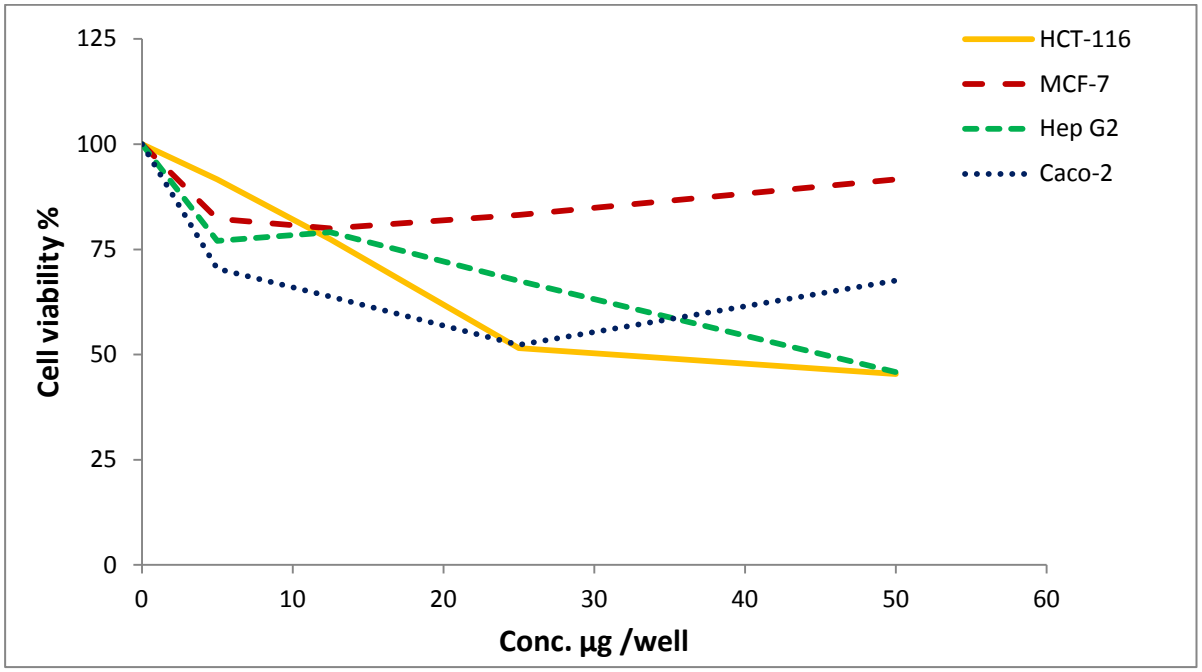

Figure 7:Cytotoxicity effect of seeds/SNPs on HCT-116, MCF-7, Hep-G2 and Caco-2 cancer cell lines using MTT assay; Seeds/SNPs showed non-significant cytotoxic activity on tested cell lines (IC_{50} > 30 µg)

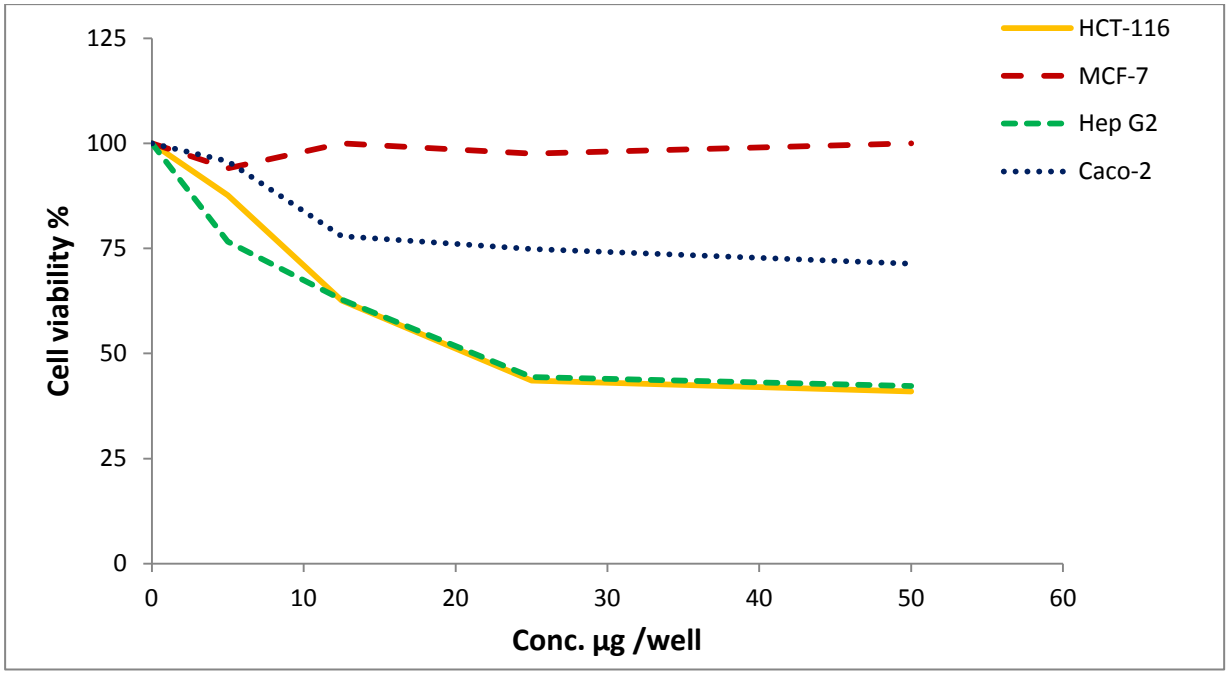

Figure 8: Cytotoxicity effect of roots/SNPs on HCT-116, MCF-7, Hep-G2 and Caco-2 cancer cell lines using MTT assay; Roots/SNPs led to significant cytotoxic effects on HCT-116 and for Hep-G2 with IC_{50} 21.2 µg and 22.4 µg respectively. Cytotoxic activity on MCF-7 and Caco-2 cell lines was non significant (IC_{50} > 30 µg)

5. Discussion

It is well known that Ag ions and Ag-based compounds have strong biological activities (Furno *et al.,* 2004), However, Ag ions or salts has only limited usefulness for several reasons, including the interfering effects of salts and the continuous release of enough concentration of Ag ion from the metal form. In contrast, these kinds of limitations can be overcome by the use of SNPs.

However, to use SNPs in various fields, it is essential to prepare the SNPs with a cost effective method. In this study we were able to prepare SNPs eco-friendly and cost effectively by using the aqueous extracts from different parts of *C. colocynthis*, and these SNPs were homogeneous (Figures 4).

Owing to their small size, SNPs impair the sulfur and phosphorus containing essential macromolecules such as proteins and DNA (Wei *et al.,* 2009), Thus, action of SNPs appears to be a consequence of adherence to and penetration inside the cell of the target cells.

The anti-proliferative effect of SNPs and Ag^+ was reported (Ahamed *et al.,* 2008; Rahman *et al.,* 2009) . Furthermore, the interaction of SNPs and Ag^+ with certain proteins has been the subject of extensive studies in recent years. In this study, anti-proliferative effect of SNPs on HCT-116, MCF-7, Hep-G2 and Caco-2 was investigated. Cells were observed to exhibit different responses after treatment with SNPs. The associate IC_{50} values as indicated in table 1 reason for

The enhanced cytotoxicity of SNPs may be due to their size which facilitates their subsequent penetration in tumor cells.

The cytotoxic effects of SNPs ,probably due to the fact that SNPs may interfere with the proper functioning of cellular proteins and induce subsequent changes in cellular chemistry (Rogers *et al.,* 2008). Zolghadri and coworkers demonstrated that SNPs provide a relatively high hydrophobicity inside bovine hemoglobin which causes a transition from alpha helixes to beta sheets and leads to partial unfolding and aggregation of the protein (Zolghadri *et al.,* 2009), other researchers suggest that SNPs are likely to interact with thiol rich enzymes (Morones *et al.,* 2005); Therefore, it is possible that once penetrated into cells, SNPs may attack functional proteins of cells which results in partial unfolding and aggregation of proteins as it is the case in the bovine hemoglobin.

Toxicity of silver nanoparticles is concentration-size-shape dependent ; In green synthesis process these factors are affected by chemical compositions of plant parts extracts, accordingly this will lead to variability in the biological activities of such extracts (Elechiguerra et al., 2005; Morones et al., 2005; Savithramma et al., 2011).

Based on the present study, the action model of SNPs may be described as SNPs making a breakthrough in the permeability of outer membrane firstly, resulting in the leakage of cellular materials. Secondly, SNPs enter the inner membrane and inactivate respiratory chain dehydrogenases, thus inhibiting respiration and growth of cells. Simultaneously, SNPs could affect some proteins and phosphate lipids and induce collapse of membrane, resulting in cell decomposition and death eventually.

These results are potentially promising because they suggest that, by using non-cytotoxic amounts of silver salt with a convenient, eco-friendly and cheap method using *C. colocynthis* aqueous extract; we can synthesize SNPs with good anticancer activities. This opens the door to prepare a suitable pharmaceutical formulation using these nanoparticles. Taking into account the mobility of SNPs into cells and their fate in a bioprocess or even in the environment, the risk aspects for the application in larger scales and in the environment as well as studies on different biological activities in different fields should be strengthened in future studies.

6. Conclusion

Silver nanoparticles with an average size between 7 to 19 nm and spherical shapes were successfully synthesized using aqueous extract of fruits, seeds, leaves and roots of *C. colocynthis*. Green synthesis of SNPs using green resources like *C. colocynthis* is a better alternative to chemical synthesis, since this green synthesis has many advantages such as, ease of applicability for large scale production, economically feasible, and eco-friendly. The anticancer studies showed great improvement in the biological activity of the extracts after SNPs synthesis.

The present study explores the potential antitumor activity of greenly synthesized SNPs on HCT-116, MCF-7, Hep-G2 and Caco-2 tumor cell lines. Our results suggest that with the aid of metal based nanoparticles,

conditional chemotherapeutic agents may have even broader range of applications in future such as direct targeting to tumor cells. Thus, a study of the exact mechanism by which SNPs inhibit signaling cascades responsible for the development and progression of the disease would be a tremendous breakthrough in the field of nanomedicines and make these agents an effective alternative in tumor and angiogenesis-related diseases.

The use of plant extracts for making SNPs is an inexpensive, easily scaled up and environmentally benign. It is especially suited for making nanoparticles that must be free of toxic contaminants as required in therapeutic applications. The plant extract based synthesis can provide nanoparticles of a controlled size and morphology. Applications in targeted drug delivery and clinical diagnostics are developing.

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LIST OF ABBREVIATIONS

TEM	Transmitted Electron Microscope
nm	nanometer
mM	millimolar
SNPs	Silver Nanoparticles
AE	Aqueous extract
DMEM	Dulbecco's Modified Eagle's Medium
ml	Milliliter
IC_{50}	50% Inhibitory concentration
Conc.	Concentration