European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 07, Issue 03, 2020

Artemisia annua L. silver nanoparticles exhibit anticancer activity by ROS mediated apoptosis in MDA-MB-231 cells.

RENUGADEVI KARTHIKEYAN[1] and JULIUS AMALDAS[2]*

[1] Renugadevi Karthikeyan Department of Biochemistry, Bharathiar University, Coimbatore - 641 046, Tamil Nadu, India

Prince shri Venkateswara Arts & Science college, gowrivakkam, Chennai, Tamil Nadu, India

[2] Julius Amaldas Sree Balaji Dental College & Hospital, Chennai - 600 100, Tamilnadu, India

**Address for correspondence*
E-mail: krenu2007@gmail.com

Abstract

Aim of this study was to investigate the growth inhibitory effect of *Artemesis annua* L. silver nanoparticles (A.annua AgNPS) on human triple negative breast cancer tumor cell line MDA-MB-231. Cells were cultured and treated with A.annua water extract and A.annua AgNPS to determine the half inhibitory concentration (IC_{50}). Oxidative stress was determined using lipid peroxidation (LPO), ROS generation and by determining the levels of antioxidants, superoxide dismutase (SOD) and reduced glutathione (GSH). Further, the mechanism of apoptotic induction was assessed by DNA fragmentation analyses. We observed that A.annua AgNPS significantly caused growth inhibition by increasing levels of LPO and ROS, in a dose dependent manner with reduced level of endogenous antioxidant enzymes. Increased shear in genomic DNA from MDA MB231 cells treated with A.annua AgNPS shows apoptosis to be the cause of cell cell death. We conclude that A.annua AgNPS can be useed as a therapeutic agent against breast cancer.

Keywords: *Artemesia annua*· Silver nanoparticle· Breast cancer· Apoptosis

1. Introduction

Breast cancer is the leading cause of death among women globally with 1 million new cases every year with an estimated prediction of 14 million new cases by 2035. Breast cancer mortality rate is gradually increasing due to conditions like poor prognosis, lack of effective treatment options and increasing drug resistance [1, 2]. Recently, breast cancer reports are increasing rapidly at an alarming rate, preceding the cervical cancer in India [3]. Breast cancer is classified into 3 major subtypes based on the molecular markers for estrogen or progesterone receptors and human epidermal growth factor 2 (HER2): hormone receptor positive (HER2) negative (70%), HER2 positive (15%-20%), and triple-negative (tumors lacking all 3 standard molecular markers; 15%). Notably, triple-negative breast cancer recur more frequently than the other 2 subtypes, and it denotes that it warrants greater attention towards identifying therapeutic regimens that can inhibit and as well prevent its recurrence.

In recent years, role of metal nanoparticles in delivering and targeting agents that are beneficial at pharmaceutical, therapeutic and diagnostic levels are gaining much importance [4]. Particularly, in disease like cancer where lack of target specificity for anticancer agents remains a greater challenge. Use of silver nanoparticle mediated drug delivery exhibit significant clinical benefits [5]. Biogenic (green chemistry) synthesis of silver nanoparticles (NPs) using plant extract were reported to exhibit useful clinical benefits than other forms of synthetic AGNPs [6]

Artemisia annua L. (commonly known as sweet wormwood, qing hao) is native to China for about 2000 years. It was popular for its unique potential as a natural antimalarial agent, as an anti-oxidant, with anti-inflammatory and anti-microbial effects [7]. The present study focused on investigating the *in vitro* cytotoxicity of biogenic AgNPs using A.annua using MDA-MB-231 cell line. Our results demonstrated that A.annua AgNPs exhibit significant cytotoxicity, which can be attributed to its ability to induce oxidative stress, mitochondrial dysfunction and apoptotic induction. We believe that investigation of nanoparticle research for treatment of cancer therapy will bring more significant clinical outcomes in near future.

2. MATERIALS AND METHODS

Chemicals and their sources:

Trypsin, Bovine serum albumin (BSA), Dimethyl sulfoxide (DMSO) 3-[4, 5-Dimethyl-2-thiazolyl]- 2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum (FBS) and antibiotics were purchased from Gibco (Carlsbad, CA, USA). FBS was stored at −20°C and then freshly prepared aliquots were used for cell culture complete medium. All the other chemicals used were of cell culture grade unless otherwise stated.

Cell line and culture:

Triple negative human breast cancer cell line MDA-MB-231 was purchased from National centre for cell science (NCCS), Pune. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-Penicillin streptomycin solution. Cells were maintained at a controlled condition of 5% CO_2, and 95% humidity. Experiments were performed in 6-well plates and 96 well plates unless stated otherwise. Cells were seeded at a density of 1×10^6 and 1×10^4 cells per well and incubated for 24 h prior to the experiments. The cells were washed with ice cold-phosphate buffered saline, (pH 7.4) for immediate use in experiments and for storage of cell pellets.

Cytotoxicity assay:
Effect of A.annua AgNPs (dose dependent) on cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide dye reduction. Briefly, MDA-MB-231 cells (1×10^4 cells) were plated onto 96-well flat bottom culture plates and treated with various concentrations A.annua AgNPs. For 24 h. After 24 h of incubation, 10 μL of MTT (5mg/mL in PBS) was added to each well, and then incubated for 2 h at 37◦C in dark. During the course of incubation, the appearance of purple precipitate in the wells was monitored using inverted microscope and then the growth medium was removed from each well including blank. Then 100 μL of DMSO per well was added to dissolve the formazan, including the blank and then mixed to obtain homogenous solution via gentle shaking at 37◦C in a plate reader, and absorbance was measured using ELISA reader at 595 nm. Finally IC50 value of A.annua AgNPs was calculated and used for further experiments. Results were expressed as mean of three independent experiments.

Intracellular ROS analysis:
Briefly, MDA-MB-231 cells were treated with A.annua AgNPs for 12 h. The net intracellular ROS measurement was carried out based on the intracellular peroxide-dependent oxidation of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, USA) into a highly fluorescent product 2',7'-dichlorofluorescein (DCF), as reported previously. MDA-MB-231 cells were seeded into 35 mm dish at a density of $1X10^5$ cells/ml and incubated overnight. Following 12 h treatment with A.annua AgNPs the cells were incubated with DCFH-DA (10 µM) for 1 h. Along with the experimental cells, freshly prepared hydrogen peroxide (H_2O_2, 0.1 mM in PBS) was used as a positive control. Then the excess DCFH-DA, from the dishes were removed and washed with PBS (twice) and suspended in 500 µl PBS. Finally the amount of ROS was estimated from fluorescence intensity of DCF production using a spectrofluorometer (excitation at 485 and emission at 530 nm).

Measurement of Lipid peroxidation Products:
Briefly, 2×10^4 cells/well were seeded in a 96-well plate (black walled) and incubated at 37°C in CO_2 incubator. MDA-MB-231 cells were treated with and without A.annua AgNPs for 24 h and harvested by centrifugation. Extent of lipid peroxidation was assessed in control and A.annua AgNPs treated cell lysate by measuring malondialdehyde (MDA) levels by colorimetric assay, according to the method described previously [8]. Reaction of two molecules of thiobarbituric acid reactive substances (TBARS) with one molecule of MDA which yields pink color from the final soluble contains the substances. Absorption of the samples was read at 532 nm using a UV–vis spectrophotometer. Amount of MDA for each treatment was normalized by protein concentration and expressed as nmol TBARS per mg of protein lysate.

Measurement of antioxidant markers:
The levels of antioxidant markers SOD and GSH were analyzed in MDA-MB-231 cells treated with and without A.annua AgNPs (dose dependently) for 12 h. Superoxide dismutase (SOD) was analyzed by kinetic absorption method prescribed previously by Aebi and Misra, [9]. SOD was expressed as units/min/mg of protein. Glutathione (GSH) was measured by previously reported method [10] and the results were expressed in terms of µg of GSH/mg of protein.

DNA fragmentation analysis:
Cells were treated with A.anuna AgNPS for 24 h in sterile tissue culture dishes and DNA fragmentation analysis was based on the method of Kasibhatla et al. (2006)[11] . Briefly, 10^6 cells/ml after treatment with AgNPS and were harvested and transferred to sterile eppendorf tubes and lysed with 20 µl Tris, EDTA-SDS lysis buffer by constant pippetting using a pipette tip. Lysates were mixed with RNase A and RNase T1 (10 µl), incubated at 37ºC for 60 min.

After incubation, the lysate is treated with proteinase K (10 µl) at 50ºC for 90 min. Finally, about 5 µl of 6X DNA loading buffer was added to each sample and electrophoretically seperated on 1.5% agarose gel (with ETBR) in TAE buffer (100 V at RT, for 20 minutes). Then the DNA laddering pattern in treated and untreated cells were visualized by a UV light transilluminator and documented.

Statistical analysis:

Experiments were performed in triplicates (n=3). The mean ± standard deviation (SD) was determined for each group. One way analysis of variance (one-way ANOVA) and Tukeys' test were used for statistical analysis. Statistical value of $p < 0.05$ was used for considering statistical difference between groups.

3. RESULTS AND DISCUSSION

Anti-cancer drugs approved by food and drug administration (FDA) has been continuously escalating ever year, however there exists lack of complete cure, coupled with adverse effects of existing anti-cancer drugs [12, 13]. Need for better therapeutics with potential anti-tumor efficacy that can reduce the unprecedented challenge in healthcare against cancer are on the rise Natural agents like plant metabolites and its derivatives have been used traditionally for various ailments; however they were not optimized in suitable formats that might enhance their efficacy [14]. Recently plant derived therapeutic agents via green amalgamation with metal nanoparticles were reported to show promising effects in mitigating cancer progression and its recurrence [15, 16]. However, very limited studies have been carried out in this aspect and hence we aimed to investigate the anticancer effect of AgNPs biosynthesized using A.annua plant extract.

A.annua AgNPs induce cytotoxicity

Biogenic A.annua AgNPs induced cytotoxicity on MDA-MB-231 cells was found to be dose dependent. Change in percentage of cell viability in MDA-MB-231 cells was observed at A.annua AgNPs concentration range (2.5 µg/ml, 5 µg/ml, 12.5 µg/ml, 25 µg/ml, and 50 µg/ml) after 24h of treatment. A.annua AgNPs exerted complete mortality at 50 µg/ml concentration, while 50 % reduction was observed at 12.5 µg/ml (IC_{50}) compared to A.annua plant extract and untreated cells (Fig 1A). However, there was no significant difference in cell viability at similar concentrations after 24 h. Hence, 12.5 µg/ml was used for further experiments. Moreover, the tested concentration range mentioned above did not show any toxicity to normal vero cell lines (supp Fig 1). Thus the exhibited effect is highly specific to cancer cells.

***A.annua* induces oxidative stress in cancer cells**

To investigate the effect of A.annua AgNPs in oxidative stress, ROS levels were measured using H2DCF-DA assay. Fig 2A shows that ROS generation was significantly elevated in cells treated with A.annua AgNPS (12.5 µg/ml) than the control cells or A.annua extract treated cells. However, upon pretreatment with an antioxidant N-acetyl cysteine (NAC), ROS generation in A.annua AgNPs and H_2O_2 treated cells was markedly reduced. Fig 2B shows that formation of TBARS was significantly increased in cells treated with A.annua AgNPs, compared to control and A.annua extract treated cells. Together, our results showed that loss of cell viability upon treatment with A.annua AgNPs is mediated via ROS generation, thereby altering the intracellular redox status which might lead to cell death via apoptosis.

Altered homeostasis in the balance between oxidants to antioxidants favors oxidative niche which leads to increased redox state of cells. Hence, to determine the status of anitoxidants during A.annua AgNPs treatment, we analysed the levels of major endogenous antioxidants GSH and SOD. A.annua AgNPs treatment to MDA-MB-231 cells caused significant dose dependent reduction in the levels of GSH and SOD compared to control and A.annua extract treated cells (Fig 3).

Excessive ROS with augmented antioxidant defense can results in oxidation of cellular macromolecules including DNA resulting in apoptotic cell death [17]. Hence, ROS generation by AgNPs was considered crucial phenomenon in its anticancer potential. In our study elevated ROS levels in MDA-MB 231 cells treated with A.annua AgNPs for 12 h denotes early induction of oxidative stress, while pretreatment with NAC reduced ROS generation and this denotes that there exists a lack of antioxidant defense in MDA-MB-231 cells during AgNPs exposure favouring oxidative niche. In addition ROS mediated oxidative stress in also substantiated by increased levels of lipid peroxidation product MDA, reduced levels of endogenous antioxidants GSH and SOD as observed in our studies. Together, our results demonstrate that A.annua AgNPs mediated cytoxicity is a ROS dependent manner and altered endogenous redox status leading to cell death.

A.annua AgNPs fragments DNA

DNA fragmentation is considered as the hall mark of apoptotic cell death, and the anti-cancer ability of therapeutic agents were tested by its ability to induce DNA fragmentation. Hence, we analysed the status of DNA fragmentation in MDA-MB-231 cells treated with A.annua AgNPs. Fig 4 shows that A.annua AgNPs induced significant DNA fragmentation as observed from increase in shear in lane 3 and also in DOX treated cells (lane 4) compared to control (lane 1), and A.annua extract (lane 2) treated cells. Together our results prove that A.annua induces apoptotic cell death in MDA-MB 231 cells. With promising results these can be tested on other tumor models and can be considered as a therapeutic agent against breast cancers.

In conclusion, we synthesized green AgNPs using A.annua extract and tested its cytotoxic potential against the breast cancer cell line MDA-MB-231.We found that the anticancer effect is mediated through oxidative stress with increased ROS production and declined antioxidant activity. Additionally apoptotic induction was mediated through DNA fragmentation. However, the ability of A.annua AgNPs in orchestrating cellular signal cascades that is responsible for its anti-cancer effects must be validated using various cancer cell lines. Such mechanistic studies will help uncover many other potential applications of AgNPs for clinical use.

4. References

1. Pilleron S, Sarfati D, Janssen- Heijnen M, Vignat J, Ferlay J, Bray F, Soerjomataram I (2019) Global cancer incidence in older adults, 2012 and 2035: A population-based study. Int J Cancer. 144(1):49-58.

2. Abbasi Gamasaee N, Admansouri M, Ghiasvand S, Shahriari F, Zare Marzouni H, Aryan H, Jangholi E, Javidi MA (2018) Hypericin Induces Apoptosis in MDA-MB-175-VII Cells in Lower Dose Compared to MDA-MB-231. Arch Iran Med. (9):387-392.

3. Das A, Narayanam MK, Paul S, Mukhnerjee P, Ghosh S, Dastidar DG, Chakrabarty S, Ganguli A, Basu B, Pal M, Chatterji U, Banerjee SK, Karmakar P, Kumar D, Chakrabarti G (2019) A novel triazole NMK-T-057 induces autophagic cell death in breast cancer cells by inhibiting γsecretase-mediated activation of Notch-signaling. J Biol Chem. 294(17):6733-6750.

4. Lee SH, Jun BH (2019) Silver Nanoparticles: Synthesis and Application for Nanomedicine. Int J Mol Sci, 20(4):865.

5. Mathur P, Jha S, Ramteke S, Jain NK (2018) Pharmaceutical aspects of silver nanoparticles. Artif Cells Nanomed Biotechnol. 46(sup1):115-126.

6. Burdușel AC, Gherasim O, Grumezescu AM, Mogoantă L, Ficai A, Andronescu E (2018) Biomedical Applications of Silver Nanoparticles: An Up-to-Date Overview. Nanomaterials (Basel, Switzerland), 8(9):681.

7. Kim WS, Choi WJ, Lee S, Kim WJ, Lee DC, Sohn UD, Shin SH, Kim W (2015) Anti-inflammatory, Antioxidant and Antimicrobial Effects of Artemisinin Extracts from Artemisia annua L. Korean J Physiol Pharmacol. 19(1):21-7.
8. Buege JA, Aust SD (1978) Microsomal lipid peroxidation, Methods Enzymol. 52:302–310.
9. Variya BC, Patel SS, Trivedi JI, Gandhi HP, and Rathod S (2015). Comparative evaluation of HMG CoA reductase inhibitors in experimentally-induced myocardial necrosis: biochemical, morphological and histological studies. Eur J Pharmacol 764, 283–291.
10. Moron MS, Depierre JW, and Mannervik B (1979) Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim. Biophys. Acta 582:67–78.
11. Shailaja Kasibhatla, Gustavo P. Amarantemendes, Deborah Finucane, Thomas Brunner, Ella Bossy-Wetzel, and Douglas R. Green (2006) Protocol Analysis of DNA Fragmentation using Agarose Gel Electrophoresis, Cold Spring Harb Protoc; 2006; Doi:10.1101/pdb.Port4429.
12. sSun J, Wei Q, Zhou Y, Wang J, Liu Q, Xu H (2017) A systematic analysis of FDA-approved anticancer drugs. BMC Syst Biol 11:87.
13. Holbeck SL, Camalier R, Crowell JA, Govindharajulu JP, Hollingshead M, Anderson LW, Polley E, Rubinstein L, Srivastava A, Wilsker D, Collins JM, Doroshow JH (2017) The National Cancer Institute ALMANAC: A Comprehensive Screening Resource for the Detection of Anticancer Drug Pairs with Enhanced Therapeutic Activity. Cancer research 77,13: 3564-3576.
14. Efferth T, Saeed M, Mirghani E, Alim A, Yassin Z, Saeed E, Khalid HE, Daak S (2017) Integration of phytochemicals and phytotherapy into cancer precision medicine. Oncotarget. 50284–50304.

15. Sleire L, Forde HE, Netland IA, Leiss L, Skeie BS, Enger PØ (2017) Drug repurposing in cancer. Pharmacol Res. 124:74-91.
16. Bayat Mokhtari R, Homayouni TS, Baluch N, Morgatskaya E, Kumar S, Das B, Yeger H (2017) Combination therapy in combating cancer. Oncotarget, 8(23). 38022–38043.
17. Song B, Zhang Y, Liu J, Feng X, Zhou T, Shao L (2016) Is neurotoxicity of metallic nanoparticles the cascades of oxidative stress? Nanoscale Res Lett. 11(1):291.

Figure 1

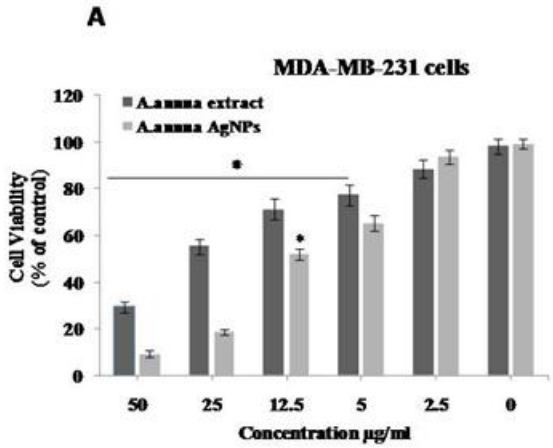


Figure 2

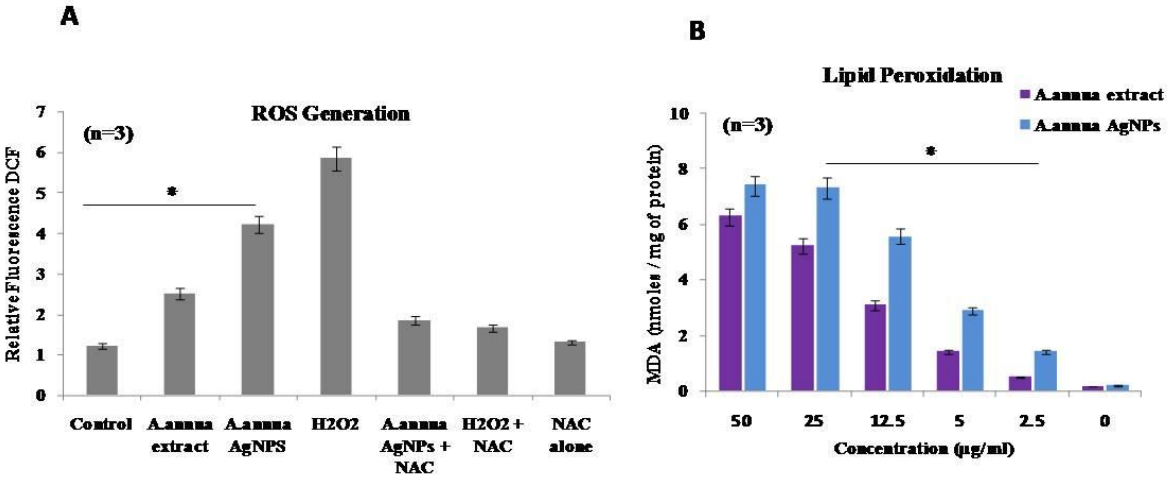


Figure 3

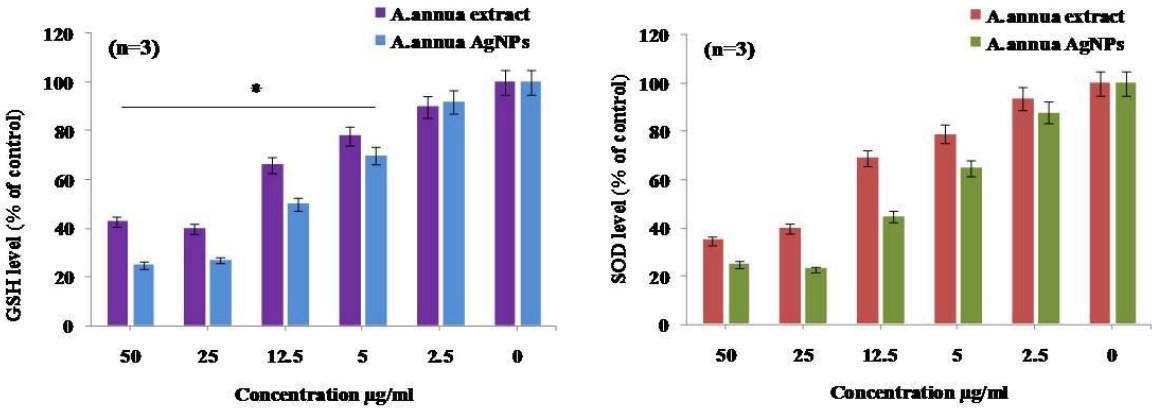
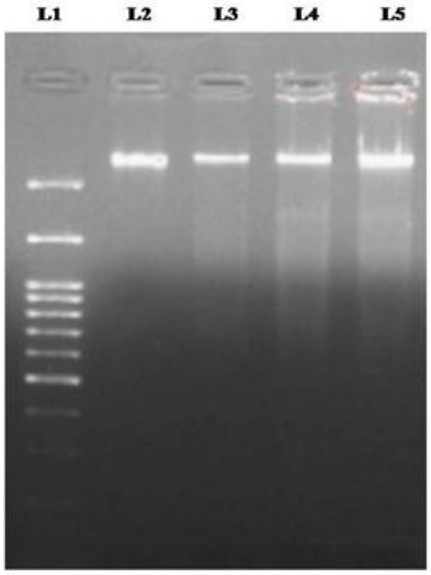
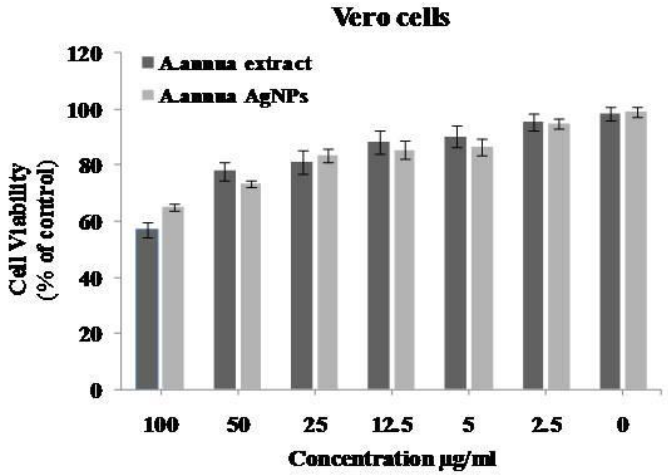

A.annua extract
A.annua AgNPs
(n=3)
*
GSH level (% of control)
120
100
80
60
40
20
0
50
25
12.5
5
2.5
0
Concentration µg/ml
A.annua extract
A.annua AgNPs
(n=3)
SOD level (% of control)
120
100
80
60
40
20
0
50
25
12.5
5
2.5
0
Concentration µg/ml

Figure 4



Supplementary Fig 1



Legends

Fig 1: Shows cytotoxicity of A.annua AgNPS. (A) Shows effect of A.annua AgNPs cell viability on MDA-MB 231 cells. Cells were treated using various concentration 2.5 µg/ml, 5 µg/ml, 12.5 µg/ml, 25 µg/ml, and 50 µg/ml for 24 h and cytotoxicity was measured using MTT assay.

Fig 2: Shows effect of A.annua AgNPS on oxidative stress. (A) Shows ROS generation in cells treated with A.annua AgNPs for 12h. At an excitation/emission wavelength, 485/530nm, the relative fluorescence of DCF was measured using spectrofluorometer. (B) MDA-MB 231 cells were treated with A.annua AgNPS for 24 h and the reaction of MDA with thioarbituric acid reactive substance (TBARs) was measured calorimetrically at a wavelength excitation/emission: 532/553nm. Mean of three independent experiments is presented here. AgNPs treated group showed significant difference ($P<0.05$) compared to control and A.annua extract alone.

Fig 3: Shows the effect of A.annua AgNPS on endogenous antioxidants in MDA-MB 231 cells. MDA-MB 231 cells were treated with various concentrations of A.annua AgNPs for 12 h and then the cell lysates were used for analysis of GSH and SOD. Concentration of GSH was expressed as milligram per gram of protein. Specific activity of SOD was expressed as units per milligram of protein. Mean of three independent experiments is presented here. AgNPs treated group showed significant difference ($P<0.05$) compared to control and A.annua extract alone.

Fig 4: Shows effect of A.annua AgNPS on DNA fragmentation in MDA-MB 231 cells. Lane 1: 100bp DNA ladder, Lane 2: A.annua extract treated cells, lane 3: A.annua AgNPs treated cells, Lane 4: DOX (5µM) treated cells. Cells were treated for 24 h and the whole genomic DNA was isolated and subjected to agarose gel electrophoresis for 20 mins at 100v. Mean of three independent experiments is presented here.

Supplementary Fig 1: Shows cytotoxicity of A.annua AgNPS. (A) Shows effect of A.annua AgNPs cell viability on Vero cells. Cells were treated using various concentration 2.5 µg/ml, 5 µg/ml, 12.5 µg/ml, 25 µg/ml, and 50 µg/ml for 24 h and cytotoxicity was measured using MTT assay. Mean of three independent experiments is presented here. AgNPs treated group showed significant difference ($P<0.05$) compared to control and A.annua extract alone.