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ANTIOXIDANT POTENTIAL OF BIOGENIC SILVER NANOPARTICLES FROM *ANACARDIUM OCCIDENTALE* LEAF IN DALTON'S ASCITES LYMPHOMA-BEARING SWISS ALBINO MICE

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ABSTRACT
Peroxidation of membrane lipids and oxidative damage of DNA and proteins are caused by free radicals and it is believed to be related to a variety of chronic pathological ailments such as cancer. The aim of this study was to evaluate the *in vivo* antioxidant potential of synthesized biogenic silver nanoparticles using aqueous extract of *Anacardium occidentale* leaf (AOAgNPs) in Dalton's ascites lymphoma (DLA)-bearing Swiss albino mice. AOAgNPs was administered intraperitoneally at the dose level of 2 and 4mg/kg body weight/day for consecutive 14 days after 24 hours of cell inoculation (1×10^6 cell) to mice using 5-fluorouracil as standard drug. Treatment with AOAgNPs decreased the levels of hepatic and renal malondialdehyde (MDA), oxidized glutathione (GSSG) and increased reduced glutathione (GSH) level, the activities of catalase (CAT), super oxide dismutase (SOD) and peroxidases in DLA-bearing mice. The study reveals that AOAgNPs exhibit prominent antioxidant activities in Dalton's ascites lymphoma-bearing Swiss albino mice.

KEYWORDS: Silver nanoparticles; *Anacardium occidentale* leaf; Dalton's ascites lymphoma; Antioxidant; Reduced Glutathione.

INTRODUCTION
Cancer is one of the disease processes facilitated by a variety of endogenous and environmental stimuli such as reactive oxygen species (ROS) and other free radicals. Endogenous and exogenous generation of ROS occurs as a function of biochemical reactions using oxygen. Least amount of ROS are required for various physiological functions including activation and modulation of signal transduction pathways, modulation of actions of redox-sensitive transcription factors and control in mitochondrial enzyme activities. High levels of reactive oxygen species are toxic and can oxidize phospholipid bilayer of cell membrane, alter protein structure and causing mutations by DNA damage leading to cancer.[1] Cell contains several antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH) and catalase (CAT) which are capable to scavenge many harmful ROS by converting into harmless product. Oxidative stress (OS) occurs when there is an imbalance between the production of ROS and decrease in antioxidant status. This stress may lead to mutagenesis, cytotoxicity and alterations in gene expression that initiate or stimulate carcinogenesis.[2] Due to the failure of cellular defense in oxidative stress, oxidative damage related diseases like cancer may be formed due to excess production of ROS and the antioxidants are necessary to scavenge ROS to protect cells against oxidative injury.[3]

Nanotechnology is an interdisciplinary approach in biomedical applications that focusing on synthesis of nanoparticles having improved antimicrobial and antioxidant properties against the degenerative diseases and cancer.[4] Silver nanoparticles (AgNPs) are one of the most commonly used Nanoparticles which have high surface area, very small size (<20 nm) and high dispersion capacity.[5] Silver is a safe and effective metal because it is non-toxic to animal cells.[6] It was reported that silver nanoparticles have antimicrobial,[7] antioxidant,[8] anticancer,[9] and anti-inflammatory[10] properties etc.

Metallic nanoparticles can be synthesized by physical, chemical or biological methods. As in biological synthesis protocol toxic chemicals are not used so it is eco-friendly and also reliable.[11] Biological synthesis of nanoparticles has got special attention also because the biologically active molecules involved in the green synthesis of nanoparticles act as functional ligands which

make these NPs more suitable in a wide range of biomedical applications.[12]

Right before the advent of civilization plants are being used extensively by mankind. Currently, research trends in natural and synthetic antioxidant led the screening and identification of new antioxidants from the plant sources. Antioxidant activity in plant extract is due to the redox potential of phytochemicals,[13] which can play an important role in quenching singlet and triplet oxygen, decomposing the peroxides or neutralizing the free radicals. Due to presence of rich functional molecules, such as phenolic compounds having high antioxidant activity many plant extracts have been considered as potent natural reducing agents.[14] In the recent years plant products are being used in nanobiotechnology extensively. Synthetic antioxidant is reported to have various properties such as anti-allergencity, anti-carcinogenicity, anti-aging activity and anti-mutagenicity.[15] It is assumed that higher antioxidant activity of biogenic nanoparticles might be due to the preferential adsorption of the antioxidant material from the extract onto the surface of the nanoparticles.

Anacardium occidentale of Anacardiaceae family, commonly called 'cashew' is a multipurpose tree possessing traditional medicinal value.[16] It is native to North-Eastern Brazil but now widely grown in India and all other climates. Phytochemical analysis revealed that *A.occidentale* leaf extracts possess phenolic compounds, flavonoids, steroids and triterpenes.[17] It has been reported that the nut and stem-bark, the shoot and the leaves of *A.occidentale* possess free radical scavenging activities.[18,19,20,21]

In our laboratory, biogenic silver nanoparticles using aqueous extract of *Anacardium occidentale* leaf (AOAgNPs) have been synthesized.

The present study was designed to explore the *in vivo* antioxidant potential of synthesized biogenic silver nanoparticles from *Anacardium occidentale* leaf (AOAgNPs) in Dalton's ascites lymphoma (DLA)-bearing Swiss albino mice.

MATERIALS AND METHODS

Chemicals and Reagents

2,4,4dithionitrobenzoic acid(DTNB), Pyrogallol, Thiobarbutaric acid(TBA), Trichloro acetic acid(TCA), Tris HCl, Potassium dihydrogen phosphate(KH_2PO_4), Hydrogen peroxide(H_2O_2), Sulfosalicyclic acid(SSA), Sodium chloride (NaCl), Hydrochloric acid (HCl) were purchased from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. All other chemicals used were analytical grade and purchased from Merck Ltd., SRL Pvt. Ltd., Mumbai, India.

Collection, preparation and extraction of plant material

Leaves of *Anacardium occidentale* were collected during March to May from Vidyasagar University campus, Midnapore, West Bengal, India and were authenticated (Identification No. CNH/2014/Tech.II/55) from Botanical Survey of India, Howrah. 500g dried pulverized leaf powder was added into 750 ml of double distilled water and kept for 72 h in room temperature. Then filtered and filtrate was lyophilized. The freeze-dried aqueous leaf extract of *Anacardium occidentale* (AOAgNPs) was stored at 4°C[22] and the yield was 8% (w/w).

Synthesis of biogenic silver nanoparticles and characterization of silver nanoparticles

In our laboratory, biogenic silver nanoparticles (AOAgNPs) was synthesized according to the method of Maity et al., 2018[23] using *Anacardium occidentale* leaf aqueous extract by a cost-effective and eco-friendly way and the characterization of AOAgNPs was done properly.

Animal maintenance

To culture Dalton's ascites lymphoma (DLA) cells, Swiss albino mice (18–25 g) were maintained under standard temperature (25 ± 2 °C) and humidity (60 ± 5%) with 12 h light /dark cycle. The animals were fed a standard pellet diet and drinking water *ad libitum.* The study was approved (approval No. IEC/7-13/C13) by the Institutional Animal Ethical Committee, registered under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests & Climate Change, Govt. of India.

Culture of cell line

Dalton's ascites lymphoma (DLA) cells were obtained from Chittaranjan National Cancer Research Institute (CNCI), Kolkata. DLA was maintained by weekly intraperitoneal transplantation in the male albino Swiss mice at the concentration of 1×10^6/cells per mouse. Washed DLA cells free from contaminating RBC were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS) and antibiotic antimycotic solution (100 U ml^{-1} penicillin, 10 mg ml^{-1} streptomycin and 4 mM L-glutamine) under 5% CO2 and 95% humidified atmosphere at 37˚C in an incubator. Viable cells (1×10^6 ml^{-1}) were used for different experiments in the present study.

Treatment schedule

Thirty male mice were divided into five groups (n=6).
Group I : Saline control
Group II : DLA control
Group III : DLA + AOAgNPs (2 mg/kg body weight)
Group IV : DLA + AOAgNPs (4 mg/kg body weight)
Group V : DLA + 5-FU (20 mg/kg body weight)

Except the first group (saline control), animals from all groups were inoculated intraperitoneally with 0.1 ml of

1×10^6 DLA cells/mouse. The second group was considered as DLA control. After 24 h of tumor inoculation, AOAgNPs was administered to III and IV groups at 2 and 4 mg/kg body weight respectively once daily and the group V was treated with reference drug 5-FU once daily for 14 consecutive days.[24] On 15th day and 18 h of fasting, the animals were sacrificed by cervical dislocation. Then, blood was collected from each mouse of all groups for the estimation of hepatic and renal oxidative stress parameters.

Determination of malondialdehyde (MDA) content

Hepatic and renal MDA content was measured[25] by mixing 1 ml of respective tissue homogenate (20 mg/ml phosphate buffer), 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of acetate buffer (20% pH 3.5) and 1.5 ml of aqueous solution of thiobarbituric acid (0.8%). After heating the mixture for 60 min at 95°C, a red pigment was formed and thatwas extracted by 5 ml of *n*-butanol-pyridine mixture (15:1) and centrifuged at 5000 rpm for 10 min at room temperature. The absorbance of supernatant was noted at 535 nm.

Estimation of reduced glutathione (GSH)

Reduced glutathione (GSH) was estimated by the method of Griffith.[26] Briefly, 100 µl sulfosalicylic acid was added with 200 µl tissue homogenate and centrifuged for 10 min at 3000 rpm. The supernatant was taken and 1.8 ml of DTNB was mixed with it and was shaken well. The reading was taken at 412 nm.

Assay of superoxide dismutase (SOD)

The SOD activity of the supernatant was estimated[27] by determining the percentage of inhibition of the pyragallol auto oxidation by SOD. 2 ml of buffer mixture (50 mM Tris HCl, 10 mM hydrochloric acid in the presence of 1 mM EDTA), 100 µl of 2 Mm pyrogallol and 10 µl of tissue homogenate were taken in a spectrophotometric cuvette, mixed and the absorbance was measured in the spectrophotometer (UV-245 Shimadzu, Japan) at 420 nm for 3 min.

Determination of catalase (CAT)

Catalase was estimated by the method of Aebi[28] by taking 1 ml of 30mM H_2O_2 and 1.9ml of 15mM PBS in 0.1ml of tissue homogenate. The readings were noted in the spectrophotometer.

Glutathione peroxidase (GP_X)

Glutathione peroxidase was assayed by the method of Rotruck et al.[29] In 0.2 ml of liver and kidney homogenate, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of 0.4 M sodium phosphate buffer, 0.1 ml of 10 mM sodium azide and 0.2 ml of 4 mM reduced glutathione were added and was incubated for 5 min at 37ºC. To stop the reaction, 0.4 ml of 10% TCA was added and centrifuged for 20 min at 3200 rpm. Then in 0.5 ml of supernatant, 1 ml of 5, 5′-dithiobisnitrobenzoic acid (DTNB) and 3 ml of disodium hydrogen phosphate (Na_2HPO_4) were mixed. The absorbance was measured at 420 nm.

Estimation of glutathione-s-transferase (GST)

Glutathione-S-transferase (GST) activity was estimated[30] by mixing 0.1 ml of tissue homogenate, 0.2 ml of 100 mM PBS, 0.05 ml of 1 mM GSH and 0.02 ml of 60 mM 1-Chloro-2, 4 dinitrobenzene (CDNB) in a cuvette and reading was taken at 340nm. The results were expressed in µmol CDNB conjugate formed/min/ mg protein.

Statistical analysis

The experimental results were expressed as the Mean ± Standard error of mean (SEM). Statistical analysis of the collected data were done by Analysis of variance (ANOVA) followed by Student's t-test. Difference was considered significant when $p<0.05$.

RESULTS

The levels of MDA were significantly ($p<0.001$) increased in DLA control group compared to saline control group. After treatment with AOAgNPs at the dose levels of 2, 4mg/kg body wt., hepatic and renal MDA was decreased significantly ($p<0.001$) compared to DLA control group (Fig 1).

Significant ($p<0.001$) decrease in hepatic and renal GSH level was observed in DLA control animals. AOAgNPs at 2 and 4 mg/kg body wt., bring back GSH level to near normal values in the liver and kidney samples (Fig 2). Most of the results regarding GSH level were found to be significant ($p<0.001$) those of observed with 5-FU treatment. Superoxide dismutase (SOD) level was decreased both in liver and kidney of DLA control animals. The treatment with AOAgNPs at 2 and 4 mg/kg body wt. doses reversed these changes to near normal values in the liver and kidney of treated mice (Fig 3). Almost similar finding was observed in case of 5-FU treated mice. AOAgNPs at 4 mg/kg body wt. dose level was found to be more effective to brought back liver SOD normal level. AOAgNPs at both doses (Fig 3) normalized the SOD activity to a good extent ($p<0.01$) compared to DLA control.

The activity of catalase (CAT) was significantly ($p<0.001$) decreased in DLA control group compared to saline control group. AOAgNPs at 2 and 4 mg/kg body wt., decreased hepatic and renal CAT activity significantly ($p<0.001$) (Fig 4). Similar finding was noted in case of 5-FU.

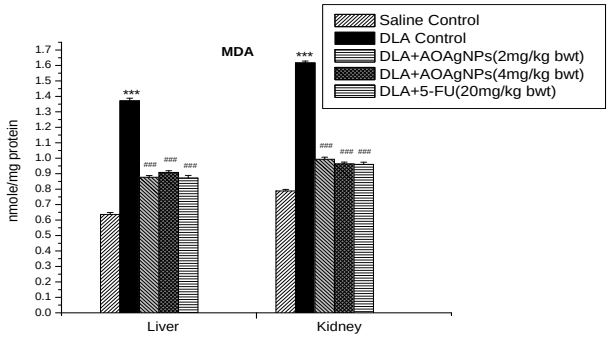

Fig. 1: shows the effect of AOAgNPs on hepatic and renal MDA content after 14 days treatment in DLA bearing mice. Data are expressed as Mean± SEM (n=6). '*'represents significant difference at (p<0.001) compared to saline control; '###'' represents significant difference at (p<0.001) compared to DLA control.**

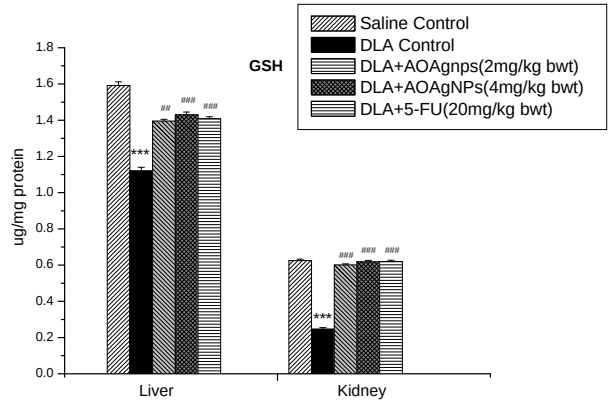

Fig. 2: Shows hepatic and renal GSH level after 14 days treatment of AOAgNPsin DLA bearing mice. Data are expressed as Mean± SEM (n=6). ''*'represents significant difference at (p<0.001) compared to saline control; '##' and '###''represents significant difference at (p<0.01) and (p<0.001) respectively compared to DLA control.**

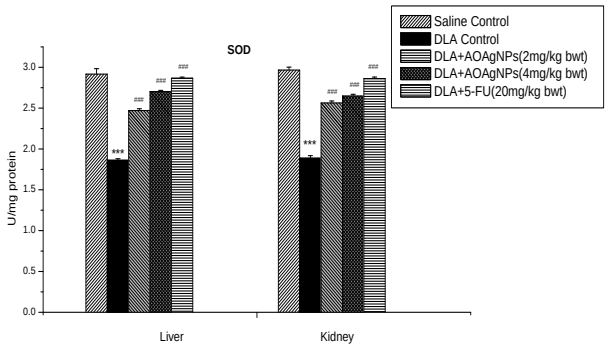

Fig. 3: shows the effect of AOAgNPs on hepatic and renal SOD activity after 14 days treatment in DLA bearing mice. Data are expressed as Mean± SEM (n=6). '*' represents significant difference at (p<0.001) compared to saline control; and '###'' represents significant difference at (p<0.001) compared to DLA control.**

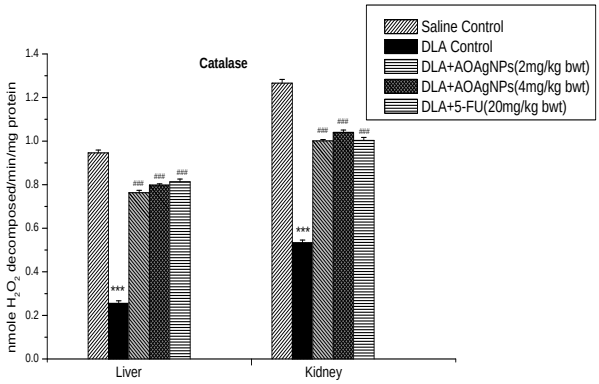

Fig. 4: shows the effect of AOAgNPs on hepatic and renal catalase (CAT) activity after 14 days treatment in DLA bearing mice. Data are expressed as Mean± SEM (n=6). '*'represents significant difference at (p<0.001) compared to saline control; '###'represents significant difference at (p<0.001) compared to DLA control.**

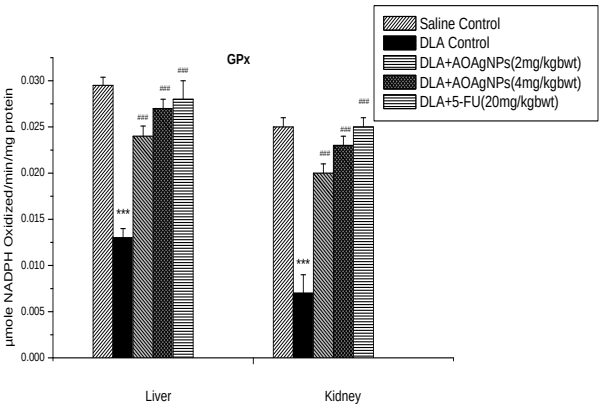

Fig. 5: shows the effect of AOAgNPs on liver and kidney GPx activity after 14 days treatment in DLA bearing mice. Data are expressed as Mean± SEM (n=6). '*'represents significant difference at (p<0.001) compared to saline control; '###'represents significant difference at (p<0.001) compared to DLA control.**

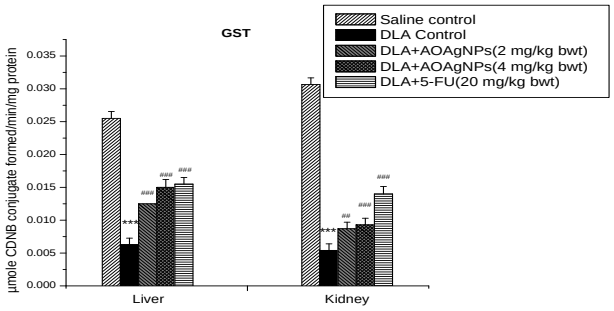

Fig. 6: shows the effect of AOAgNPs on liver and kidney GST after 14 days treatment in DLA bearing mice. Data are expressed as Mean± SEM (n=6). '*'represents significant difference at (p<0.001) compared to saline control; '##'represents significant difference at (p<0.01) compared to DLA control,'###' represents significant difference at (p<0.001) compared to DLA control.**

The activity of glutathione peroxidase (GPx) was significantly ($p<0.001$) decreased in liver and kidney of DLA control animals compared to saline control group. After treatment with AOAgNPs, GPx activity increased significantly ($p<0.001$) in the liver and kidney (Fig 5).

Pronounced ($p<0.001$) decrease in liver and kidney GST activity was also observed in DLA control animals. The treatment with AOAgNPs at the above mentioned dose levels increased the GST level significantly ($p<0.001$) to near normal level in treated groups (Fig 6).

DISCUSSION

The present study was carried out to evaluate the antioxidant activity of silver nanoparticles synthesized from aqueous extracts of *Anacardium occidentale* leaves (AOAgNPs) in DLA-bearing mice.

AOAgNPs decreased the lipid peroxidation in term of hepatic and renal MDA content and increases free radical scavenging GSH as well as antioxidant enzymes catalase and SOD in tumour bearing mice to near normal levels. There is a reduction of peroxidase enzyme level in DLA control group which was normalized by 14 days AOAgNPs treatment.

It is reported that implication of free radicals occurred in carcinogenesis.[31] Excessive free radicals production causes oxidative stress which may lead to damage of macromolecules such as lipids and can induce lipid peroxidation *in vivo.* Increased lipid peroxidation causes degeneration of tissues. MDA is the end product of lipid peroxidation and present in the carcinomatous tissue at higher concentration than normal tissue.[32] In this experiment it is noted that treatment with AOAgNPs decreased the levels of lipid peroxidation in DLA bearing mice. So, it is clear that AOAgNPs have antioxidant activity.

Glutathione is a potent inhibitor of neoplastic process. It plays an important role as an endogenous antioxidant system. It is present in higher concentration particularly in liver and is known to have key function in the protective process.[33] It is noted that in the DLA control group, reduced glutathione level decreased may be due to its utilization by the excessive amount of free radicals, whereas in treated group GSH level was significantly increased compared to the untreated DLA bearing mice.

The SOD level also decreased significantly in tumor bearing mice compared to control group, this degradation is increased by the treatment with 5-FU, and AOAgNPs. It was reported that a small amount of catalase is present in tumor cells.[34] It was also reported that different antioxidant enzymes i.e. catalase and SOD activities were inhibited as a result of tumor growth.[35] In the present study similar findings were observed in DLA bearing mice. The administration of AOAgNPs at two different doses increased the SOD and CAT compared to DLA control as well as it restored the peroxidase and GSH content to near normal levels. It indicated that the antioxidant and free radical scavenging property of AOAgNPs in Dalton's ascite tumor bearing mice.

During oxidative stress, detoxification pathway is triggered involving SOD catalyzing the first step and then CAT and various peroxidases eliminating hydrogen peroxide from cell in order to inhibit the generation of free radicals. These enzymes are safeguards for DNA from oxidative stress and hinder the individual's risk of cancer susceptibility.[36]

CONCLUSION

Silver nanoparticles synthesized from aqueous extracts of *Anacardium occidentale* leaves (AOAgNPs) exhibited excellent antioxidant potential. This may be due to the presence of phytochmicals in *A. occidentale leaf extract* which are used in the synthesis of AOAgNPs.

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