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SUMMARY AND CONCLUSION

Nanobiotechnology is an upcoming branch of nanotechnology which have been playing an important role in the field of medical science. Nanotechnology refers broadly to a field of applied science and technology whose unifying theme is the control of matter on the atomic and molecular scale. Rapidly developing field of nanoscience had raised the possibility of using therapeutic NPs in the diagnosis and treatment of human cancers. Nanoscale particles and molecules are a potential alternative for treatment of cancer because they have unique biological effects based on the structure and size, which differ from traditional small molecule drugs. In general, silver NPs should serve as one of the best ways of treating diseases that involve cell proliferation and cell death.In the last few years, several pharmaceuticals companies had obtained approval from the US Food and Drug Administration (FDA) for the development of nanotechnology based drugs.

Cancer is considered as the second leading cause of death worldwide. Cancer is the cause of more than six million deaths each year in the world. Cancer can be characterized by the failure in the regulation of tissue growth results in the uncontrolled multiplication of the normal cells to form tumors which further invades into nearby parts of the body. The cancer can be caused by both carcinogenic factors and also hereditary. The cancer is considered as a preventable disease since the majority of the cancer was caused by the environmental including lifestyle factors. Currently, Chemotherapy and Radiotherapy treatments were followed for the treatment of various cancers, but are found to be having limited survivability and possess various side effects. Hence, there is an urge for the development of new anticancer drug for its treatment and prevention. Natural products from plants play a dominant role in the discovery of such new drugs. It has been estimated that about 60% of approved drugs were of natural origin.

Antioxidants are micronutrients that have gained importance in recent years due to their ability to neutralize free radicals or their actions. Antioxidant based drugs and formulations for the prevention and treatment of complex diseases like Alzheimer's disease and cancer have appeared during last three decades. Recent studies have shown that a number of plant products including polyphenols, alkaloids terpenes and various plant extracts exerted an antioxidant action against certain forms of cancer. There is currently immense interest in natural antioxidants and their role in human health and nutrition. Considerable amount of data have been generated on antioxidant properties of plants around the globe.

In this work, we have explored an inventive contribution for the synthesis of AgNPs using *Gloriosa superba* and their antioxidant and antitumorigenic potential.

The present research entitled **"Antioxidant and Antitumorigenic efficacy of methanolic extracts of *Gloriosa superba* and Silver Nanoparticles of methanolic extracts of *Gloriosa superba* to DLA tumor cells"** was carried out and the results are summarized in this chapter.

The study was carried out in three phases.

In phase I, identification of the phytochemical constituents of MGsSTL and characterization of MGsSTL and AgMGsSTL were carried out. In phase II, *in vitro* antioxidant and antitumorigenic effects by cytotoxic and apoptotic role of MGsSTL and AgMGsSTL were assessed. In phase III *in vivo* antioxidant and antitumorigenic effects of MGsSTL and AgMGsSTL were evaluated in DLA induced Swiss albino mice.

In phase I to determine the bioactive constituents of MGsSTL, the preliminary phytochemical screening was carried out which revealed the presence of major secondary metabolites such as Carbohydrates, Alkaloids, Glycosides, Flavanoids, Steroids, Terpenoids and Phenolics in MGsS;

Carbohydrates, Alkaloids and Flavanoids in MGsT and Carbohydrates and Steroids in MGsL.

Since MGsSTL showed alkaloids as major constituent the HPTLC analysis was carried out to confirm the presence of alkaloids and their derivatives in MGsSTL using colchicines as standard. The results showed the presence of colchicine (Rf values of 0.48), colchicines derivatives (Rf values of 0.34 and 0.83) and 11 unknown alkaloids in MGsS; two colchicine derivatives (Rf values of 0.46 and 0.50) and 13 unknown alkaloids in MGsT and 10 unknown alkaloids in MGsL.

To characterize the presence of AgNPs in the synthesised AgMGsSTL the synthesized AgNPs were analyzed periodically using UV- Visible (UV-Vis) spectrophotometer. The absorbance of the NPs was measured in the range 400-800nm. Absorbance maxima of AgMGsS at 360 and 440 nm with remarkable broadening of peak indicated that the particles are polydispersed. The absorption peak of the AgMGsT and AgMGsL was found at 350 nm. This clearly indicated the interaction between AgNPs and biomolecules present in the MGsSTL.

Functional groups of the plant secondary metabolites involved in the reduction and capping of NPs were identified by FTIR technique. The FTIR spectrum of MGsS showed 28 peaks; MGsT showed 21 peaks and MGsL showed 20 peaks. The FTIR results confirmed the major involvement of CH_2- groups, –CO– groups and –NH_2– groups of colchicine, colchicines derivatives and also other phytoconstituents of MGsS, MGsT and MGsL are responsible for capping and efficient stabilization and reduction of silver ions and formation of the AgNPs. The present study, thus showed a simple green route for rapid and economical synthesis of silver NPs.

The size of the AgNPs was determined by the Scanning Electron Microscopy (SEM) and elemental composition of the NPs was further confirmed by the Energy Dispersive X-ray (EDX) analysis. The sizes of AgMGsS and

AgMGsT ranged from 35 - 42 nm and 44 - 90 nm. The EDX plot and picture of AgMGsS and AgMGsT confirmed the development of silver nanostructures.

The TEM analysis used to characterize the shape, size, and morphology of AgMGsSTL. TEM analysis of AgMGsS and AgMGsT showed the spherical and polydispersed AgNPs at a range of 20-69 nm and capped by plant constituents that prevented their aggregation. Inherent capping offers additional advantage of the stability in the green chemical synthesis. The TEM images also revealed that NPs are not in physical contact but are separated by uniform interparticle distance. It was noticeable that the edges of the particles were lighter than the centers, suggesting that the functional groups of some bioorganic compounds (such as alkaloids (colchicines and its derivatives) and other phytochemical constituents in MGsS and MGsT) involved in capping of AgNPs.

The particle size distribution as characterized in Dynamic Light Scattering (DLS) was found to be in the range of 3-110 nm; 3-229 nm and 6-477 nm for AgMGsS, AgMGsT and AgMGsL respectively. The highest fraction of AgMGsS, AgMGsT and AgMGsL were present in the solution at 34nm, 61nm and 71nm respectively. From the plot it was evident that the NPs in solution were having various sizes which are indeed in agreement of the results obtained by SEM analysis.

Zeta-potential measurements were performed to study the stability of NPs. Zeta-potential analysis demonstrated that the presence of negative charges in AgMGsS (−57.0mV), AgMGsT (−47.3mV) and AgMGsL (−27.0mV). DLS and Zeta-potential analysis showed the presence of minimum nanosized particles and stability of the AgMGsSTL. AgMGsS and AgMGsT were found to be more sufficiently charged to maintain stable dispersion at room temperature in solution over several months of time than that of AgMGsL.

The morphology and the nanocrystalline size of AgNPs were determined by the characteristic peaks obtained from the XRD image. The data obtained was matched with the database of Joint Committee on Powder Diffraction

Standards (JCPDS) file No. 04-0783. The presence of peaks at 2θ values at 38.15°, 46.28°, 77.34° for AgMGsS showed the number of strong Bragg reflections which correspond to (111), (200), (311) planes of silver; 2θ values at 38.60°, 46.32, 64.48°, 77.34° for AgMGsT showed the number of strong Bragg reflections which correspond to (111), (200), (220), (311) planes of silver and 2θ values at 38.30° showed the number of strong Bragg reflections which correspond to (111) plane of silver for AgMGsL. From the Scherrer equation the average crystalline size of AgMGsS, AgMGsT and AgMGsL were found to be 5.17 nm, 3.05nm, 5.17nm, 3.08 nm; 4.83 nm, 4.26nm, 4.67 nm and 6.03 nm respectively. The XRD study confirmed the crystalline face-centered cubic lattice of AgNPs of AgMGsSTL.

Phase II results of the present study showed the dose dependent free radical scavenging efficacy of MGsSTL and AgMGsSTL. The free radical scavenging efficacy of MGsS was found to be high with minimum concentration of the extract {DPPH (37 µg) > $^{\cdot}O_2^-$ (45 µg) > H_2O_2 (50 µg) > NO (61 µg) > $OH^{\cdot}$ (71 µg)} which was followed by MGsT {DPPH (53 µg) > $^{\cdot}O_2^-$ (61 µg) > H_2O_2 (62 µg) > NO (72 µg) > $OH^{\cdot}$ (76µg)} > MGsL {H_2O_2 (68 µg) > $^{\cdot}O_2^-$ (72 µg) > DPPH (80 µg) > NO (83 µg) > $OH^{\cdot}$ (84 µg)} when compared to standard Vitamin C {DPPH (40 µg) > $^{\cdot}O_2^-$ (42 µg) > H_2O_2 (58 µg) > NO (60 µg) > $OH^{\cdot}$ (63 µg)}.

Similarly the free radical scavenging efficacy of AgMGsS was found to be high with minimum concentration of the extract {DPPH (30 µg)> $^{\cdot}O_2^-$ (33 µg) > H_2O_2 (41 µg) > NO (58 µg) > $OH^{\cdot}$ (61 µg)} which was followed by AgMGsT {DPPH (50 µg) > $^{\cdot}O_2^-$ (52 µg) > H_2O_2 (58 µg) > NO (70 µg) > $OH^{\cdot}$ (74 µg)} > AgMGsL {H_2O_2 (66µg) > $^{\cdot}O_2^-$ (69 µg) > DPPH (74 µg) > NO (80 µg) > $OH^{\cdot}$ (82 µg)} when compared to standard Vitamin C {DPPH (40 µg) > $^{\cdot}O_2^-$ (42 µg) > H_2O_2 (58 µg) > NO (60 µg) > $OH^{\cdot}$ (63 µg)}. The free radical scavenging efficacy of AgMGsS was found to be higher than that of Vitamin C, AgMGsT and AgMGsL.

The dose dependent free radical scavenging activity of MGsSTL, AgMGsSTL were found to be comparable to the standard Vitamin C and indicated their *in vitro* antioxidative potential.

AgMGsS showed higher scavenging activity than that of MGsS which was followed by AgMGsT> MGsT> AgMGsL> MGsL. Thus, the free radical scavenging efficacy of AgMGsS was found to be higher than that of all other extracts (MGsSTL and AgMGsTL).

In vitro antitumorigenic effect of MGsSTL and AgMGsSTL was evaluated against DLA tumor cells by MTT cell proliferation assay, trypan blue exclusion method and also apoptotic effect to DLA cells by flow cytometry.

The results of MTT assay showed that MGsSTL and AgMGsSTL mediated a concentration dependent increase in toxicity towards DLA cells with the ED_{50} values of 29 µg, 39 µg and 80 µg for MGsS, MGsT, MGsL respectively and of 21 µg, 24 µg and 77 µg for AgMGsS, AgMGsT, AgMGsL respectively and these concentrations were used in *in vivo* studies. Thus, AgMGsSTL showed more toxicity towards DLA cells than that of MGsSTL.

In trypan blue assay, incubation of DLA tumor cells with MGsSTL and AgMGsSTL produced a concentration dependent cytotoxic effect which was indicated by the increase in number of dead cells with increasing concentrations of MGsSTL and AgMGsSTL. DLA tumor cell proliferation was significantly inhibited by MGsSTL with the ED_{50} values of (40µg, 53 µg, 88 µg) and AgMGsSTL with the ED_{50} values of (30 µg, 37 µg and 82 µg) respectively. Thus, the synthesized AgMGsSTL were found to be the more potent cytotoxic agent to DLA tumor cells than that of MGsSTL.

Flow cytometry analysis using Annexin V-Biotin and propidium iodide staining confirmed the apoptotic effect of MGsSTL and AgMGsSTL to DLA cells. AgMGsS with DLA showed 6.47%; AgMGsT with DLA showed 4.26% and AgMGsL with DLA showed 2.50%. Annexin V-Biotin and PI positive cells when compared with 0.99%, of Annexin V-Biotin and PI negative cells detected in

untreated cells. Results of Annexin V-Biotin and PI positive cells detected in treated tumor cells were statistically significant when compared with annexin V-Biotin and PI negative cells detected in untreated tumor cells. The *in-vitro* anticancer study reported that the MGsSTL and AgMGsSTL have cidal activity to DLA cancer cells in a sustained manner. This study revealed that MGsSTL and AgMGsSTL have the potency to bring about apoptotic inducing effect to DLA cells.

Knowing the antioxidative and antitumorigenic effect of MGsSTL and AgMGsSTL in *in vitro* studies, a long term *in vivo* studies were carried out to confirm the above results in Phase III.

In vivo antioxidative and antitumorigenic potential of MGsSTL and AgMGsSTL individualy and in DLA tumor induced Swiss albino mice was evaluated by assessing the activities of enzymic antioxidants, levels of non enzymic antioxidants and, the levels of MDA in 17 groups with 6 mice in each treatment groups (Group 1- PBS, Group 2- DMSO, Group 3- Paraffin oil, Group 4- Silymarin, Group 5- MGsS, Group 6- AgMGsS, Group 7- MGsT, Group 8- AgMGsT, Group 9- MGsL, Group 10- AgMGsL, Group 11- DLA+MGsS, Group 12- DLA+AgMGsS, Group 13- DLA+MGsT, Group 14- DLA+AgMGsT, Group 15- DLA+MGsL, Group 16- DLA+AgMGsL and Group 17- DLA) in 20 days and 60 days treatment periods.

Administration of silymarin, MGsSTL and AgMGsSTL significantly increased the activities of enzymic antioxidants CAT, SOD, GST, GPx and GR , the levels of non-enzymic antioxidants Vitamin A, C, E and GSH and siginificantly decreased the levels of MDA in 20 days treatment period. DLA tumor induced mice showed a significant decrease in the activities of enzymic antioxidants and the levels of non-enzymic antioxidants and increase in the levels of MDA in 20 days of treatment period. Coadministration of MGsSTL and AgMGsSTL to the DLA induced mice significantly enhanced the activities of

enzymic antioxidants and the levels of non-enzymic antioxidants and significant declined MDA levels in 20 days treatment period. Similar trend was noticed on 60 days treatment period when compared to 20 days treatment period. These findings revealed the *in vivo* antioxidative and antitumourigenic role of MGsSTL and AgMGsSTL. The over all antioxidative role of the MGsSTL and AgMGsST was found to be more significant than that of the standard antioxidant silymarin.

The above *in vivo* antitumorigenic efficacy was also confirmed by evaluating the increase in average life span of mice administered with MGsSTL and AgMGsSTL individually and to DLA induced mice. The DLA tumor bearing mice life span was found to be 15-25 days with the average life span of 20 days. Coadministration of MGsS, MGsT, MGsL, AgMGsS, AgMGsT and AgMGsL inhibited the growth of DLA tumor cells by detoxifying the tumor cells and increased the life span to 75 days, 72 days, 58 days, 75 days, 74 days and 60 days respectively and indicated their antitumorigenic effect.

The histopathological observations of liver section of all 17 groups of control and experimental mice were evaluated to support the above results of MGsSTL and AgMGsSTL individually and in DLA tumor induced mice. Control groups (PBS, DMSO, Paraffin oil) showed normal lobular architecture with intact central vein and sinusoids and normal portal triad. Liver sections of Swiss albino mice treated with the MGsSTL, AgMGsSTL and silymarin showed normal lobular pattern, contain a large spherical nucleus as compared to the normal control group. DLA induced animals showed severe necrosis, surrounding fibrosis, perivenular inflammation and vacuole formation. However, DLA tumor induced mice treated with MGsSTL and AgMGsSTL showed reduced vacuole formation and inflammation and almost normal hepatocellular architecture. Histopathological examinations also showed the protective effect *of* MGsSTL and AgMGsSTL. The prevention of necrosis by the treatment of MGsSTL and AgMGsSTL may be due to the diminution of oxidative stress by the their antioxidative potential.

From the *in vitro* and *in vivo* studies it can be hypothesised that the antioxidative role of MGsSTL and AgMGsSTL could be due to the antioxidative potential as revealed by the

i Dose dependent radical scavenging effect,

ii Significant induction of the in the enzymic antioxidants (CAT, SOD, GST, GPx and GR), levels of non enzymic antioxidants (vitamin A, vitamin C, vitamin E and GSH) and

iii Significant decrease in the rate of LPO

Antioxidative potential of MGsSTL and AgMGsSTL is responsible for the antitumorigenic effect and was confirmed by their

1. Dose dependent cytotoxic effect and apoptotic inducing effect to DLA tumor cells.

2. Effect on the increase in life span of DLA tumor induced mice.

The observed antitumorigenic property may be attributed to the bioactive antitumor principles present in the MGsSTL and AgMGsSTL.

Conclusion

Overall findings of the study emphasized that colchicine, colchicine derivatives and also other phytoconstituents of MGsSTL and AgMGsSTL are responsible for the antioxidative and antitumorigenic effect by their ability to

i. Prevent oxidative damage by scavenging of free radicals produced

ii. Induce cytotoxicity and apoptosis to DLA tumor cells

Iii. Induce enzymic and non enzymic antioxidants

iv. Inhibit Lipid peroxide formation and

v. Increase the life span of DLA tumor induced mice

Our results revealed that AgMGsSTL are more effective than MGsSTL. The proposed null hypothesis was rejected and it was proved that there is

significant difference at 5% level between MGsSTL and AgMGsSTL towards antioxidative and antitumorigenic effect. So, AgMGsSTL can be recommended as antioxidants and antitumorigenic agents to the individuals under oxidative stress such as inflammation, aging and cancer.

The present research work leaves the following future prospects:

Detailed mechanism of action of AgMGsSTL on

- Apoptosis
- Immunomodulatory role
- Tumor suppressor genes/ proteins
- The expression of xenobiotic phase I and phase II enzymes may be elucidated.